Principles of Bone Biology

THIRD EDITION

Volume 1

EDITED BY

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Dedication

Gideon A. Rodan

We pay special tribute in this Third Edition of *Principles of Bone Biology* to one of the original three editors, Gideon Rodan, who passed away after a long illness on January 1, 2006. Gideon was a wonderful scientist who made outstanding contributions to our understanding of bone cell biology and to the treatment of metabolic bone diseases. His quiet but highly effective leadership style, superb intellect and major scientific achievements brought together bone and mineral investigators from all over the world. He was a beloved friend whose insight, empathy and sense of humor enriched our lives. Gideon's wisdom and breadth of knowledge were invaluable in selecting and evaluating the contributions to the first two editions of this book.

Gideon's education in mathematics and basic sciences in Israel, and his PhD at the Weitzman Institute on physicochemical aspects of mineral metabolism, provided the fuel for a career of sustained achievement and scholarship. He began his academic career at the University of Connecticut Dental School, rapidly became Chairman of the Department of Oral Biology, and built a program of research that brought that School to great prominence. He was a mentor supreme, with a large number of students, post-doctoral trainees and close colleagues who went on to have successful careers. They remained intensely loyal to him. A former President of the American Society of Bone and Mineral Research (ASBMR), Gideon was also the first recipient of the ASBMR Excellence in Mentorship award, an Award that has been named for him in perpetuity. After moving to the pharmaceutical industry in 1984 to lead research and development in bone biology and osteoporosis at Merck, Gideon fulfilled one of his obligations to that position many times over by selecting and then developing alendronate as a treatment for osteoporosis. This achievement set the bar for all future drug development programs in osteoporosis. Most remarkably at Merck, however, Gideon retained and developed even further the rigorous academic approach to bone biology that had always characterized him, wherever he was. In his never-ending quest to teach, to train, and to learn, Gideon was helped enormously by his wife, Sevgi, also his lifelong co-worker.

We all owe much to the innovative thought that Gideon brought to all levels of bone and mineral research. His great contributions directed our thinking and our concepts for an entire generation that has followed him. Gideon would have contributed as much to this third Edition as he did to the first two Editions. It is with the greatest admiration and respect that we dedicate this Third Edition of *Principles of Bone Biology* to his memory.

T. J. Martin, L. G. Raisz and J. P. Bilezikian.

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Preface to the Third Edition

The two previous editions of *Principles of Bone Biology* have been well received. They have not only provided a resource for investigators already working in the field, but have helped new investigators rapidly “get up to speed” in developing their projects and grant proposals. The rapid progress in our field in the last 6 years mandates that we update this text, so that it can continue to serve as a basic resource.

In this third edition, most of the chapters have been prepared by authors of the previous edition but the chapters have been extensively revised and updated. In addition a number of new authors have gracefully consented to join us. This has involved consolidation, reconfiguration, and reorganizing the information being presented. The two-volume format has been retained along with approximately the same text length. The loss of Gideon Rodan has been deeply felt by all of us and these volumes are dedicated to him. In the spirit that Gideon would have applauded, we are delighted that his close colleague and friend Jack Martin has joined us as Editors to continue this work to which Gideon contributed so much. Finally we would like to acknowledge the help of the staff at Elsevier-Academic Press who have worked valiantly to maintain schedules and have enabled us to complete this third edition. We trust that the book will be successful in providing a complete repository of the most current and accurate information in the field of bone biology.

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Preface to the First Edition

The world of modern science is undergoing a number of spectacular events that are redefining our understanding of ourselves. As with any revolution, we should take stock of where we have been, where we are, and where we are going. Our special world of bone biology is participating in and taking advantage of the larger global revolution in modern science. Often with shocking but delightful suddenness, we are gaining new insights into difficult issues, discovering new concepts to explain old observations, developing new approaches to perennial mysteries, and applying novel technological advances from other fields to our own. The pace with which the bone world is advancing is impressive not only to the most ardent optimists, who did not expect so much so soon, but also to the more sober minded who, only several years ago, would have brushed off the notion that progress could come with such lightening speed.

The rationale for this book is rooted in the recognition of the revolution in bone biology. We need a new repository of knowledge, bringing us both to the core and to the edge of our universe. Our goal is to provide complete, truly up-to-date, and detailed coverage of this exciting and rapidly developing field. To achieve this, we assembled experts from all over the world and asked them to focus on the current state of knowledge and the prospects for new knowledge in their area of expertise. To this end, *Principles of Bone Biology* was conceived. It is designed to be useful to students who are becoming interested in the field and to young investigators at the graduate or postgraduate level who are beginning their research careers. It is also designed for more established scientists who want to keep up with the changing nature of our field, who want to mine this lode to enrich their own research programs, or who are changing their career direction. Finally, this book is written for anyone who simply strives for greater understanding of bone biology.

This book is intended to be comprehensive but readable. Each chapter is relatively brief. The charge to each author has been to limit size while giving the reader information so complete that it can be appreciated on its own, without necessary recourse to the entire volume. Nevertheless, the book is also designed with a logic that might compel someone to read on, and on, and on!

The framework of organization is fourfold. The first 53 chapters, in a section titled "Basic Principles," cover the cells themselves: the osteoblast, the osteoclast, and the osteocyte; how they are generated; how they act and interact; what turns them on; what turns them off; and how they die. In this section, also, the biochemistry of collagenous and noncollagenous bone proteins is covered. Newer understandings of calcium, phosphorus, and magnesium metabolism and the hormones that help to control them, namely, parathyroid hormone, vitamin D metabolites, calcitonin, and related molecules, are presented. A discussion of other systemic and local regulators of bone metabolism completes this section.

The second section of this book, "Molecular Mechanisms of Metabolic Bone Diseases," is specifically devoted to basic mechanisms of a variety of important bone diseases. The intention of these 17 chapters is not to describe the diseases in clinical, diagnostic, or therapeutic terms but rather to illustrate our current understanding of underlying mechanisms. The application of the new knowledge summarized in Part I to pathophysiological, pathogenetic, and molecular mechanisms of disease has relevance to the major metabolic bone disorders such as osteoporosis, primary hyperparathyroidism, and hypercalcemia of malignancy as well as to the more uncommon disorders such as familial benign hypocalciuric hypercalcemia, pseudohypoparathyroidism, and osteopetrosis.

The third section of this book, "Pharmacological Mechanisms of Therapeutics," addresses the great advances that have been made in elucidating how old and new drugs act to improve abnormalities in bone metabolism. Some of these drugs are indeed endogenous hormones that under specified circumstances are useful therapies: estrogens, vitamin D, calcitonin, and parathyroid hormone are representative examples. Others agents such as the bisphosphonates, fluoride, and calcium are reviewed. Finally, agents with therapeutic potential but still in development such as calcimimetics, insulin-like growth factors, transforming growth factor, bone morphogenetic protein, and fibroblast growth factor are presented with a view to the future. The intent of this 12-chapter section is not to provide step-by-step "how-to" instructions for the clinical uses of these agents. Such prescribing information for established

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therapies is readily found in other texts. Rather, the underlying mechanisms by which these agents are currently believed to work is the central point of this section.

The fourth and final section of this book, "Methods in Bone Research," recognizes the revolution in investigative methodologies in our field. Those who want to know about the latest methods to clone genes, to knock genes out, to target genes, and to modify gene function by transfection and by transcriptional control will find relevant information in this section In addition, the selection and characteristics of growth conditions for osteoblastic, osteoclastic, and stem cells; animal models of bone diseases; assay methodologies for bone formation and bone resorption and surrogate bone markers; and signal transduction pathways are all covered. Finally, the basic principles of bone densitometry and bone biopsies have both investigative and clinical relevance. This 15-chapter section is intended to be a useful reference for those who need access to basic information about these new research technologies.

The task of assembling a large number of international experts who would agree to work together to complete this ambitious project was formidable. Even more daunting was the notion that we would successfully coax, cajole, and otherwise persuade authors of 97 chapters to complete their tasks within a six-month period. For a book to be timely and still fresh, such a short time leash was necessary. We are indebted to all the authors for delivering their chapters on time.

Finally, such a monumental undertaking succeeds only with the aid of others who helped conceive the idea and to implement it. In particular, we are grateful to Jasna Markovac of Academic Press, who worked tirelessly with us to bring this exciting volume to you. We also want to thank Tari Paschall of Academic Press, who, with Jasna, helped to keep us on time and on the right course. We trust our work will be useful to you whoever you are and for whatever reason you have become attracted to this book and our field. Enjoy the book. We enjoyed editing it for you.

John P. Bilezikian
Lawrence G. Raisz
Gideon A. Rodan

Part I

Basic Principles

Chapter 1

Modeling and Remodeling

The Cellular Machinery Responsible for the Gain and Loss of Bone's Material and Structural Strength

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INTRODUCTION

Propulsion against gravity requires levers. Bones are levers and must be stiff, that is, they must resist deformation. Impact loading imparts energy to bone. Because energy cannot be destroyed, it must be stored or dissipated. Thus, bone must also be flexible in order to absorb energy by changing shape; it must be able to shorten and widen in compression and lengthen and narrow in tension without cracking (Currey, 2002). Bone must also be light to allow mobility.

The elastic properties of bone allow it to absorb energy by deforming reversibly when loaded (Lanyon *et al.,* 1976; Turner *et al.,* 2006). If the load imposed exceeds bones' ability to deform elastically, plastic deformation occurs but this is irreversible; it is accompanied by a permanent change shape with accumulation of microcracks that allow energy release (Currey, 2002). The ability to develop microdamage is a defense against the alternative, namely, a complete fracture, but microcracks compromise strength as they accumulate (Burr *et al.,* 1998). If both the elastic and plastic zones of deformation are exceeded, structural failure – fracture – occurs.

Bone achieves the paradoxical properties of stiffness yet flexibility, strength yet lightness through its material composition and its structural design – the way this material is fashioned in three-dimensional space containing "nothing" – void space. Excavation of a marrow cavity during growth confers strength in tubular bones like the femur or tibia, which function mainly as levers, by shifting the mineralized cortical bone radially increasing resistance to bending (Ruff and Hayes, 1988). It also confers lightness by minimizing the mass needed to achieve this resistance to bending.

For structures like the vertebral body that must have greater flexibility than long bones, nature again takes advantage of void to achieve a different type of strength – the ability to deform (tolerate strain or change in length) without cracking. Lightness is achieved by fashioning the mineralized bone material with many voids; as a porous sponge-like structure of trabecular plates and sheets. Stiffness and the ability to tolerate large loads is sacrificed in favor of greater ability to deform – peak loads achievable are less than in tubular bones but the ability to absorb energy by changing length without cracking is greater.

Material Strength

Type 1 collagen is tough: It is distensible in tension but lacks resistance to bending so it needs to be stiffened. This is achieved by creating a composite of collagen plus mineral but more mineral is not necessarily better. Greater the mineral content produces greater the material stiffness, but the ability to deform and so absorb and store energy decreases as a result. For a given increase in the percentage mineral ash, stiffness increase fivefold but work to fracture decreases fourteen-fold (Currey, 2002) (Fig. 1, upper panel).

Nature selects the mineral concentration most suited to the particular function a given bone *usually* performs. Ossicles in the ear are over 80% mineral, a feature selected for so that they can vibrate like tuning forks without storing energy in deformation (Fig. 1, lower panel). These bones sacrifice the ability to deform in favor of stiffness to transmit sound with high fidelity. The slightest deformation and they crack but deformation is unlikely because they are protected safely in the skull. On the other hand, deer antlers are less densely mineralized to facilitate deformation so energy can be absorbed like springs during head butting in mating season. Greater energy-absorbing ability of antlers is favored over stiffness but they do not need stiffness; they are not load-bearing (Currey, 1969).

The organization of the composite of mineral and collagen is incompletely understood. Although the mineral is the material that stiffens bone, it is also the most brittle component and must be protected. Collagen fibers contain

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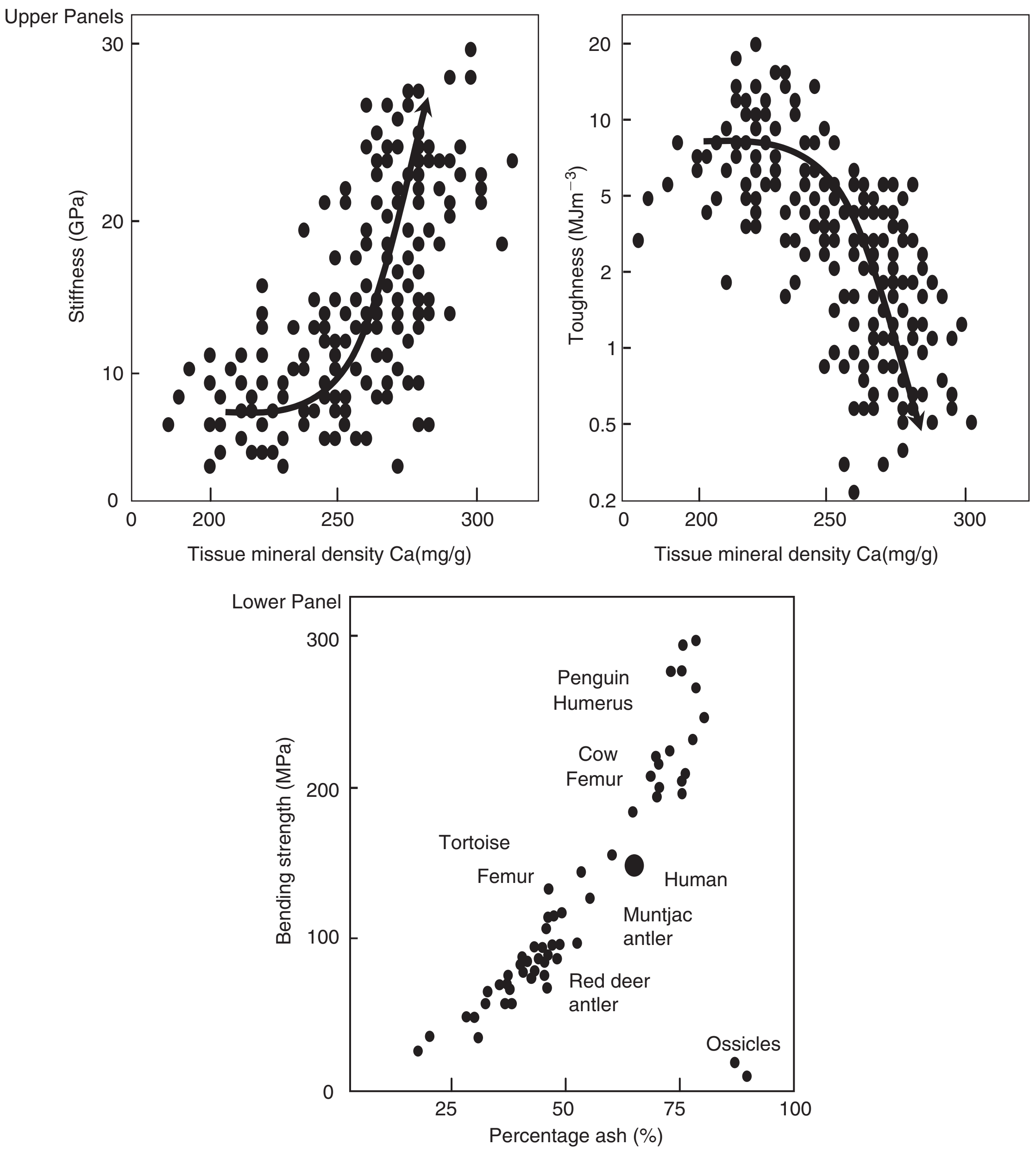

FIGURE 1 Upper panels: As tissue mineral content increases stiffness increases but toughness decreases. Adapted from Currey (2002). Lower panel: Ossicles of the ear are 90% mineral. They function as sound transducers and have little resistance to bending; they are brittle and will crack when slightly deformed. Antlers are about 40% mineral allowing them to deform without cracking. Adapted from Currey (1969).

mineral, interfibrillary matrix, and mineralized fibrils. The mineralized fibrils are composed of platelets of mineral and an intrafibrillary matrix phase of noncollagenous proteins. The brittle mineral confers stiffness and is protected during loading by energy absorption by collagen deformation and by noncollagenous proteins that dissipate energy by reversibly breaking intrahelical bonds that are "sacrificed" to provide "hidden" length (Fantner *et al.,* 2005; Gupta *et al.,* 2006) (Fig. 2). Stresses at the tissue, fiber, and mineral levels decrease in proportions of 12:5:2.

Structural Strength

During growth, the bone with its appropriate material composition is fashioned into three-dimensional masterpieces of biomechanical engineering. Although there is variability in the material composition of bone, this composition is similar in land-dwelling mammals (Keaveney *et al.,* 1998), so that most of the diversity in bone strength is the result of structural diversity that is obvious at the macroscopic level from bone to bone but the diversity in cross-sectional size,

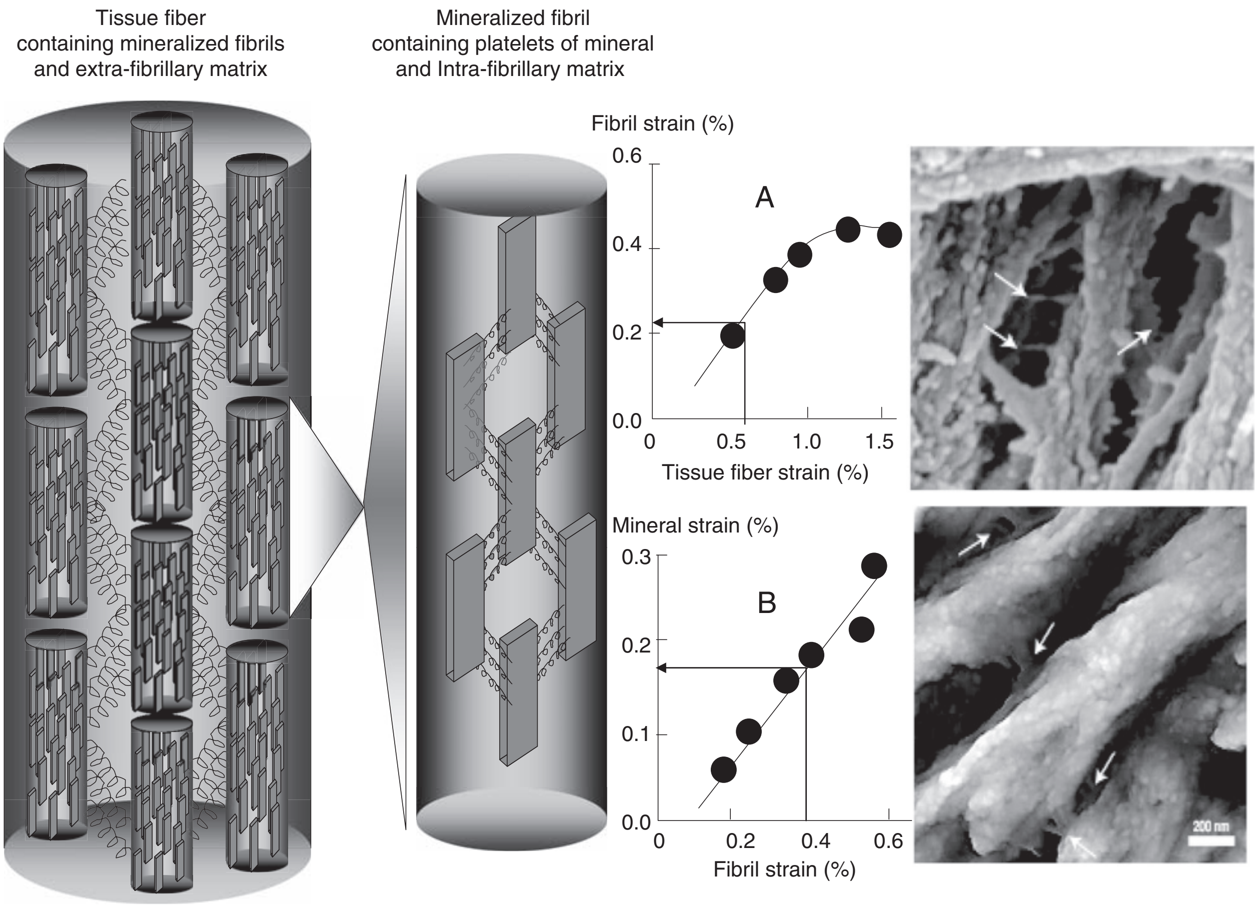

FIGURE 2 A collagen tissue fiber contains mineralized fibrils. The fibrils contain mineral platelets bound by noncollagenous proteins, helical structures that can absorb and dissipate energy during tensile strain by the breakage of sacrificial intra-helical bonds allowing uncoiling to provide "hidden length." This avoids overloading the mineral platelets. Graph A: Tissue fiber strain distributed to fibrils. Graph B: Fibrils absorb strain minimizing mineral strain. Adapted from Gupta *et al.* (2006). Images of noncollagenous glue like proteins. From Fantner *et al.* (2005).

shape, and the way its architecture is distributed in three-dimensional space from cross-section to cross-section as cortical and trabecular bone along a bone has only recently been given attention (Zebaze *et al.*, 2005; Zebaze *et al.*, 2007).

Structural diversity is largely due to individual differences in genetic makeup rather than individual differences in life style (Pocock *et al.*, 1987; Christian *et al.*, 1989). Fetal limb buds removed *in utero* and grown *in vitro* develop the shape of the proximal femur implying that bone shape is "imprinted" in the genetic material (Murray and Huxley, 1925). Studies in families and twins support this view (Seeman *et al.*, 1989; Seeman *et al.*, 1996). Although the many genes responsible for the diversity in bone's structural strength, and the contribution of environmental factors to this diversity, are largely undefined, the final pathway mediating genetic and environmental influences on structural diversity is the cellular machinery of bone modeling and remodeling (Parfitt, 1989).

BONE MODELING AND REMODELING DURING GROWTH AND THE ATTAINMENT OF PEAK STRENGTH

Bone *modeling* (construction) is the process by which bone is formed by osteoblasts without prior bone resorption. This process is vigorous during growth and produces changes in bone size and shape. Bone *remodeling* (reconstruction) occurs throughout life. Bone is first resorbed by osteoclasts and then formed in the same location by osteoblasts. These cells form the basic metabolic unit (BMU) that reconstructs bone in distinct locations on the three (endocortical, intracortical, and trabecular) components of its inner (endosteal) envelope and to a much lesser extent on the outer (periosteal) envelope (Orwoll *et al.*, 2003; Blizoites *et al.*, 2006).

Bone modeling and remodeling achieve strength for loading and lightness for mobility in two ways: by strategically depositing bone in locations where it is needed to modify bone size and shape, and by removing bone from where it is not needed to avoid bulk. The enormous capacity of this cellular machinery to modify structure during growth is seen in the morphological differences between the playing and nonplaying arm of tennis players. Modeling and remodeling modifies bone size, shape, and mass distribution of the humerus of the playing arm without changing its mass (Haapasalo *et al.,* 2000; Bass *et al.*, 2002; Seeman, 2002). However, this ability to adapt structure to its loading circumstances after the completion of longitudinal growth is limited because periosteal apposition decreases precipitously and the age-related changes in remodeling occur that produce structural decay (see Section III).

The Purpose of Modeling and Remodeling During Growth – Optimizing Strength and Minimizing Mass

If bone had only to be strong it could achieve this with bulk – more mass, but mass takes time to grow, is costly to maintain and limits mobility. Bone also must serve a second need – lightness to facilitate mobility. Longer tubular bones need more mass to construct their length than shorter bones do, but wider and narrower cross-sections do not necessarily differ in the absolute amount of material needed to construct them (Zebaze *et al.,* 2007).

Although it seems obvious that the total cross-sectional area (cortical area plus marrow area) of a wider femoral neck or femoral shaft must be assembled with more mass, this is not the case. The total cross-sectional area of a tubular bone and its bone mass are independent; wider and narrower bone cross-sections are assembled using a similar amount of material (Fig. 3). Thus, larger cross-sections are assembled with less material relative to their size producing a lower apparent volumetric bone mineral density (vBMD) and so avoiding bulk. Smaller cross-sections are assembled with more material relative to their size, producing a higher vBMD while avoiding the fragility of slenderness.

Bulk is avoided in larger cross-sections by greater endocortical resorption, which excavates a correspondingly larger marrow cavity so that the endocortical envelope approximates the periosteal envelope; wider tubular bones are assembled with a relatively thinner cortex (producing the same cortical bone area because the thinner "ribbon" of cortex is distributed around a larger perimeter). By analogy, constancy of mass achieves a wider or narrower cylinder by rolling a sheet of paper with fewer or more rolls of the sheet.

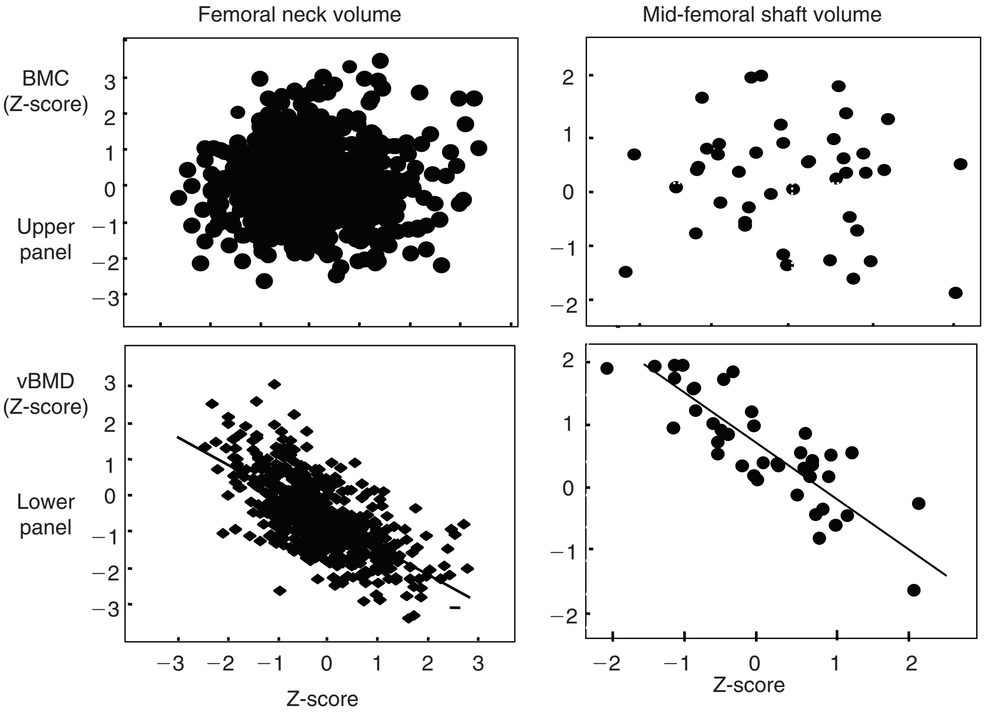

FIGURE 3 Upper panels: There is no association between the bone mineral content (BMC) Z-score and the volume of a femoral neck (FN) or femoral midfemoral shaft (FS) cross-sectional slice (including marrow volume). Lower panels: Larger cross-sections are assembled with relatively less mass and so have a lower volumetric apparent bone mineral density (vBMD). ("Apparent" refers to the vBMD of the whole cross-section, bone plus marrow areas.) Adapted from Zebaze *et al.* (2007).

Diversity in Bone Size, Shape, and the Spatial Distribution of its Mass

Long bones are not drinking straws with the same dimensions throughout their length; long bones do not have a single cross-sectional diameter, the same cortical thickness or marrow cavity diameter. Group means obscure variance – the diversity in structure and mass distribution so critical to determining diversity in bone strength. Bone strength and lightness are also achieved by altering bone shape. Diameters of a cross-section differ at each degree around the periosteal perimeter creating differences in the external shape of the cross-section. Differences in the medullary diameters at corresponding points around the endocortical perimeter determine the shape of the marrow cavity and the proximity of these two envelopes, which in turn then determine cortical thicknesses around the perimeter of the cross-section and the distance the cortical mass is placed from the neutral axis (Zebaze *et al.,* 2007).

This diversity in bone size, shape, and mass distribution is the result of differing degrees of focal bone formation at each point around the periosteal perimeter and resorption at the corresponding point on the endocortical surface during growth. Bone strength is optimized, not by using a greater net amount of mass, but by strategically modifying bone size, shape, and the distribution of mass using the minimum net amount of bone needed to do so.

For example, total cross-sectional area of the femoral neck is greatest adjacent to the shaft of the femur and smaller nearer the femoral head but the amount of bone in each cross-section is no different (Fig. 4). What differs is the way this bone is distributed in space as cortical and trabecular bone. Adjacent to the femoral shaft, the femoral neck cross-section is elliptical with long axis in the supero-inferior direction. The marrow cavity shape follows the external shape, but not identically; the greater periosteal apposition superiorly and inferiorly relative to mediolaterally produces the elliptical shape. Differences in periosteal

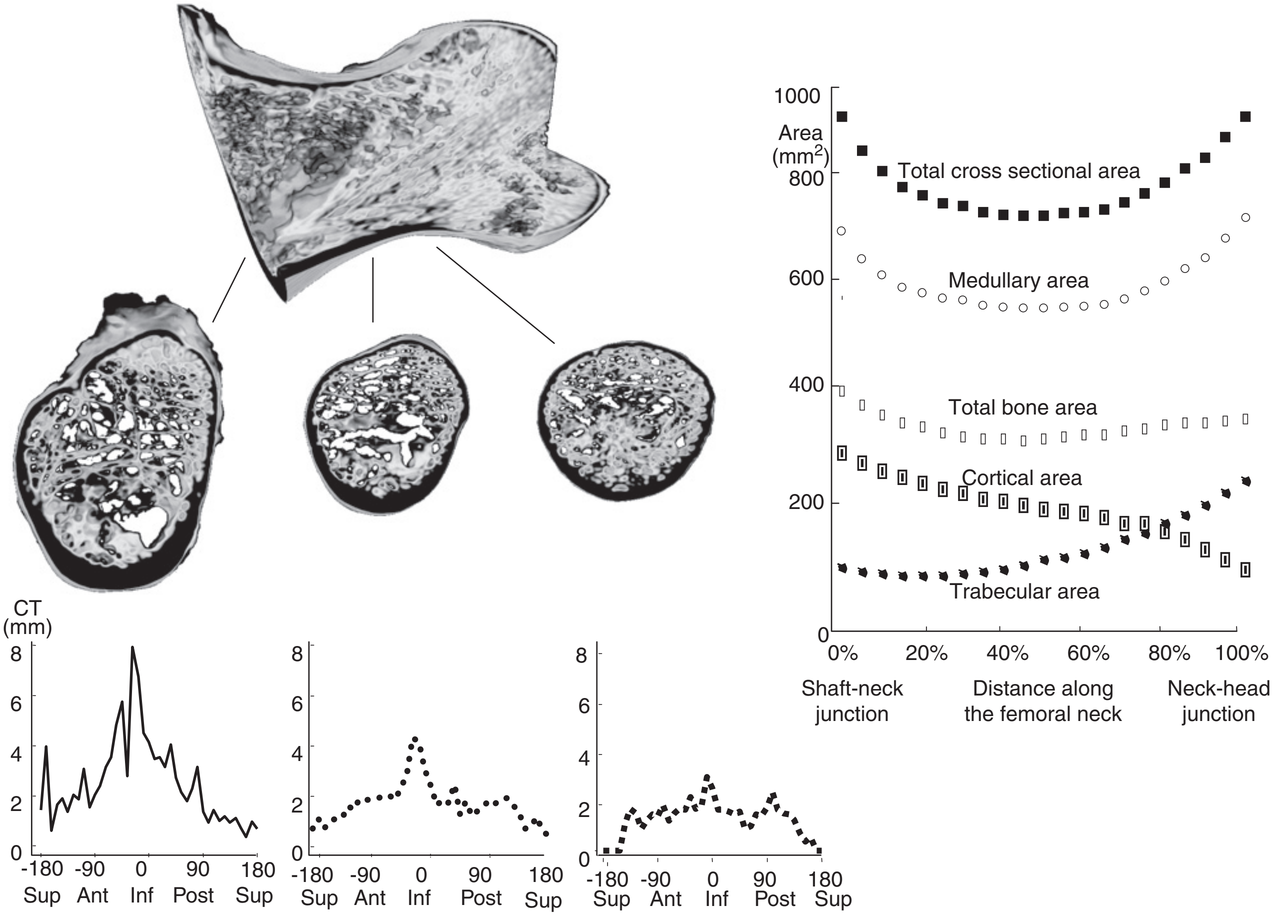

FIGURE 4 Femoral neck (FN) size and shape varies along its length. A similar amount of bone material is used to assemble each cross-section despite each cross-section varying in total cross-sectional area (CSA), shape, and proportions of cortical and trabecular bone. Adjacent to the femoral shaft the FN shape is elliptical and the bone is mainly cortical with varying cortical thickness (CT) at each point around the perimeter. At the mid-FN and adjacent to the femoral head the shape is more circular, there is more trabecular bone, reciprocally less cortical bone, which is similar in thickness around the perimeters. Adapted from Zebaze *et al.* (2007).

apposition and endocortical resorption produce a thicker cortex inferiorly and a thinner cortex superiorly (Zebaze *et al.*, 2007).

The bone in the cross-section at the junction of the femoral neck with the femoral shaft is largely cortical. Moving proximally, femoral neck shape becomes more circular reflecting similar degrees of periosteal apposition around the perimeter and the bone mass is distributed progressively more as trabecular and less cortical bone while cortical thickness is similar around the perimeter (as can be seen by the similar distribution profile in the lower part of Fig. 4).

The relative contributions of genetic factors and loading circumstances to this diverse structural organization is uncertain but modeling, by deposition bone, and remodeling by removing bone, assemble very different structures along the length of the femoral neck to accommodate differing loading patterns using similar net amounts of material.

This principle of optimizing strength and minimizing mass is illustrated in a prospective study of growth of a tibial cross-section assessed using quantitative computed tomography (Wang *et al.*, 2005; Wang *et al.*, 2007). In prepubertal girls, tibial cross-sectional shape was already elliptical at age 10. During two years, focal periosteal apposition increased the ellipticity by adding twice the amount of bone anteriorly and posteriorly than added medially and laterally. Consequently, estimates of bending strength increased more in the anteroposterior (Imax) than mediolateral direction (Imin) (Fig. 5). Marrow area changed little so more mass was distributed as a thicker cortex anteroposteriorly due to periosteal apposition without concurrent endocortical resorption. Resistance to bending increased by 44% along the principal axis (Imax) with a 22% increase in mass. If cortical thickness increased by the same amount of periosteal apposition at each point around the tibial perimeter, the amount of bone producing the same increase in bending resistance would be 205 mg, fourfold more than observed.

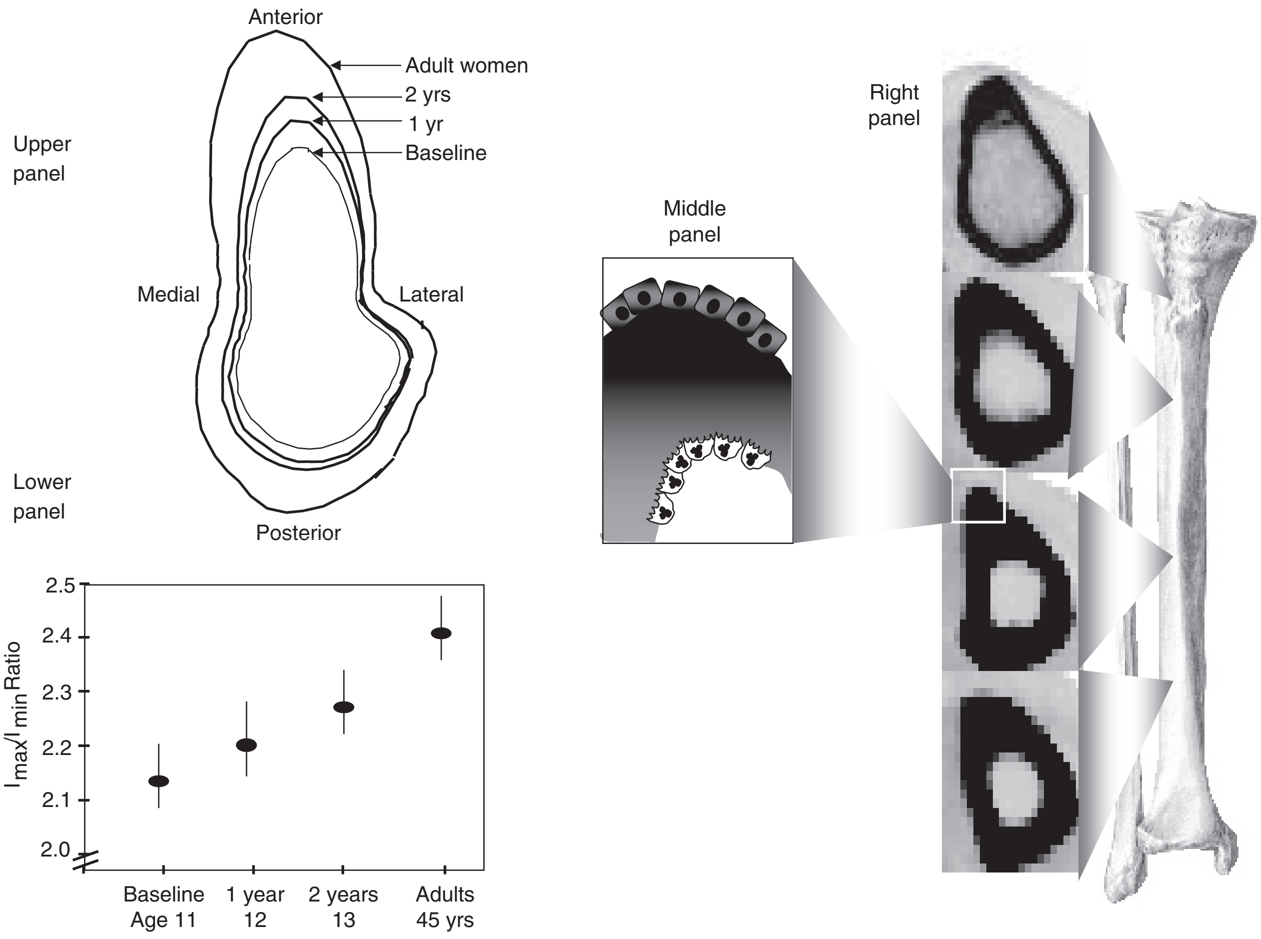

FIGURE 5 Left upper and lower panel: Bone mass distribution around the center of the tibial cross-section. More bone is deposited anteriorly and posteriorly than medially and laterally during two years of growth, increasing the ellipticity of the cross-section. Bending resistance increases more along the anteroposterior axis (Imax) than mediolateral axis (Imin) as reflected in the increasing ratio. Middle and right panel: Diagram depicting periosteal apposition and endocortical resorption modifying cortical thickness. Shape of the tibial cross-sections varies along the length of the tibial shaft. Adapted from Wang *et al.* (2007).

It is also intuitive that a bone with a larger cross-sectional area must be constructed with more periosteal bone than a smaller cross-section. The contrary was observed. During two years, the absolute amount of bone deposited on the periosteal surface of the tibial cross-section was similar in children with baseline tibial total cross-sectional area in the upper, middle, and lower tertile at age 10. Thus, larger cross-sections were assembled with less mass *relative* to their starting cross-sectional size avoiding bulk, and smaller cross-sections were assembled with more mass *relative* to their starting total cross-sectional size offsetting the fragility associated with slenderness.

Deposition of similar amounts of bone on the periosteal surface of larger and smaller cross-sections (and so less in relative terms on the former and more on the latter) was possible because the differences in bone size were established early, probably in utero (see later discussion). Consequently, the deposition of the same amount of bone on the periosteal surface of an already larger cross-section confers more bending resistance than deposition of the same amount of bone on a smaller cross-section because resistance to bending is proportional to the fourth power of the distance from the neutral axis (Ruff and Hayes, 1988).

This ability of bone to increase its strength in response to loading by adapting its design rather than increasing its mass is convincingly documented in racket sports. During growth, greater loading of the playing arm achieves greater bone strength by modifying its external size, shape, and the spatial distribution of its internal architecture. Focal periosteal apposition and endocortical resorption at some locations but endocortical bone formation at others changes the distribution of bone in space without a net change in its mass to accommodate loading patterns so that vBMD does not change; bending strength increases without increasing bulk, the latter hardly conducive to a good forehand volley (Haapsalo *et al.,* 2000; Bass *et al.,* 2002).

Trait Variances in Adulthood Originate Before Puberty

Although adults have larger skeletons than children, differences in bone size and mass in adult life probably have their origins established early in life. In a 3-year prospective study of growth in 40 boys and girls, Loro *et al.* report that the variance at Tanner stage 2 (prepuberty) in vertebral cross-sectional area and volumetric trabecular BMD, femoral shaft cross-sectional area (CSA) and cortical area was no less than at Tanner stage 5 (maturity); 60–90% of the variance at maturity was accounted for by the variance present before puberty. Thus, the magnitude of trait variances (dispersion around the age-specific mean) is largely established before puberty (Loro *et al.,* 2000).

The ranking of individual values at Tanner stage 2 was unchanged during 3 years in girls (Fig. 6). These traits tracked so that an individual with a large vertebral or femoral shaft cross-section, or higher vertebral vBMD or femoral cortical area before puberty retained this position at maturity. The regression lines for each of the quartiles did not cross during three years. Similar observations were made in boys (not shown).

Similar observations have been reported using peripheral computed tomography of the tibia in 258 girls. The magnitude of variance at ages 10–13 did not differ from that two years later, and did not differ from that of their premenopausal mothers (Wang *et al.,* 2007). Likewise, Garn *et al.* monitored 744 women and men during 25 years. About 90% of the variance in cortical thickness in adulthood was accounted for by variance at completion of growth 25 years earlier (Garn *et al.,* 1992). Emaus *et al.* reported distal and ultra distal radius size and mass tracked during 6.5 years follow-up of 5,366 women and men ages 45–84 (Emaus *et al.,* 2005; Emaus *et al.,* 2006).

Finding that the magnitude of the trait variances at maturity is no different from the magnitude of their variances before puberty suggests that growth in larger and smaller bones occurs at the same rate (Wang *et al.,* 2007). (If larger bones deposit more bone during growth than smaller bones, variance will increase.) In addition, the constant variance and tracking also suggests that environmental factors are likely to contribute little to total variance of a trait in the population.

If variance is established before puberty, when is growth more rapid in some individuals than others to give rise to these large variances in bone size and mass (1 SD = ~10% of the mean)? Do bones from individual to individual begin by being the same size then some grow more rapidly (deposit more bone per unit time than others) to form the upper tertile of a trait while others grow more slowly forming the middle and lower tertile?

The answer to this question is unknown. In infants and children between ages 1 and 10, the variance in diaphyseal diameter and muscle diameter was established at 1–2 years of age (Maresh, 1961). In a cross-sectional study of 146 stillborn fetuses ages 20–41 weeks' gestation, the percentage of a femur, tibia, and humerus diaphyseal cross-section that was cortical area was about 80–90% at 20 weeks' gestation and remained so across the 20 weeks of intrauterine life, suggesting that as bone size increased during advancing intrauterine life, the proportion of bone within the cross-section remained constant and was established prior 20 weeks' gestation (Rodriguez *et al.,* 1992). By contrast, one cross-sectional study using three-dimensional ultrasound suggested variance in femoral volume doubled during intrauterine growth (Chang *et al.,* 2007).

The divergence of data points of graphic analyses of growth creating the impression of increasing variance may be more apparent than real because larger numbers differ by larger absolute amounts. Further studies are needed to define the magnitude to trait variances by sex and race and to define the genetic and environmental components of that variance.

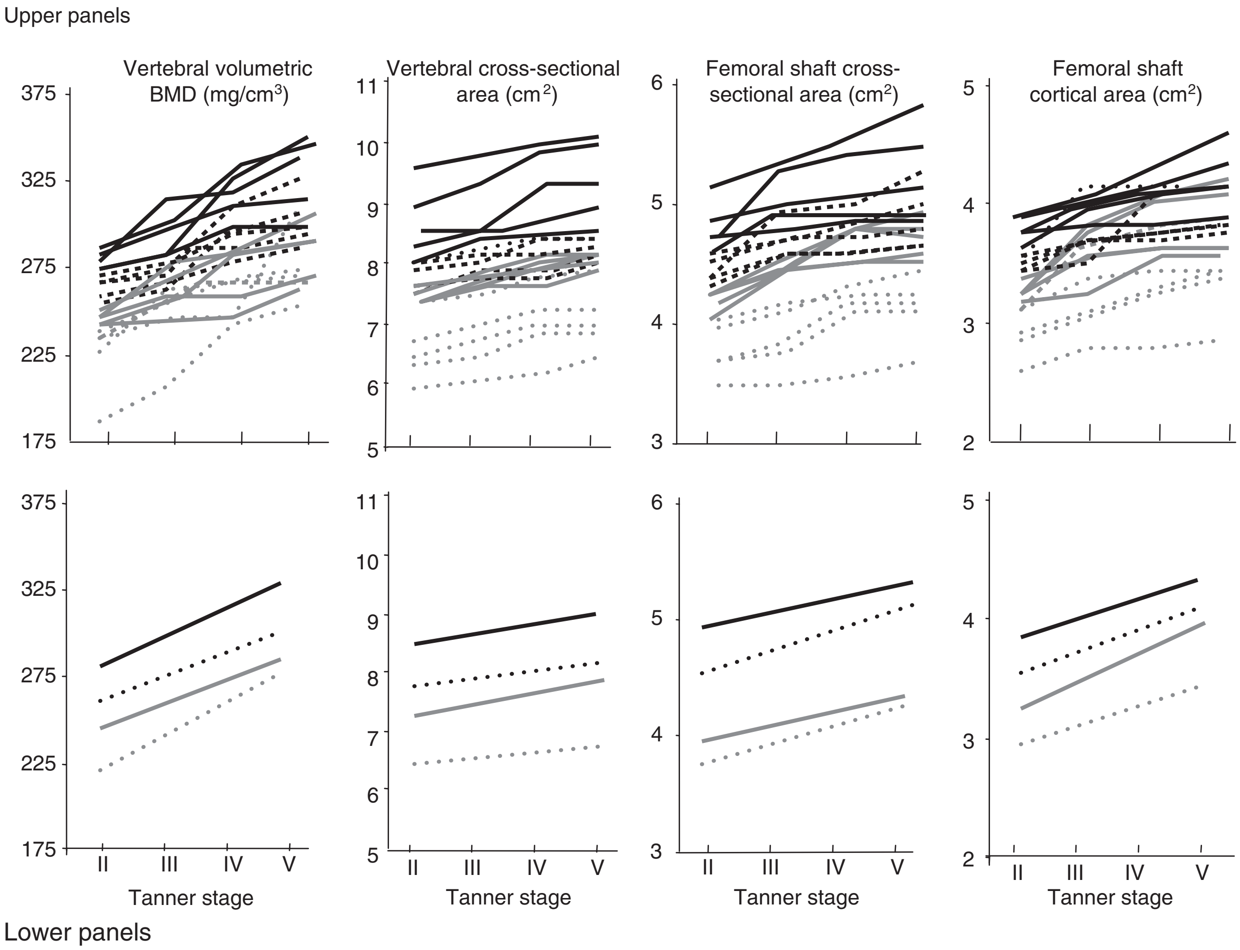

FIGURE 6 Upper panels: Variances in vertebral volumetric bone mineral density (vBMD) and cross-sectional area, femoral shaft total cross-sectional area and cortical area are established before puberty in girls. Individual values track retaining their percentile of origin during 3 years. Lower panels: The regression lines for each quartile do not overlap during 3 years. Adapted from Loro *et al.* (2000).

The obvious inference from the early establishment and constancy of trait variances is that genetic rather than environmental factors account for this variance. Studies in family members, twins, birth cohorts followed for many decades, and studies of fetal limb buds grown *in vitro* support this view (Murray and Huxley, 1925; Pocock *et al.,* 1987; Seeman *et al.,* 1996). However, this does not mean that traits *in an individual* are immutably fixed.

This flawed notion confuses variance in a population and the effect of environmental or disease on a trait in an individual. Muscle paralysis in utero, exercise during growth, or effects of disease in adulthood all have profound effects on bone structure in individuals (Bass *et al.,* 2002; Pitsillides, 2006). Lifestyle change can influence the population mean of a trait as documented many times by secular increases in height, a highly heritable trait (Bakwin, 1964; Meredith, 1978; Cameron *et al.,* 1982; Tanner *et al.,* 1982; Malina and Brown, 1987). However, under stable conditions, lifestyle differences within a population make only a small contribution to trait variances compared with genetic differences in that population.

Sex and Racial Differences in Axial and Appendicular Structure

For the vertebrae, increasing bone size by periosteal apposition builds a wider vertebral body in males than in females and in some races than in others (Seeman, 1998). Trabecular number per unit area is constant during growth. Therefore, individuals with a low trabecular number in young adulthood are likely to have had lower trabecular numbers in childhood (Parfitt *et al.,* 2000). The age-related increase in trabecular density is the result of increased thickness of existing trabeculae. Before puberty there is no difference in trabecular density in boys and girls of either Caucasian or African American origin (Gilsanz *et al.,* 1988; Gilsanz *et al.,* 1991). This suggests that both vertebral body size and the mass within its periosteal envelope increase in proportion until Tanner stage 3 (Fig. 7).

At puberty, trabecular density increases by race and sex, but within a race there is no sex difference in trabecular density. This increase is probably the result of cessation of external growth in bone size but continued bone formation

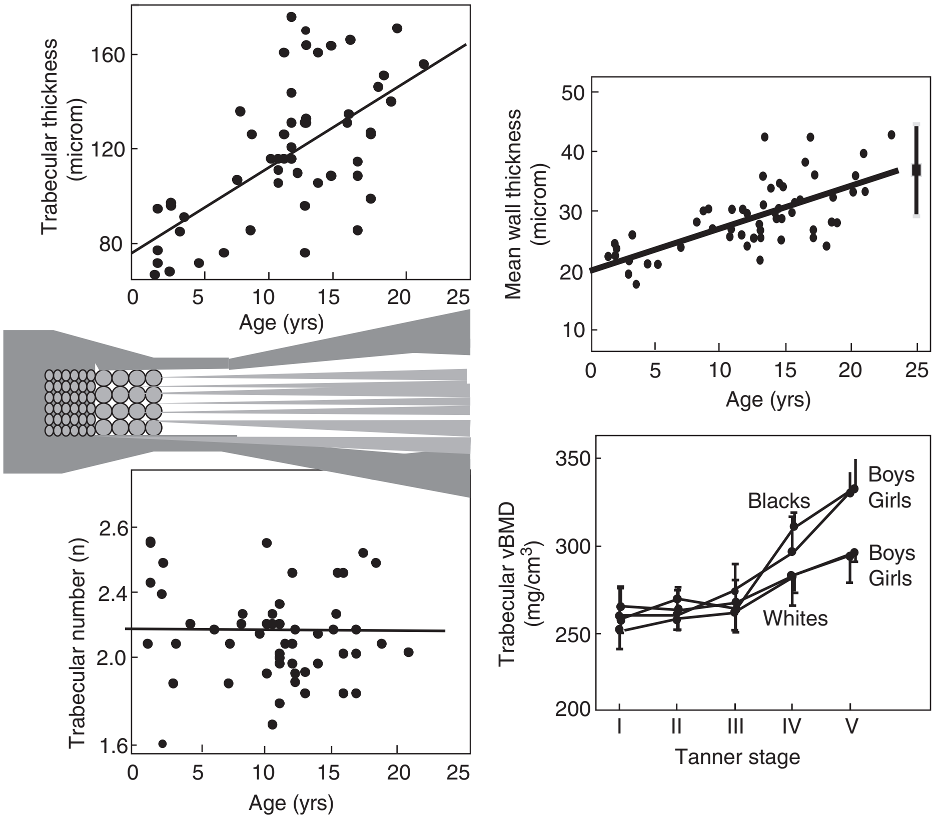

FIGURE 7 Trabecular volumetric bone mineral density (vBMD) increases with advancing age due an increase in trabecular thickness not numbers. This occurs by an increase in bone formation reflected in the increase in mean wall thickness. Adapted from Parfitt *et al.* (2000). Before puberty, trabecular vBMD is no different by sex or race, increases at Tanner stage 3 similarly by sex within a race but more greatly in African Americans than Caucasians. Adapted from Gilsanz *et al*. (1991).

on trabecular and endocortical envelopes resulting in more bone within the periosteal envelope of the bone – higher vBMD. Thus, growth does not build a "denser" vertebral body in males than females; it builds a bigger vertebral body in males. Strength of the vertebral body is greater in young males than females because of size differences. Within a sex, African Americans have a higher trabecular density than whites due to a greater increase in trabecular thickness (Han *et al.,* 1996). The mechanisms responsible for the racial dimorphism in trabecular density but resemblance in males and females within a race are yet to be defined. The greater trabecular thickness in African Americans accounts for the lower remodeling rate in adulthood because there is less surface available for remodeling (Han *et al.,* 1996).

Sex differences in appendicular growth are partly the result of differences in timing of puberty (Fig. 8). Before puberty there are already sex differences in diaphyseal diameter (Iuliano *et al.,* 2008). As long bones increase in length by endochondral apposition, periosteal apposition widens the lengthening long bone. Concurrent endocortical resorption excavates the marrow cavity but as periosteal apposition is greater than endocortical resorption, the cortex thickens. In females, earlier completion of longitudinal growth with epiphyseal fusion and earlier inhibition of periosteal apposition produces a smaller bone.

Bone length continues to increase in males and periosteal apposition increases cortical thickness. However, cortical thickness is similar in males and females because endocortical apposition in females contributes to final cortical thickness (Garn, 1970; Bass *et al.,* 1999). Cortical thickness is similar by race and sex. What differs is the position of the cortex in relationship to the long axis of the long bone (Wang *et al.,* 2005; Duan *et al.,* 2005). It is not clear whether the wider diaphysis in males than females is the result of accelerated periosteal apposition in males as commonly believed, or is the result of continued longitudinal growth in males as they enter puberty one to two years after females (Garn, 1970).

In summary, the cellular machinery of bone modeling and remodeling adapt bone size, shape, and mass distribution to its loading circumstances throughout the whole of growth ensuring that strength is optimized by depositing bone where it is needed and mass is minimized by removing bone from where it is not. The magnitude of the trait variances in adulthood are largely expressed in childhood. Traits track in their percentile of origin established at some time before puberty, if not in utero. Thus, differences in bone size and mass from individual to individual

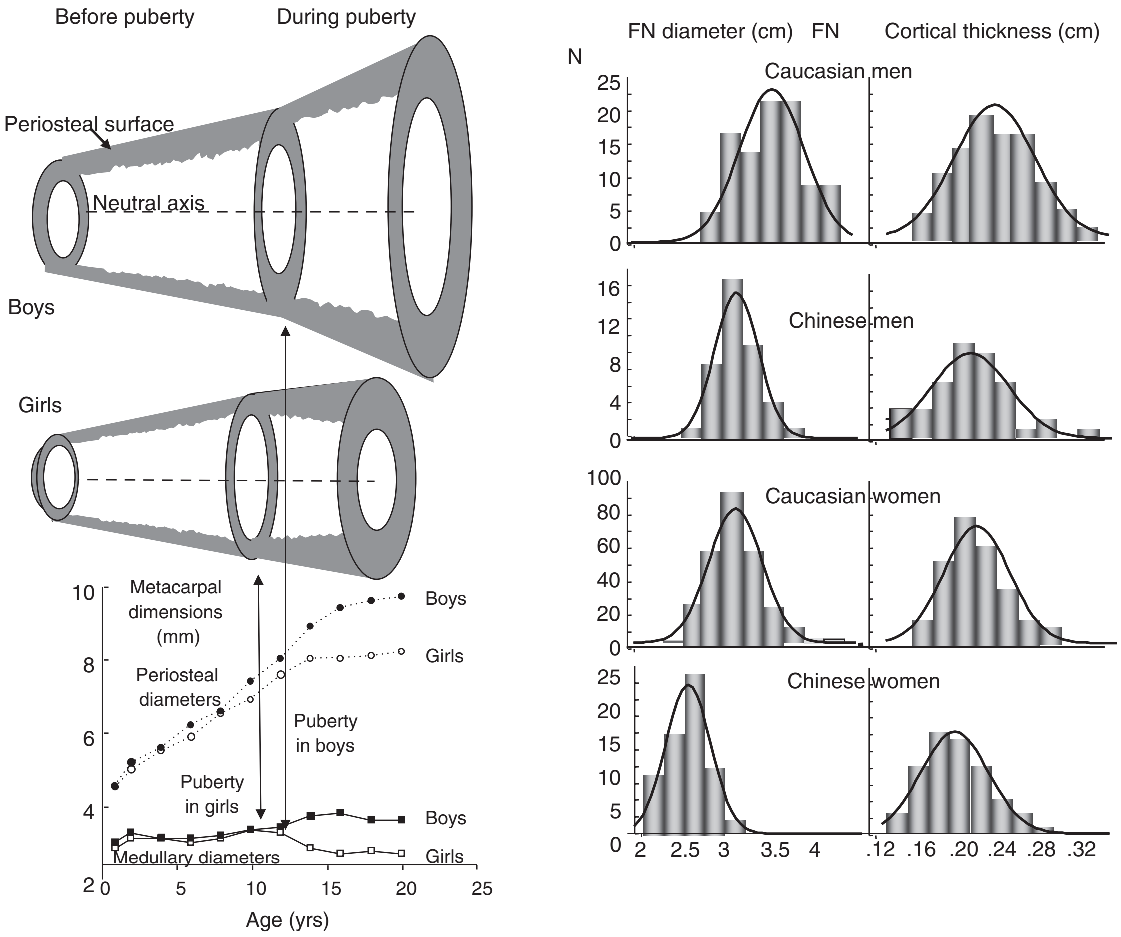

FIGURE 8 Long bone diameter is already greater in males than females before puberty. Growth in length and width continues longer in males because puberty occurs later. Endocortical apposition in females contributes to cortical thickness so that final cortical thickness is similar in males and females but displaced further radially in males. Adapted from Garn (1970). Distribution of femoral neck (FN) diameter differs by sex and race but femoral neck cortical thickness is similar by sex and race. E. Seeman with permission.

in adulthood are likely to be already evident early in life. This is obvious for cross-sectional size because periosteal apposition is minimal after completion of longitudinal growth. It is less obvious that the amount of bone within the periosteal envelope in adulthood is also largely established during growth (Zebaze *et al.,* 2007).

Variance in bone mass at completion of growth is an order of magnitude greater than variance in rates of bone loss during aging (1 SD = 10% versus 1%, respectively) (Parfitt, 1996). Thus, bone size, architecture, and mass attained during growth is likely to play an important role in determining the relevance of bone loss during advancing age (Hui *et al.,* 1999; Seeman *et al.,* 2001). For example, in children with larger tibial cross-sections, the advantage of assembling the larger bone with a relatively thinner cortex (to avoid bulk) may be a disadvantage when age-related bone loss occurs. Women with hip fractures and their daughters have larger femoral neck diameters and reduced vBMD (Filardi *et al.,* 2004). In smaller bones, the fragility of slenderness is offset by constructing them with more mass relative to their size as less endocortical resorption excavates a smaller marrow cavity, leaving a relatively thicker cortex. This balance may be compromised as bone loss produces cortical thinning and intracortical porosity which reduce compressive strength and resistance to bending.

BONE MODELING AND REMODELING IN ADULTHOOD AND THE EMERGENCE OF BONE FRAGILITY

The Purpose of Modeling and Remodeling in Adulthood – Maintenance of Bone Strength

The purpose of modeling and remodeling during growth is to achieve the skeleton's peak strength. The purpose of bone remodeling during adulthood is to maintain bone strength by removing damaged bone. *Bone, like roads, buildings, and bridges, develops fatigue damage during repeated loading but only bone has a mechanism enabling it to detect the location and magnitude of the damage, to remove it, replace it with new bone, and thus to restore bone's material composition, micro- and macroarchitecture (Parfitt, 1996; Parfitt, 2002).

Bone resorption is not bad for bone unless it becomes excessive and untargeted. On the contrary, the resorptive phase of the remodeling cycle removes damaged bone and is essential to bone health. Indeed, prolonged suppression of remodeling using potent anti-resorptive therapy may result in microdamage accumulation, fractures, and reduced bone healing (Mashiba *et al.,* 2000; Odvina *et al.,* 2005). The formation phase of the remodeling cycle restores bone's structure provided that the volume of damaged bone removed is replaced by the same volume of normal bone. This process depends on the normal production, work, and life span of osteoclasts and osteoblasts, but the BMU is a *multicellular* unit and many cell types participate in the remodeling cascade.

The Pivotal Role of Osteocyte Death in Bone Remodeling

The osteocyte is one of these cells and is likely to play a pivotal role in bone modeling and remodeling. Osteocytes are the most numerous, longest-lived, and least studied cells of bone. There are about 10,000 cells per cubic millimeter and 50 processes per cell (Marotti *et al.,* 1990). These processes connect osteocytes with each other and with flattened lining cells on the endosteal surface. Thus, bone with its haversian and Volkmann canals and its lacunar-canalicular system is no less intricate in design than the hepatobiliary, bronchoalveolar, or glomerulotubular communication systems (Fig. 9, Panel 1). The dense lace-like network of osteocytes with their processes ensures that no part of bone is more than several microns from a lacuna containing its osteocyte suggesting that these cells are part of the machinery guarding the integrity of the composition and structure of bone (Parfitt, 2002).

Microcracks sever osteocyte processes in their canaliculi, producing osteocyte apoptosis (Hazenberg *et al.,* 2006) (Fig. 9, Panel 2). Apoptotic osteocytes may also be a form of damage, perhaps reducing the energy absorbing/dissipating capacity of bone when lacunae mineralize. Estrogen deficiency and corticosteroid therapy result in apoptosis (Manolagas, 2006). The increased remodeling rate in midlife in women may be partly the result of osteocyte death. Alternatively, or in addition, osteocyte apoptosis can produce damage to surrounding mineralized matrix producing bone fragility (independent of bone loss). Corticosteroid-treated mice have large osteocyte lacunae surrounded by matrix with a 40% reduction in mineral and reduced elastic modulus (Lane *et al.,* 2006). Genetic ablation of osteocytes produces bone fragility and failed mechanotransduction (Tatsumi *et al.,* 2007). Prevention of osteocyte death may be an attractive therapeutic target if they are damage or produce damage (Keller and Kneissel, 2005; Manolagas, 2006). Fragility can be prevented using anti-apoptotic agents (O'Brien *et al.,* 2004; Manolagas, 2006).

Whether apoptotic osteocytes are a consequence of damage, are the damage itself, or produce matrix damage, the number of dead osteocytes provides the topographical information needed to identify the location and size of damage (Verborgt *et al.,* 2000; Taylor 1997; Schaffler and Majeska, 2005) (Fig. 9, Panel 3). Osteocyte apoptosis is likely to be one of the first events signaling the need for remodeling. It precedes osteoclastogenesis (Clark *et al.,* 2005). *In vivo*, osteocyte apoptosis occurs within three days of immobilization and is followed within two weeks by osteoclastogenesis (Aguirre *et al.,* 2006). *In vitro*, death of the osteocyte-like MLO-Y4 cells induced by scratching results in the formation of TRACP positive (osteoclast-like) cells along the scratching path (Kurata *et al.*, 2006).

Thus, just as the spider knows the location and size of its wriggling prey by signals sent along its vibrating web, the need for reparative remodeling is likely to be signaled by osteocyte death via their processes connected by gap junctions to flattened osteoblast lining the inner or endosteal surface of bone where remodeling takes place. The nature of the signal from the osteocyte remains unknown.

The Pivotal Role of the Bone Remodeling Canopy in Bone Remodeling

It is not yet feasible to study the life of a BMU *in vivo*, documenting its birth, daily work in resorption, and formation to its end as an ossified osteon or hemi-osteon; the "fossilized" record of that remodeling cycle. Inferences regarding the sequence of events and their molecular regulation must be made with trepidation because observations are based on histomorphometric "snapshots" and *in vitro* studies of cell systems.

Bone remodeling occurs on the endocortical, trabecular, and intracortical components of the endosteal envelope. The endocortical and trabecular surfaces are adjacent to marrow. The intracortical surface forms the wall of haversian canals. While remodeling occurs on these endosteal surfaces, damage occurs deep to them, within the matrix of osteons or the interstitial bone between osteons in the case of cortical bone or within hemi-osteons in the case of trabecular bone. So, information concerning the location and size of damage must reach these surfaces and cells involved in remodeling must reach the site of damage beneath the endosteal surface. This anatomical arrangement makes the flattened lining cells conduits transmitting the health status of the bone matrix to the bone marrow environment, which in turn is a source of the cells of the BMU, but not the only source.

Apoptotic osteocytes signal the location and size of the damage burden to the flattened lining cells of the endosteal

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Central regulation of bone remodeling and the role of remodeling in energy metabolism will not be discussed (Ducy *et al.,* 2000; Lee *et al.,* 2007).

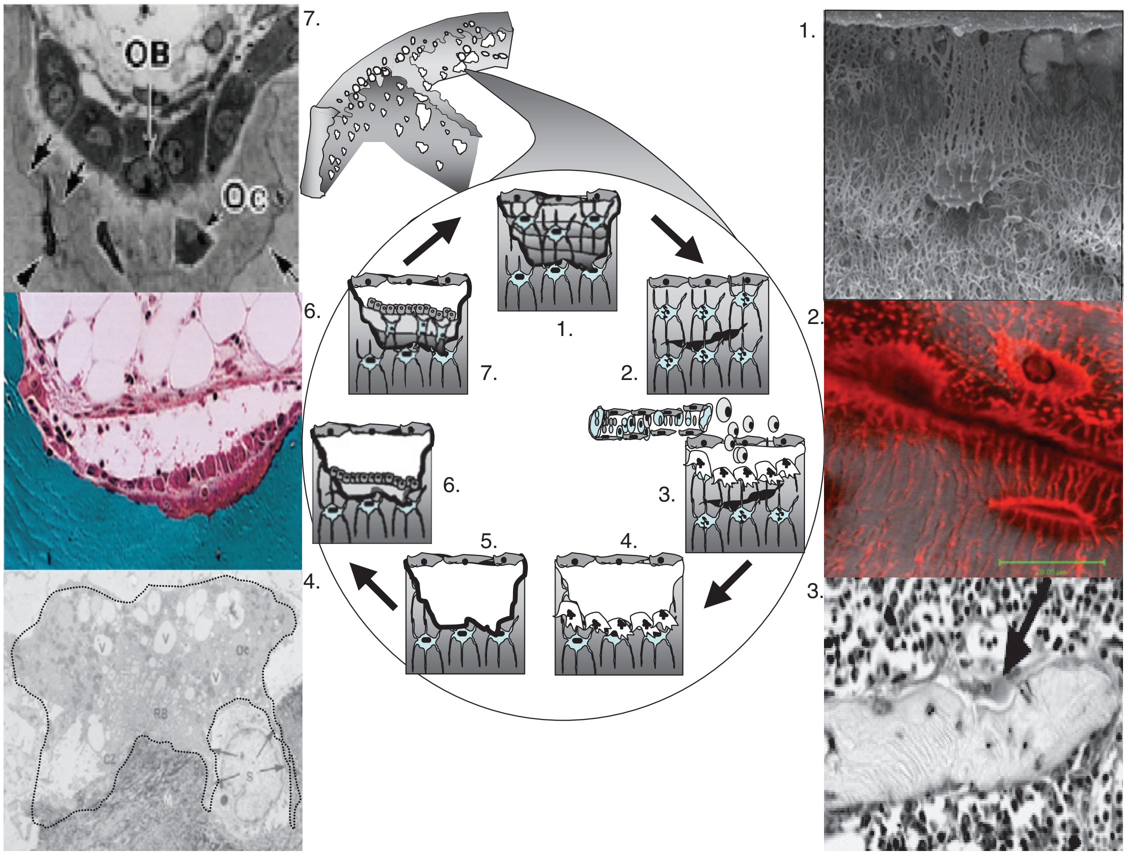

FIGURE 9 (1) Osteocytes are connected to each other and to lining cells on the endosteal surface adjacent to the marrow; (2) Damage to osteocytic processes by a microcrack produces osteocyte apoptosis. Courtesy J. Hazenberg *et al.* (2006); (3) The distribution of apoptotic osteocytes provides the topographical information needed to target osteoclasts to the damage. Courtesy M. Schaffler and R. Majeska (2005) (3) Collagenase from lining cells digests unmineralized collagen exposing mineralized bone and creates the bone remodeling cavity within which progenitors for osteoclastogenesis and osteoblastogenesis are delivered via the marrow or blood supply or locally (see text); (4) Osteoclasts (Oc) resorb bone and remove damage as well as phagocytosing osteocytes with its cytoplasmic extensions (arrows) inserted between lacunar wall and osteocyte (S). RB ruffled border, CZ clear zone, V vacuole. From Elmardi *et al.* (1990); (5) The reversal phase and formation of a cement line follows; (6) Osteoblasts deposit osteoid; and (7) Some osteoblasts are entombed in the osteoid they deposit and differentiate into osteocytes reconstructing the osteocytic canalicular network. From Suzuki *et al.* (2000). (See plate section)

surface leading to the formation of a bone remodeling compartment (BRC), which confines and targets remodeling to the damage minimizing removal of normal bone (Hauge *et al.*, 2001) (Fig. 9, cartoon and especially Panel 5). The regulatory steps between osteocyte apoptotic death and creation of the BRC are not known. Bone lining cells express collagenase mRNA (Fuller and Chambers, 1995). An early event creating the BRC may be collagenase digestion of unmineralized osteoid to expose mineralized bone, a requirement for osteoclastic bone resorption to proceed.

The flattened bone lining cells are probably osteoblasts. They express markers of the osteoblast lineage, particularly those forming the canopy over the BRC (Hauge *et al.*, 2001; Parfitt, 2001). These canopy cells also express markers for a range of growth factors and regulators of osteoclastogenesis such as RANKL suggesting that the canopy has a central role in the differentiation of precursor cells of marrow stromal origin, monocyte-macrophage origin, and vascular origins toward their respective osteoblast, osteoclast, or vascular phenotypes.

The Multidirectional Steps of the Remodeling Cycle

Although the two classical events of remodeling – resorption of a volume of bone by osteoclasts and formation of a similar volume of bone by osteoblasts occur sequentially (Hattner *et al.*, 1965), the cellular and molecular regulatory events leading to these two fully differentiated functions may not be sequential. Some may be contemporaneous and multidirectional; osteoblastogenesis and its regulators determine osteoclastogenesis and the volume of bone resorbed whereas osteoclastogenesis and the products of the resorbed matrix regulate osteoblastogenesis, while both may be regulated to some extent by osteocytes and its products (e.g., sclerostin). How this cellular and molecular traffic is orchestrated from beginning to end is far from clear.

Signaling from apoptotic osteocytes to cells in the canopy expressing the osteoblast phenotype may influence further differentiation toward osteoblast precursors expressing RANKL and fully differentiated osteoid-producing osteoblasts. So even at this stage, regulation of osteoclastogenesis and osteoblastogenesis is occurring simultaneously through osteoblast precursors. In the MLO-Y4 cell line, damaged osteocyte-like cells have been reported to secrete M-CSF and RANKL (Kurata *et al.*, 2006). Whether this occurs in human subjects *in vivo* is not known but raises the possibility that osteocytes participate in the differentiation of monocyte-macrophage precursor cells toward the osteoclast lineage. Both osteoblast and osteoclast precursors circulate and so may arrive at the BRC via the circulation and via capillaries penetrating the canopy (Eghbai-Fatourechi *et al.*, 2005; Eghbai-Fatourechi *et al.*, 2007; Fujikawa *et al.*, 1996). The contribution of precursors from the canopy, the marrow via sinusoids or capillaries is not well-defined.

Angiogenesis is essential to bone remodeling. Osteoprogenitor cells are associated with vascular structures in the marrow and several studies suggest there may be common progenitors giving rise to cells forming the blood vessel and the perivascular cells that can differentiate toward cells of multiple lineages (Doherty *et al.*, 1998; Howson *et al.*, 2005; Sacchetti *et al.*, 2007; Matsumoto *et al.*, 2006; Kholsa, 2007; Otsura *et al.*, 2007; Khosla *et al.*, 2008).

Once differentiated, teams of osteoclasts resorb a volume of damaged bone but little is known of the factors determining the volume of bone resorbed, particularly how resorption stops after the damaged region has been resorbed. Osteoclasts phagocytose osteocytes and this may be one way the signal for resorption is removed (Fig. 9, Panel 4).

Products from the osteoclasts independent of their resorption activity, and products from the resorbed matrix partly regulate osteoblastogenesis and bone formation (Suda *et al.*, 1999; Martin and Sims, 2005; Lorenzo, 2000). In addition, products from the osteocyte may contribute to regulation of bone formation. For example, sclerostin is secreted by osteocytes and perhaps other cells as well. It is a product of the ScleroSteosis (SOST) gene and inhibits bone formation. Its inhibition is permissive to bone formation. Whether osteoblast precursors are generated before resorption has occurred, either from the canopy, or by products of the osteoclast before it started matrix resorption is not known. If so, these cells form preemptive teams of cells ready to deposit bone, die, become lining cells or osteocytes depending on later signals from osteoclasts, the resorbed matrix or products of the osteocyte such as sclerostin or cell–cell contact (Zhao *et al.*, 2006).

After the reversal phase, osteoblasts deposit osteoid partly or completely filling the trench cross-section (establishing the size of the negative BMU balance in that cross-section) and forming the lamellae that then undergo primary and secondary mineralization. In a given cross-section, how the osteoblasts change polarity to produce the differently orientated collagen fibers from lamella to lamella is not known. Most osteoblasts die, others become lining cells whereas others become entombed in the osteoid they formed to become osteocytes which communicate with each other to "rewire" the osteocytic canalicular communicating system for later mechanotransduction, damage detection, and repair (Han *et al.*, 2004).

In summary, bone remodeling may not be exclusively damage-driven but if it is, the osteocyte appears to play a pivotal role in initiating this remodeling cycle and perhaps participating in the regulation of the volumes of bone ultimately resorbed and formed by the BMU. Many of the advances that have taken place raise more questions than they answer. Some very fundamental questions concern the role of remodeling in intermediary metabolism, the link between central control of remodeling and regulation of remodeling for regional structural adaptation to loading and focal damage removal.

Even the question of what is "damage" betrays many areas in need of exploration. Damage at the nano- or microstructural level has not been comprehensively categorized in morphological terms so that the causes of damage, biomechanical effects, biochemical and structural means of detecting, signaling, and repairing different types of damage remain unstudied (Akkus *et al.*, 2004; Burr *et al.*, 1998; Danova *et al.*, 2003; Diab *et al.*, 2006; Diab and Vashisha, 2005; Garnero *et al.*, 2006; Landis, 2002; Ruppel *et al.*, 2006; Silva *et al.*, 2006; Taylor, 1997).

Age-Related Changes in Modeling and Remodeling Adulthood

Although bone can accommodate loading circumstance by adaptive modeling and remodeling during growth, this capacity diminishes because four age-related changes in the cellular machinery of bone modeling and remodeling compromise bone's material properties and structural design. Bone's ability to adapt to loading is impaired because each time a remodeling event occurs there is loss of bone and some structural decay (Seeman and Delmas, 2006).

Remodeling rate is rapid during growth because each remodeling event deposits only a small moiety of bone (Parfitt, 2002). As growth nears its "programmed" completion, rapid remodeling is no longer needed and remodeling rate slows. With the completion of longitudinal growth, the only requirement for bone formation is the repair of micro- and macrodamage so there is a decline in bone formation, a mechanism proposed to be responsible for bone fragility over 65 years ago (Albright *et al.*, 1941).

Thus, the first age-related change in this machinery is a reduction in bone formation at the cellular level by each BMU (Lips *et al.*, 1978; Vedi *et al.*, 1984) (Fig. 10). The second abnormality is also a reduction in bone formation but at the tissue level – bone modeling on the periosteal envelope slows precipitously after completion of

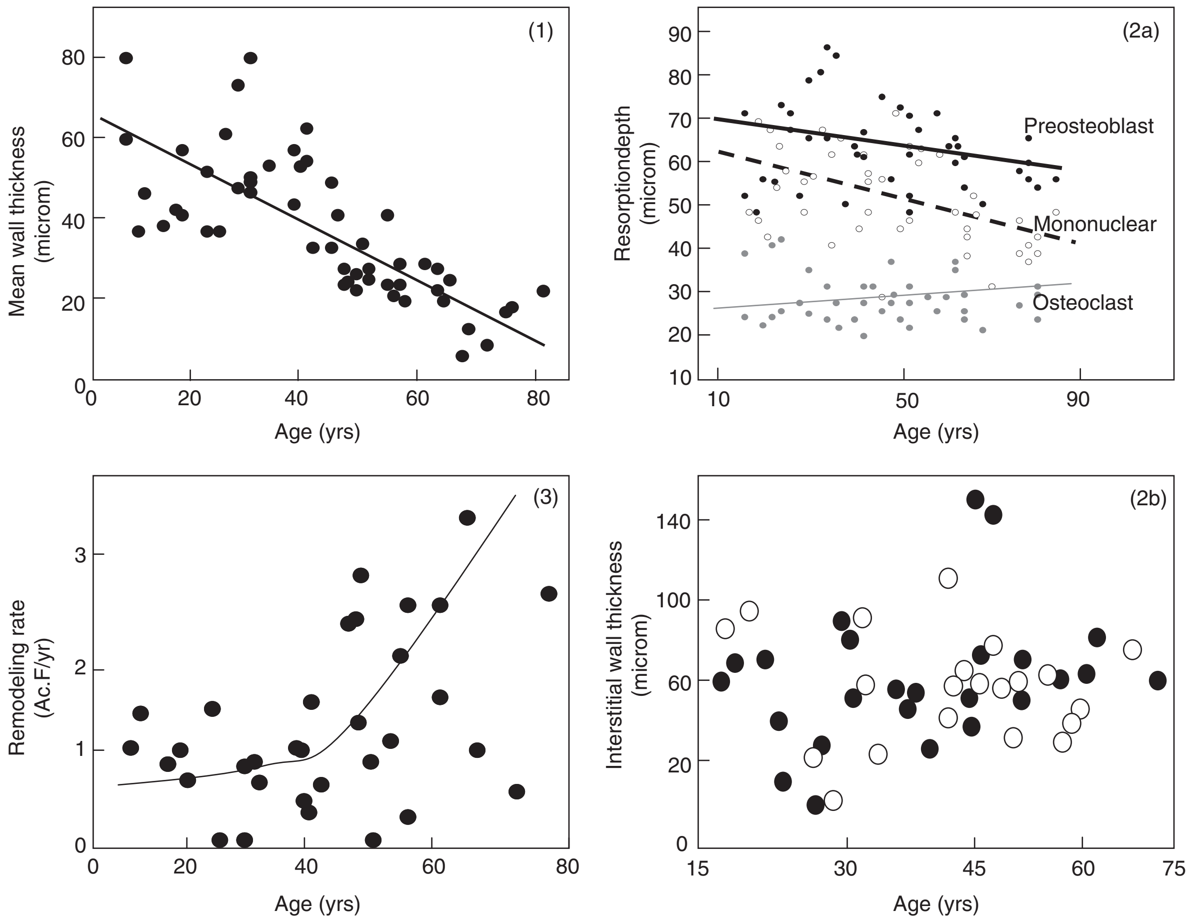

FIGURE 10 Endosteal bone loss is the result of: (1) a reduction in the volume of bone formed in each basic metabolic unit (BMU) reflected in a reduction in mean wall thickness with age. Adapted from Lips *et al.* (1978); (2) A fall or little change in the volume of bone resorbed in each BMU. This is reflected in (2a) as little change in erosion depth defined by preosteoblasts, mononuclear cells, or osteoclast surfaces (Adapted from Ericksen *et al.*, 1985) and (2b) and no change in interstitial wall thickness (females black symbols) Adapted from Vedi *et al.* (1984); and (3) Increased remodeling rate (activation frequency). Courtesy J. Compston.

longitudinal growth but continues slowly so that bone diameters enlarge, but no more than a few millimeters during the next 60 years.

The mechanisms responsible for the reduction in the volume of bone formed in each BMU are not well-defined but may include a reduction in stem cell precursors of osteoblasts, a reduction in differentiation of stem cells to the osteoblast lineage, reduced osteoid production of individual cells, and a reduction in the life span of these cells (Bonyadi *et al.*, 2003; Nishida *et al.*, 1999; Stenderup *et al.*, 2001; Oreffo *et al.*, 1998).

The third abnormality in remodeling is believed to be an increase in the volume of bone resorbed by the BMU but this may be confined to a brief period following sex hormone deficiency (Ericksen, 1986; Ericksen *et al.*, 1999; Manolagas, 2000; Compston *et al.*, 1995). The opposite may occur across the whole of life – the volume of bone resorbed by each BMU appears to decrease as reflected in a lower resorption cavity depth and an age-related increase, rather than decrease, in interstitial thickness (Croucher *et al.*, 1991; Ericksen *et al.*, 1999). (If resorption depth increased with age, interstitial wall thickness, the distance between cement lines of opposing hemi-osteons in trabecular bone, should decrease.)

The fourth age-related abnormality in the cellular machinery contributing to structural decay is an increase in the rate of bone remodeling after menopause. This is accompanied by worsening of the negative bone balance in each BMU as the volume of bone resorbed increases and the volume of bone formed decreases in the many more BMUs now remodeling bone on the three (endocortical, intracortical, and trabecular) components of its endosteal envelope (Manolagas, 2000).

Bone Loss During Young Adulthood

If the volume of bone resorbed decreases to the same degree as the decrease in the volume of bone formed by the BMU there will be no net negative BMU balance at the completion of a remodeling cycle so remodeling events will not produce any permanent bone loss or structural decay. However, at some stage in midlife or early, there is a net negative bone balance as the volume of bone resorbed exceeds that formed (irrespective of the absolute decrease in both) and this negative BMU balance is the necessary and sufficient requirement for loss of bone from the skeleton, structural decay, and bone fragility.

There is evidence using noninvasive methods such as densitometry or computed tomography for a decline in bone mass in young adulthood in women and in men (Riggs *et al.,* 1986; Gilsanz *et al.,* 1987; Riggs *et al.,* 2007). Assuming the decline is not an artifact produced by an increase in marrow fat with age (Bolotin and Sievänen, 2001), this decline is likely to be the result of bone loss driven by a decline in bone formation. More definitive statements cannot be made due to lack of histomorphometric data in premenopausal women and young adult men.

Riggs *et al.* report a decline in trabecular volumetric density prior menopause in a 3-year prospective study of 553 women and men (Riggs *et al.,* 2007). Before age 50, women lose 37% and men 42% of the total trabecular bone lost across life, and 6% and 15% of lifetime cortical bone loss. The structural and biomechanical consequences are likely to be less than bone loss later in life because (1) remodeling rate is slow, (2) trabecular bone loss probably proceeds by reduced bone formation rather than increased bone resorption in the BMU, (3) bone loss proceeds by trabecular thinning rather than loss of connectivity so a given decrement in trabecular BMD produces less loss of strength than produced by loss of connectivity (van der Linden *et al.,* 2001), and (4) continued periosteal apposition partly offsets endocortical bone loss shifting the cortices radially maintaining cortical area and resistance to bending (Szulc *et al.,* 2006).

Bone Loss During Menopause and Advancing Age

Variance in the positive BMU balance on trabecular surfaces during growth is small compared with the variance in the rate of remodeling so that the rate of gain in bone mass is driven more by the remodeling rate. Similarly, the variance in the negative BMU balance during aging is small compared with the variance in the rate of remodeling so the rate of bone loss during menopause and aging is driven more by the remodeling rate.

Thus, the higher rate of bone remodeling is a most important determinant of bone loss and the increase in remodeling rate in midlife associated with estrogen deficiency is responsible for accelerated bone loss. Perimenopausal women with remodeling rates in the lowest quartile lose little bone (Szulc *et al.,* 2006). Estrogen deficiency also increases the volume of bone resorbed by each BMU by prolonging the life span of osteoclasts, and reduces the volume of bone formed by each BMU by reducing the life span of osteoblasts, thereby aggravating the negative BMU balance (Manolagas, 2000). Whether the changes in the life span of the cells is permanent or temporary is not known but the combination of a rapid remodeling and a more negative BMU balance than observed before menopause accelerates bone loss and structural decay after menopause.

Before menopause, remodeling is slow. The birth rate of new BMUs creating resorption cavities is matched by slow completion of previously created BMUs in their formation phase. At menopause, this steady state is perturbed by an increase in the birth rate of new BMUs on bone's endosteal envelope. The now many BMUs remove bone while the fewer BMUs created before menopause complete remodeling by depositing bone. This perturbation produces a net acceleration in bone loss and a rapid decline in BMD (Fig. 11).

This is the remodeling transient, a reversible loss of bone mass and bone mineral that is a consequence of the normal delay in onset and slower progression of the formation phase of the remodeling cycle in the many remodeling foci created after menopause (Parfitt, 1980). The temporary deficit in bone mass and mineral has three components: the excavation site that lacks osteoid and mineral, the osteoid that lacks mineral, and bone that has undergone primary but not secondary mineralization. Primary mineralization occurs rapidly, secondary mineralization, the slow enlargement of crystals of calcium hydroxy-apatite-like mineral takes many months to years to go to completion (Akkus *et al.,* 2003). At any time, there are osteons created in the immediate postmenopausal period and fewer, earlier created, osteons at various stages of completing secondary mineralization.

Bone loss slows in the three to five years following menopause, not because remodeling rate slows. It doesn't. The rate of bone loss slows because steady state is restored at the new higher remodeling rate. Now the large numbers of BMUs excavating resorption cavities are matched by completion of remodeling by bone formation the large numbers of BMUs created in early menopause. Bone loss continues at a faster rate than before menopause but at a slower rate than immediately after menopause because BMU balance is negative, perhaps more negative than before menopause producing a permanent deficit in bone mass and mineral mass. The higher the remodeling rate and the more negative the BMU balance, the greater the bone loss and structural decay. If the worsening BMU balance produced by changes in the life span of osteoclasts and osteoblasts is temporary, and the negative BMU balance lessens but persists, the rate of loss will also lessen, but it will persist because bone loss is driven by the high remodeling rate.

Remodeling occurs on bone surfaces (envelopes), much more on the endosteal envelope than the periosteal envelope (Balena *et al.,* 1992; Orwoll, 2003), and more on the

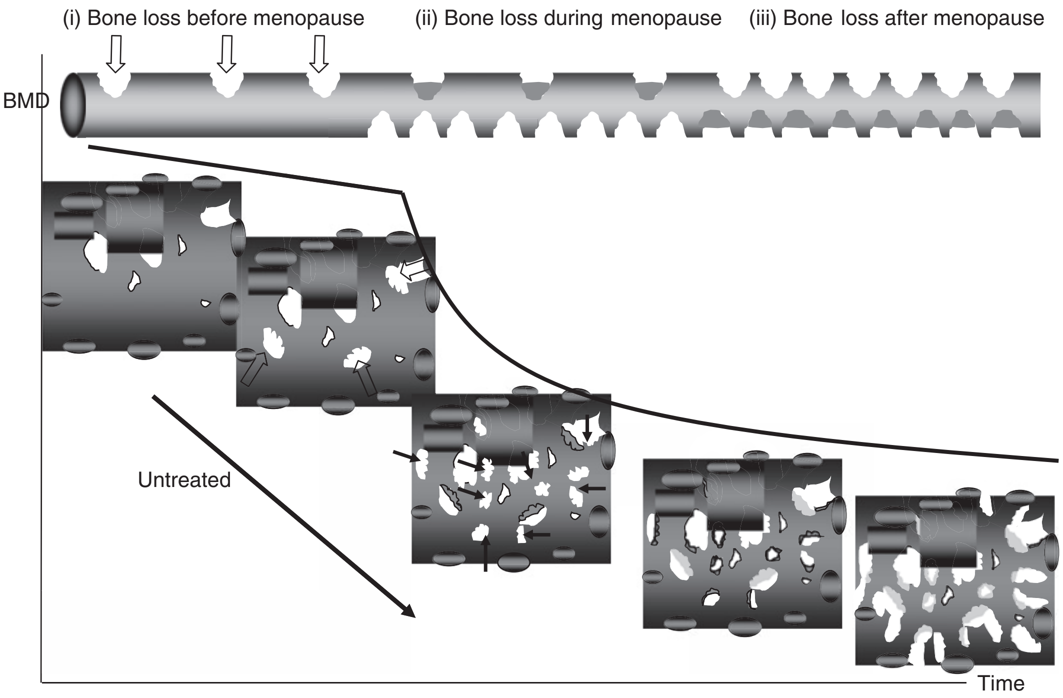
(i) Bone loss before menopause
(ii) Bone loss during menopause
(iii) Bone loss after menopause
BMD
Untreated
Time

FIGURE 11 (i) Bone loss is slow before menopause because remodeling is slow; only a few sites on the trabecular surface remove bone (open arrows). (ii) Bone loss accelerates at menopause as remodeling rate increases. Now many basic multicellular units (BMUs) remove bone (black arrows) while the three BMUs initiated before menopause deposit bone. (iii) Bone loss after menopause slows because steady state is restored. The many BMUs removing bone at menopause are now in their formation phase but as many new BMUs are created and resorb bone. Bone is lost because each remodeling event removes bone from bone. E. Seeman with permission.

trabecular than endocortical and intracortical surfaces of the endosteal envelope. Trabecular bone has more surface per unit bone volume than cortical bone so that trabecular bone is more likely to be remodeled than cortical bone. Excavated resorption sites create stress "concentrators," that focus stresses to a single point (as a small cut in a test tube makes it easy to snap) (Hernandez *et al.,* 2006) (Fig. 12, upper panels). The high remodeling rate and negative BMU balance produces trabecular thinning and complete loss of trabeculae. Increased resorption depth is more likely to produce perforation and complete loss of trabeculae than either greater numbers of resorption cavities or reduced formation in the BMU in women (Parfitt, 1996). A 10% loss of trabecular density by perforation reduces strength more greatly than the same loss by trabecular thinning (Fig. 12, lower panel).

As remodeling continues, trabeculae are lost so the trabecular surface available for resorption decreases but remodeling on endocortical surface continues increasing the endocortical surface (like the folds of a curtain) (Parfitt, 1984; Brown *et al.,* 1987; Arlot *et al.,* 1990; Foldes *et al.,* 1991). Remodeling on the intracortical surface (haversian canals) increases intracortical porosity (Martin, 1984; Brockstedt *et al.,* 1993; Yeni *et al.,* 1997) (Fig. 13). Increased porosity due to increased numbers of pores and/or increased size of pores by coalescence of adjacent remodeling cavities increases the surface available for remodeling "trabecularizing" the cortex. Either total bone surface does not change (increasing in cortical bone, decreasing in trabecular bone) or increases (in regions of cortical bone only) so that late in life, bone loss is more cortical than trabecular in origin.

As age advances and remodeling continues at the same intensity due to estrogen deficiency and perhaps secondary hyperparathyroidism, the extent of coalescence of pores increases so the number of pores in cortical bone decreases but the total area of porosity increases, and perhaps more so in patients with hip fractures than controls (Bell *et al.,* 1999). Cortices porosity reduces the ability of bone to limit crack propagation so that bone cannot absorb the energy imparted by a fall and so it is released in the most undesirable way by fracturing (Martin, 1984; Yeni *et al.,* 1997). The continued remodeling at a similar intensity with its negative BMU balance, on the same amount or more surface, removes the same amount of bone from an ever-decreasing amount of bone accelerating the loss of bone and structural decay.

Rapid remodeling also modifies the material properties of bone increasing fracture risk. More densely mineralized bone is removed and replaced with younger, less densely mineralized bone, reducing stiffness (Boivin and Meunier, 2002; Boivin *et al.,* 2003). Increased remodeling impairs isomerization of collagen reducing bone strength (Viguet-Carrin *et al.,* 2006; Garnero *et al.,* 1996). Interstitial bone deep to surface remodeling becomes more densely mineralized and more highly cross linked with advanced glycation

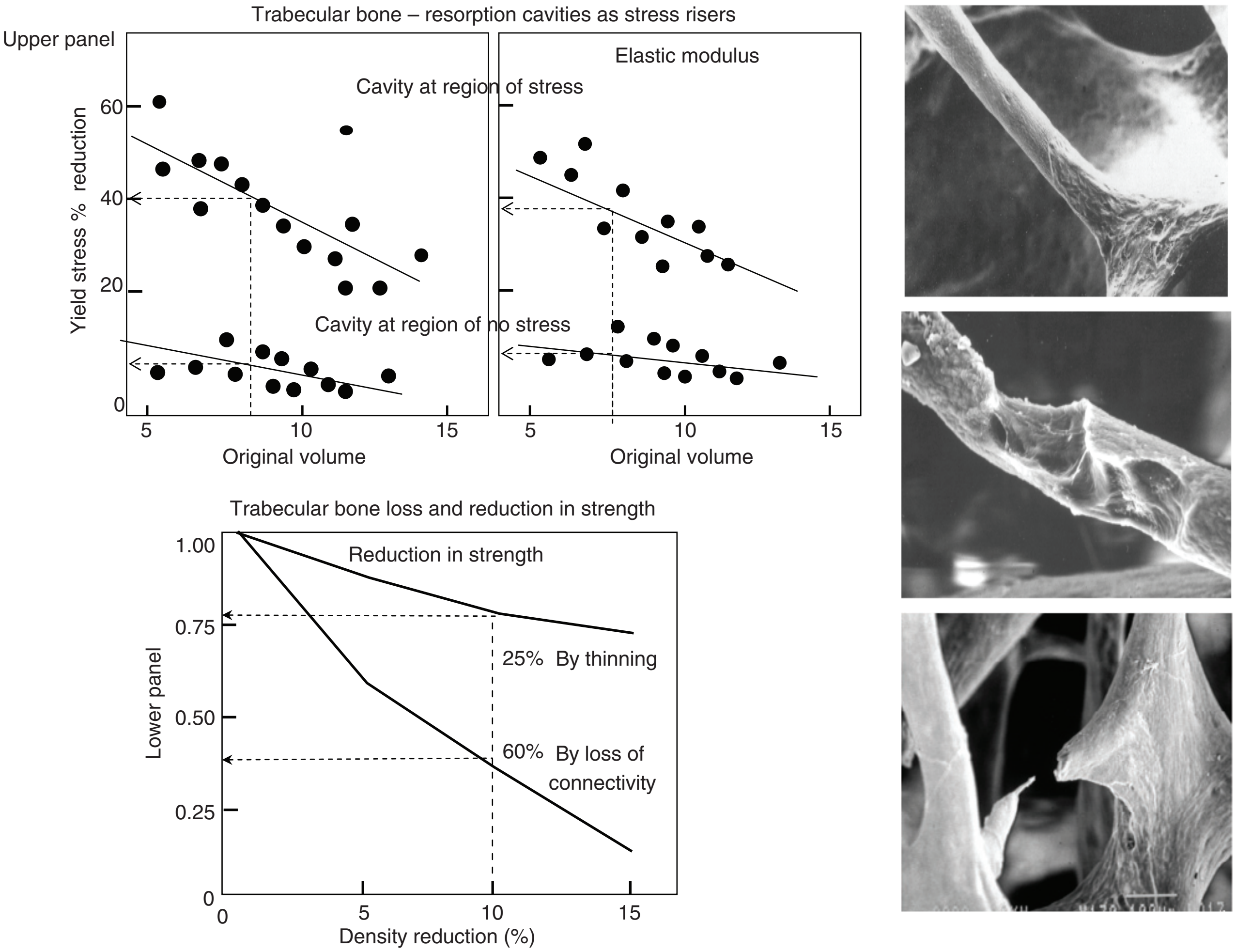

FIGURE 12 Upper panels: When resorption cavities occur in regions of stress on trabecular bone the decline in yield stress and elastic modulus is greater than produced by cavities in unstressed regions. Adapted from Hernandez *et al.* (2006). Lower panel: Reduction in strength produced by 10% deficit in trabecular density is greater when this deficit is produced by loss of trabecular connectivity than by thinning. Adapted from Van der Linden *et al.* (2001). Images show an intact trabecula, a resorption cavity, and loss of connectivity. Mosekilde and Mosekilde, 1990 With permission from the publisher.

products (AGEs) like pentosidine, both processes reducing bone toughness; it is easier for microcracks to travel through homogeneously mineralized bone and lengthen. Interstitial bone (between osteons) has reduced osteocyte numbers, accumulating microdamage (Bailey *et al.,* 1999; Banse *et al.,* 2002; Nalla *et al.,* 2004; Qui *et al.,* 2005; Yeni *et al.,* 1997).

Net Effects of Reduced Periosteal Bone Formation and Endosteal Bone Loss

The challenges regarding identifying the existence of periosteal apposition during adulthood, its site specificity, magnitude, and sex differences are considerable. In cross-sectional studies, secular changes in bone size may obscure or exaggerate periosteal apposition. These problems are not necessarily resolved by adjusting for height. Secular increases in stature occur in one or both sexes, in some races but not others and may occur in the skelton of the upper or lower body (Bakwin, 1964; Meredith, 1978; Cameron *et al.,* 1982; Tanner *et al.,* 1982; Malina and Brown, 1987). These secular trends can produce misleading inferences when increments or lack of increments in bone diameters are used as surrogates of periosteal apposition.

For example, in cross-sectional studies, absence of an increment in periosteal diameter across age may not mean periosteal apposition was absent. Earlier born individuals (the elderly in a cross-sectional sample) may have been shorter and had more slender bones than later born individuals (young normals in a cross-sectional sample). When periosteal apposition occurs, earlier born with more slender bones have an increase in bone diameter that comes to equal that in later born group (who have not yet had age-related periosteal apposition) leading to the flawed inference that there was no periosteal apposition in the cross-sectional sample.

When comparisons are made between sexes (or races) in cross-sectional studies, if the truth is that periosteal

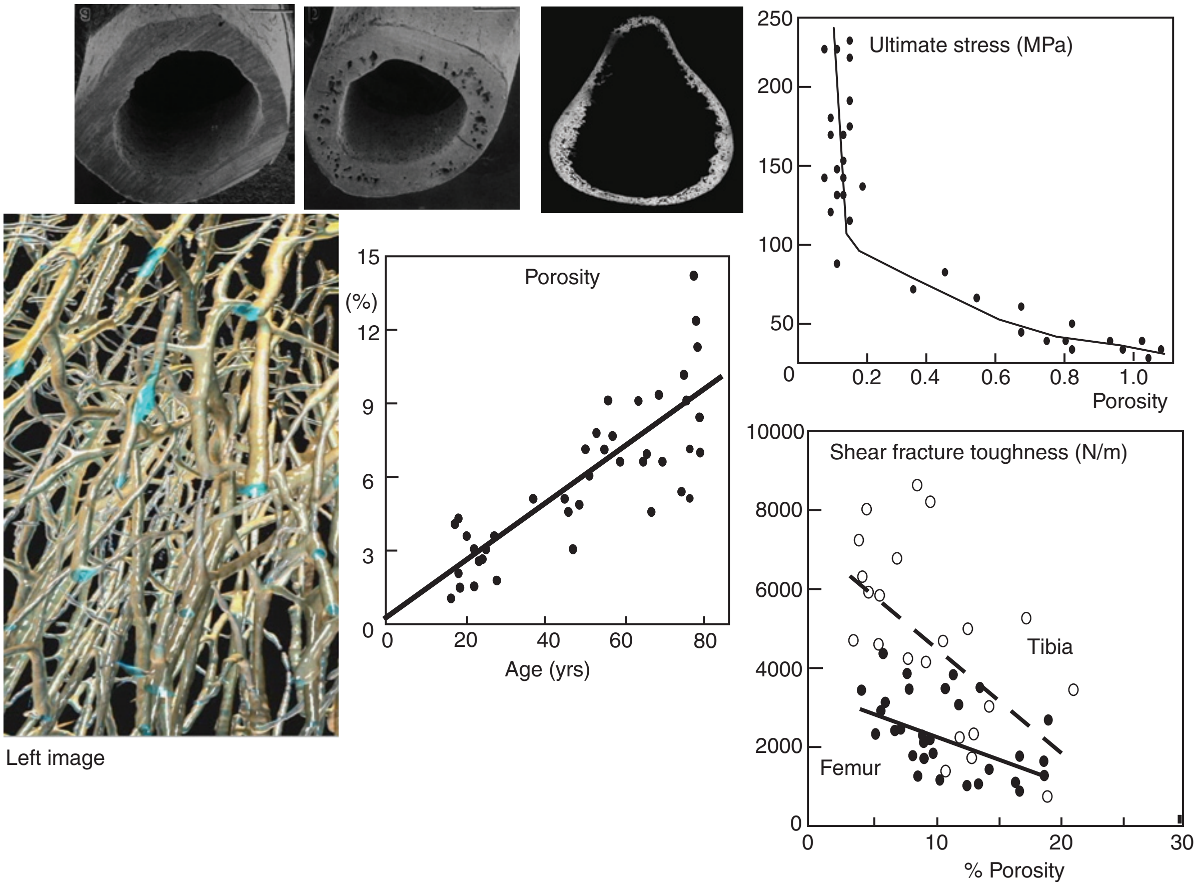

FIGURE 13 Cortical porosity increases as age advances (Brockstedt *et al.* 1993). This is associated with a decline in ultimate stress (adapted from Martin, 1984) and reduction in toughness (adapted from Yeni *et al.*, 1997). Upper images illustrate increasing porosity and thinning. Left image shows haversian canals in longitudinal section responsible for porosity in cross-section. Courtesy M. Knackstedt, Australian National University, Canberra. (See plate section)

apposition is greater in men than women but men have a secular increase in bone size and women do not, then the secular increase in men will blunt the increment in bone width across age in men and make it appear that the age-related increase in vertebral and femoral neck diameters (and so periosteal apposition) is similar in women and men. Longitudinal studies are also problematic because changes in periosteal apposition during aging are small (Balena *et al.*, 1992). The precision of methods to determine bone diameter, usually bone densitometry, and problems with edge detection when bone mineral density is changing limit the credibility of these measurements.

Periosteal apposition is believed to increase as an adaptive response to compensate for the loss of strength produced by endocortical bone loss, so there will be no *net* loss of bone, no cortical thinning, and no loss of bone strength (Alhborg *et al.*, 2003). In a 7-prospective study of over 600 women, Szulc *et al.* report that endocortical bone loss occurred in premenopausal women with concurrent periosteal apposition (Szulc *et al.*, 2006) (Fig. 14). As periosteal apposition was less than endocortical resorption, the cortices thinned but there was no *net* bone loss because the thinner cortex was now distributed around a larger perimeter conserving total bone mass. Moreover, resistance to bending increased despite bone loss and cortical thinning because this same amount of bone was now distributed further from the neutral axis. So bone mass alone is a poor predictor of strength because resistance to bending is determined by the spatial distribution of the bone.

Endocortical resorption increased during the perimenopausal period, yet periosteal apposition decreased – it did not increase as predicted if the notion that periosteal apposition is a compensatory mechanism is correct. The cortices thinned as periosteal apposition declined further. Nevertheless, bending strength remained unchanged – despite bone loss and cortical thinning because periosteal apposition was still sufficient to shift the thinning cortex outwards.

Bone fragility emerged only after menopause when accelerated in endocortical bone resorption and deceleration in periosteal apposition produce further cortical thinning. As periosteal apposition was now minimal, there was little outward displacement of the thinning cortex so cortical area now declined as did resistance to bending. Endocortical

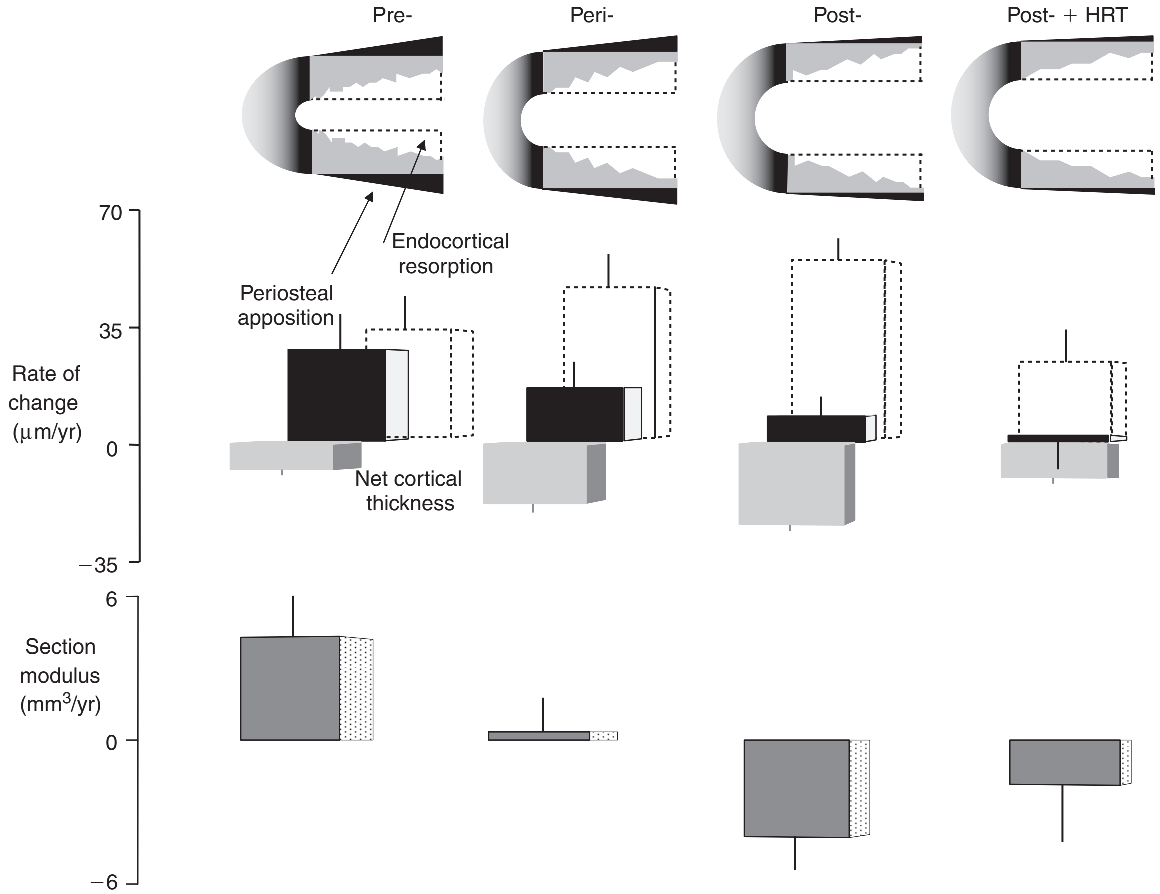

FIGURE 14 The amount of bone resorbed by endocortical resorption (open bar) increases with age. The amount deposited by periosteal apposition (black bar) decreases. The net effect is a decline in cortical thickness (grey bar). In premenopausal women, the thinner cortex is displaced radially increasing section modulus (Z). In perimenopausal women Z does not decrease despite cortical thinning because periosteal apposition still produces radial displacement. In postmenopausal women, Z decreases because endocortical resorption continues, periosteal apposition declines and little radial displacement occurs. In women treated with hormone replacement therapy (HRT), resorption is decreased with no effect on periosteal apposition. Z is less reduced than in untreated women. Adapted from Szulc *et al.,* 2006.

resorption was reduced but not abolished in women receiving hormone replacement therapy while periosteal apposition was no different to untreated women; cortical thinning was reduced and the resistance to bending occurred but less than in untreated women.

Periosteal envelope is regarded exclusively as a bone-forming surface. This is incorrect (Balena *et al.,* 1992). During growth, bone resorption is critical for the in-wasting that produces the fan-shaped metaphyses (Rauch *et al.,* 2001). Blizoites and colleagues report that bone resorption occurs in adult nonhuman primates (Blizoites *et al.,* 2006). Femur specimens from 16 intact adult male and female nonhuman primates showed that periosteal remodeling of the femoral neck in intact animals was slower than in cancellous bone but more rapid than at the femoral shaft. Gonadectomized females showed an increase in osteoclast number on the periosteal surface compared with intact controls. If these data are correct, adult skeletal dimensions may decrease in size as age advances.

Thus, even though the genius of bone biology Fuller Albright suggested over 65 years ago that osteoporosis was a disorder of reduced bone formation (Albright *et al.,* 1941), research into the pathogenesis of bone fragility during the last 40 years has focused on the role of increased bone resorption. During aging, both increasing endocortical bone resorption and reduced periosteal apposition cause *net* bone loss, alterations in the distribution of the remaining bone, and the emergence of the bone fragility. The cellular basis of the vigor of bone formation during growth and progressive decline in vigor during aging on the periosteal surface and within each BMU is yet to be defined.

Sex and Racial Differences in Trabecular and Cortical Bone Loss

A greater proportion of women than men sustain fragility fractures during their lifetime. The reasons for this sexual dimorphism are not clear. Men have a larger skeleton than women do so that resistance to bending is greater in men than women. Bone loss in most, but not all, men is the result of a negative BMU balance produced by reduced formation rather than increased resorption by the BMUs, so trabecular bone loss occurs by thinning rather than loss of connectivity (Aaron *et al.,* 1987). Men do not have a midlife decline in sex hormones and increase in remodeling rate that drives structural decay produced by the negative BMU balance. Better preservation of trabecular bone in elderly men leaves more trabecular surfaces for remodeling to occur upon so trabecular bone loss continues longer in men (Aaron *et al.,* 1987). Net trabecular bone loss across age is only slightly greater in women than men (Riggs *et al.,* 2004), or is similar (Aaron *et al.,* 1987; Meunier *et al.,* 1990; Kalender *et al.,* 1989; Mosekilde and Mosekilde, 1990; Seeman, 1997; Seeman *et al.,* 2001). However, the same deficit in trabecular density produced by thinning (as occurs in men) produces less reduction in strength than produced by loss of connectivity (as occurs in women) (Van der Linden *et al.*, 2001).

Marrow cavity expansion occurs in both sexes but whether it is greater in women than men is uncertain (Riggs *et al.,* 2004). Cortical porosity increases less in men than in women because remodeling rate is lower in men and so crack propagation in cortical bone is probably better resisted in men than in women. Periosteal apposition is reported to be greater in men than in women in some (Duan *et al.,* 2001, Duan *et al.,* 2003, Duan *et al.,* 2005, Wang *et al.,* 2005, Seeman *et al.,* 2001) but not all studies (Riggs *et al.,* 2004).

Thus, methodological issues must temper the inferences that can be made regarding the basis of sexual dimorphism in bone strength (Seeman *et al.,* 2004). The absolute risk for fracture in women and men of the same age and BMD is similar (Kanis *et al.,* 2001; Kanis *et al.,* 2005). The lower fracture incidence in men than in women is likely to be the result of lower proportion of elderly men than elderly women having material and structural properties (cortical thinning, porosity, trabecular thinning, loss of connectivity, microdamage) below the critical level at which the loads on the bone are greater than the bone's net ability to tolerate them. Structural failure occurs less in men because the relationship between load and bone strength is better maintained in men than in women (Riggs *et al.,* 2006, Bouxsein *et al.,* 2006).

The Heterogeneous Material and Structural Basis of Bone Fragility in Patients with Fractures

Patients with fractures are grouped by having "one or more minimal trauma vertebral fractures," or sustaining a fall from "no greater than the standing position." However, the pathogenesis and structural basis of the bone fragility underlying the fractures is heterogeneous. Patients with vertebral fractures may have high, normal, or low remodeling rates (Brown *et al.,* 1984; Arlot *et al.,* 1990; Delmas, 2000). Some have a negative BMU balance due to reduced formation, increased resorption, or both, or no negative BMU balance at all (Ericksen *et al.,* 1990). Some patients with vertebral fractures have increased, whereas others have reduced, tissue mineral density (Ciarelli *et al.,* 2003) (Fig. 15). Some patients have reduced osteocyte density; others do not (Qui *et al.,* 2003; Qui *et al.,* 2005). Contemporary therapeutics gives no consideration to the underlying pathogenesis or structural abnormalities present in an individual. Whether anti-fracture efficacy can be improved from its current values of 50% for vertebral and hip fractures and 20% for nonvertebral fractures (Delmas, 2002) by defining the pathogenesis and structural basis in an individual remains uncertain, but it is worthy of exploration.

SUMMARY AND CONCLUSION

The purpose of modeling and remodeling during growth is to optimize bone strength by depositing bone where it is needed and to minimize mass by removing it from where it is not needed. Bone must be stiff – resistant to deformation, yet flexible – able to store energy in elastic deformation or to dissipate it. Otherwise, energy will be released by structural failure – fracture.

These paradoxical properties are achieved by bone's material composition and structural design. Material composition is similar among mammals so differences in bone strength in adulthood are largely the result of structural diversity. This diversity is already expressed before puberty. The magnitude of the variance in bone size and mass in prepubertal children is similar to that in their parents. Individuals with traits in the 5th, 50th, or 95th percentile in adulthood occupied these positions in early life because traits track along their percentile of origin. Long bones with a larger cross-section have a biomechanical advantage so the same periosteal apposition on a larger cross-section (i.e., less relative to size) confers greater stiffness than on a smaller cross-section. Endocortical resorption excavates a larger marrow cavity shifting the cortex radially, increasing stiffness and minimizing mass; larger bone cross-sections have a lower volumetric bone mineral density (vBMD). In slender bones, higher vBMD is the result of similar amounts of periosteal apposition (more relative to size) and less endocortical resorption, which excavates a smaller marrow leaving a relatively thicker cortex to offset the fragility of slenderness. Varying cellular activity around the periosteal and endocortical envelopes fashions the diverse shapes of adjacent cross-sections. Vertebral bodies are fashioned as a honeycomb of trabecular plates and void spaces conferring flexibility and lightness.

Modeling and remodeling are successful during growth, not adulthood. The purpose of modeling and remodeling during adulthood is to maintain bone strength by damage

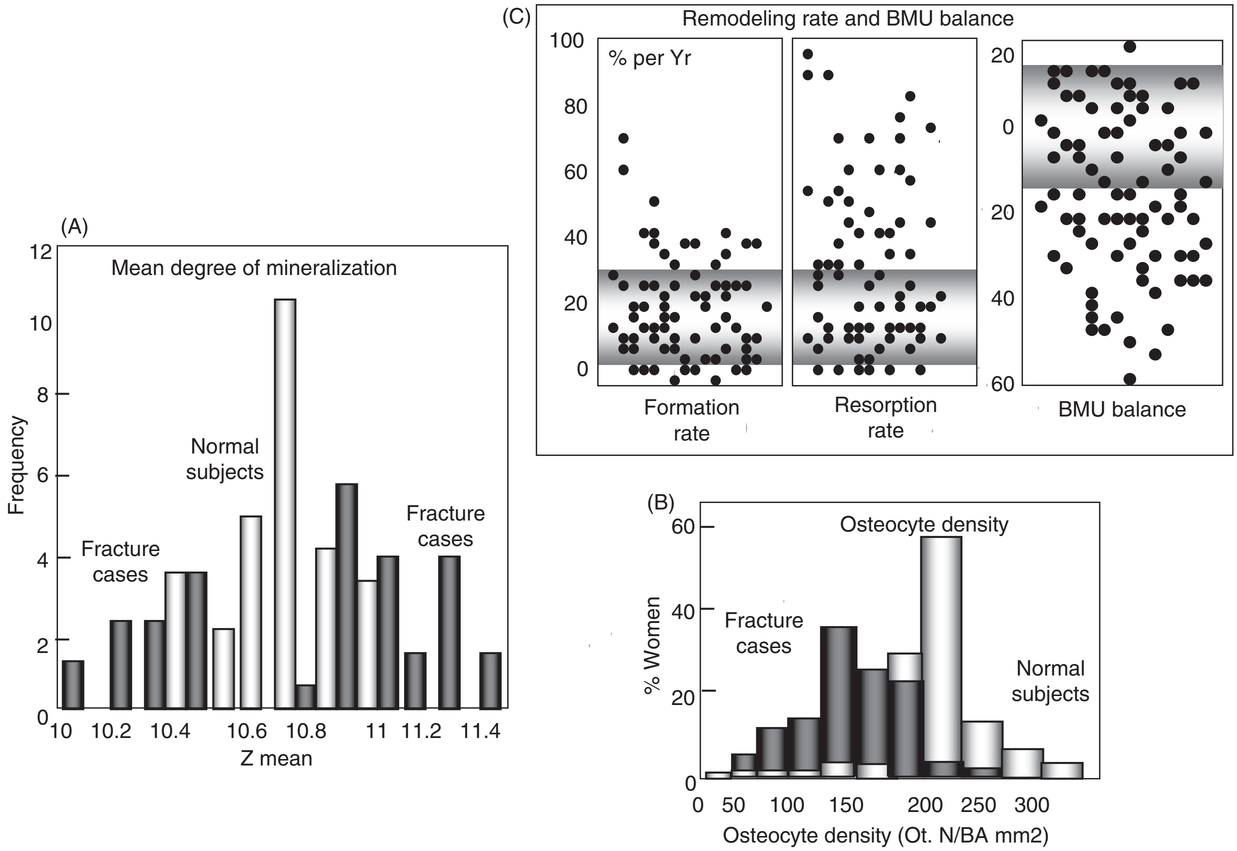

FIGURE 15 Bone fragility in patients with fractures has a heterogenous pathogenesis and structural basis. Patients have tissue mineral density in the upper or lower part of the normal distribution (adapted from Ciarelli *et al.,* 2003). Some have reduced or normal osteocyte density (adapted from Qui *et al.,* 2003). Formation and resorption rates may be lower normal or high bone balance in the basic multicellular level (BMU) may be normal or negative (adapted from Ericksen *et al.,* 1990).

repair but four age-related changes compromise bone's material composition and structure; a decline in periosteal bone formation, a decline in the volume of bone formed by each basic multicellular unit (BMU), continued resorption by each BMU, and high remodeling. Bone loss occurs in early adulthood but the structural and biomechanical consequences are modest because the negative BMU balance is driven by reduced bone formation not increased resorption, remodeling is slow and modest periosteal apposition offsets endocortical bone loss shifting the thinner cortex radially. After menopause, increased remodeling, worsening negative BMU balance, and a decline in periosteal apposition accelerate cortical thinning and porosity, trabecular thinning, and loss of connectivity. Interstitial bone, deep to surface remodeling, becomes more densely mineralized, has few osteocytes, greater collagen cross-linking, and accumulating microdamage. Late in life secondary hyperparathyroidism sustains high remodeling producing further cortical thinning and porosity. These age-related changes produce the material and structural abnormalities responsible for bone fragility.

Recent advances raise many questions concerning the uni-, bi- and multidirectional regulation and steps in remodeling, how resorption and formation phases are regulated and co-regulated, how osteocytogenesis occurs and the lacunar-canalicular system is reestablished for mechanotransduction and damage detection. Damage removal may not be the only reason bone remodels but is likely to be one of its main purposes in adulthood. However, the nature of "damage" has not been systematically defined and so questions remain concerning the determinants of damage production, its biomechanical consequences, and how different types of damage are signaled for repair. Thus, our understanding of why or how bones fail at the material and structural level remains incomplete. This is an essential direction of enquiry if we are to provide targeted approaches to drug therapy.

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Chapter 2

Biomechanics of Bone and Age-Related Fractures

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INTRODUCTION

The bones that make up the skeleton function in many important capacities. Bones are the primary structural elements in the body, protecting vital organs and providing a rigid framework for locomotion. In addition, the skeleton participates in mineral homeostasis and is a primary site of hemopoiesis. Through remodeling, our bones are capable of self-repair and of adapting their structure in response to changes in mechanical, biological, and biochemical demands. Many of these demands differ across anatomic sites, and thus is it not surprising that the microstructure of bone is remarkably varied, ranging from the dense, composite fiber-reinforced structure of the diaphyseal cortex to the honeycomb-like, open-cell architecture of the trabecular bone in metaphyseal regions.

One primary reason for studying the mechanics of bone is to improve our understanding of how and why bones fracture. From an engineering viewpoint, fractures represent a structural failure of the bone whereby the forces and/or moments applied to the bone exceed its load-bearing capacity. Therefore, when investigating the biomechanics of age-related fractures, one must consider the mechanical properties of bone as well as the applied loads and how these two factors change with age.

In this chapter we define and explain some of the basic terms and concepts used in bone mechanics with particular emphasis on the concepts most relevant to the study of age-related fractures. The next portion of the chapter presents a review of the current understanding of the mechanical behavior of cortical and trabecular bone and of whole bones. This information provides a foundation for the final portion of the chapter that focuses on the biomechanics of age-related fractures. In this final section, we consider the roles of both skeletal loading and bone fragility as they relate to hip and spine fractures and identify important directions of future research in these areas.

BASIC CONCEPTS AND DEFINITIONS

Material versus Structural Behavior

In studying the mechanical behavior of bone, it is important to distinguish between the mechanical behavior of a whole bone as a structure (structural behavior) and the mechanical behavior of the bony tissue itself (material behavior). The material behavior of a specimen is independent of its geometry (size and shape), and it reflects the intrinsic properties of the material itself. Typically, the material behavior of a specimen is determined by conducting mechanical tests on standardized specimens under simple loading conditions. In contrast, the structural behavior of a specimen, determined by conducting mechanical tests on whole-bone specimens subjected to physiological or traumatic loading conditions, reflects both the morphology and the material properties of the specimen. Bone fractures observed clinically are likely the result of material failure of the bone tissue that leads to catastrophic failure of the bone structure.

In a hierarchical composite material such as bone, the definition of material properties requires additional clarification. In describing the properties of bone as a tissue, one could consider the mechanical properties of single trabeculae, the calcified bone matrix, single osteons, or small specimens of cortical or trabecular bone. For purposes of

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this chapter, we consider bone "material" to include the calcified bone matrix, the marrow spaces in trabecular bone, and Haversian and Volkmann's canals in cortical bone. Thus, we take a continuum mechanics approach, in that the specimen is small enough to be homogeneous (uniform), but large enough to include a sufficient number of trabeculae (for trabecular bone) or osteons (for cortical bone) to characterize the material behavior of trabecular bone and cortical bone. We note that this scope necessarily excludes the rapidly growing body of research in which techniques such as microtensile and bending tests (Ascenzi and Bonucci, 1967, 1968; Ascenzi *et al.*, 1985, 1994; Choi and Goldstein, 1992; Hernandez *et al.*, 2005; Rho *et al.*, 1993; Townsend *et al.*, 1975), pushout tests (Ascenzi and Bonucci, 1972; Dong and Guo, 2004), nanoindentation (Fan *et al.*, 2002; Hengsberger *et al.*, 2002; Hoc *et al.*, 2006; Hofmann *et al.*, 2006; Rho *et al.*, 1999; Turner *et al.*, 1999; Zysset *et al.*, 1998), and acoustic microscopy (Katz and Meunier, 1993; Litniewski, 2005) have been used to characterize the micro- and nanoscale mechanical properties of bone.

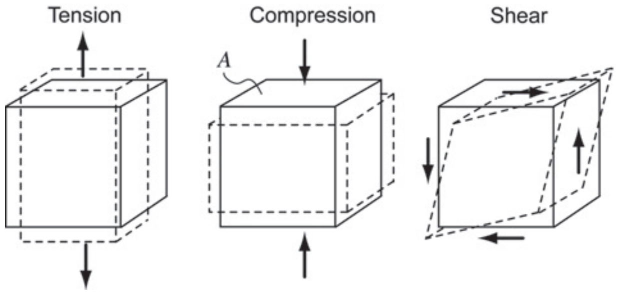


FIGURE 1 Normal and shear stresses acting on a specimen produce normal and shear strains. The dotted lines represent the specimen that is deformed under the action of the applied forces. (From Morgan *et al.*, 2008, with permission).

Biomechanical Properties

The primary biomechanical properties describe the relationship between forces, or loads, applied to the bone or bone specimen and the deformations that result from these applied forces. It is interesting to note that, *in vivo*, the forces applied to bones have several possible origins, including external forces acting on the body (such as a ground reaction force during walking or an impact force owing to a fall), internal forces created by ligament tension or muscle contraction, and internal bone-on-bone contact forces. The resistance within the bone that develops in response to these applied forces is known as stress, and it represents local force intensity with dimensions of force per unit area. The local deformation that results from the applied forces is referred to as strain, which is defined as a relative change in size or shape and therefore is often expressed in terms of percent. When forces are applied to a bone, a complex and varied distribution of stresses and strains arise throughout the bone structure. These stresses and strains can be categorized as normal stresses and strains or shear stresses and strains (Fig. 1). For a given plane within the bone structure, normal stresses act perpendicular to that plane (i.e., tension and compression) and shear stresses act parallel to that plane. Normal strains represent elongation or shortening, whereas shear strains represent distortion.

Bone mechanical properties can be defined with the aid of a load–deformation curve or stress–strain curve. The load–deformation curve (Fig. 2) describes the amount of load needed to produce a unit of deformation and reflects the structural behavior of the bone. Thus, the shape of this curve depends on both the size and shape of the bone, as well as the properties of the tissue that comprise it. In general, load and deformation are linearly related until the yield point is reached, at which time the slope of the load–deformation curve is reduced. Before the yield point, the bone is considered to be in the elastic region, and if unloaded, would return to its original shape with no residual deformation. The slope of the load–deformation curve in this elastic region defines the structural stiffness of the bone. In contrast to a bone's behavior in the elastic region, in the postyield region, the bone undergoes permanent deformation and will not return to its original shape even when the load is removed completely. If the load continues to increase, the ultimate or failure load is reached, after which the structure often fails catastrophically. The energy required to cause failure of the structure is computed as the area under the load–displacement curve, and is termed the work to failure.

The stress–strain curve (Fig. 3) is analogous to the load–deformation curve but reflects the material behavior of the bone tissue. Typically, a stress–strain curve is generated by conducting a mechanical test on a specimen of standardized geometry, such that one can easily compute stress from force and strain from displacement. The slope of the stress–strain curve in the elastic region is the elastic (or Young's) modulus. The values of stress and strain at the yield point are the yield stress and yield strain, respectively. Similarly, the values of stress and strain at the ultimate point are the ultimate stress and ultimate strain. The ultimate stress and strain may or may not be equal to the fracture stress and fracture strain, where the latter is a measure of the ductility of the specimen. Materials that sustain little deformation before fracture are brittle materials, whereas those that undergo significant deformation before fracture are considered to be ductile materials. The area under the stress–strain curve is the amount of work per unit volume of material required to fracture the specimen

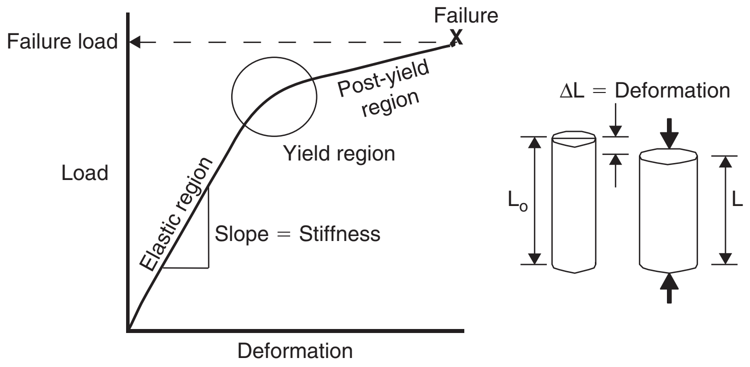


FIGURE 2 The load versus deformation plot is used to describe the structural behavior of a specimen. The elastic region is distinguished from the plastic region by the yield region. In the elastic region, when the load is removed there will be no residual deformation and the bone will return to its original shape. In contrast, in the postyield region, the bone will undergo permanent deformations that will remain even if the load is removed.

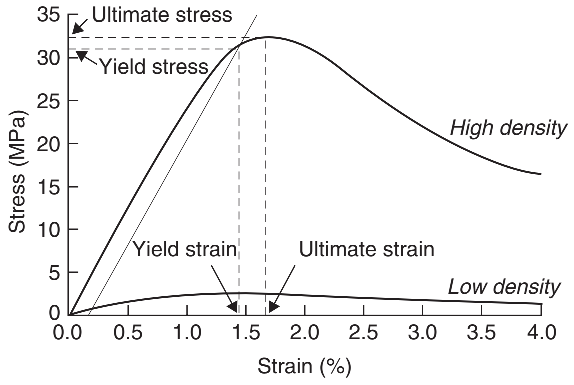


FIGURE 3 Representative stress–strain curves for high- and low-density trabecular bone specimens in compression. The elastic modulus, yield stress, yield strain, ultimate stress, and ultimate strain are indicated for the high-density specimen. Owing to the smoothness of the stress–strain curve, the yield point is typically defined via the 0.2% offset method: the intersection of the curve and a line with slope equal to the elastic modulus but with an *x*-intercept of 0.2% strain marks the yield point. Trabecular bone does not exhibit a well-defined fracture point in compression but rather continues to compact in on itself. For tensile loading, the fracture point is defined as the point just before the stress drops to zero as a consequence of the specimen pulling apart.

and is a measure of the toughness of the specimen. Tough bone will be more resistant to fracture, but it may yield at a lower stress and, according to that measure, be considered weaker. Indeed, a loose inverse relationship between toughness and strength exists for most engineering materials. A second measure of the toughness of a material is the fracture toughness, which is the resistance of the material to the initiation and propagation of cracks.

Additional material properties that are relevant in the study of bone include fatigue properties and viscoelastic properties. These properties characterize the response of the tissue to repeated loading and the time-dependent material behavior, respectively. A primary outcome of fatigue testing is the speed at which properties such as the elastic modulus and yield or ultimate stress decrease with increasing numbers of loading cycles, as this quantifies the rate of material degradation. The intrinsic properties of viscoelastic materials, such as bone, depend on loading rate and can differ substantially under quasi-static loading compared with the higher loading rates that occur during trauma.

MECHANICS OF BONE TISSUE

Role of Bone Composition and Microstructure

The mechanical properties of cortical bone are heavily dependent on porosity and the degree of matrix mineralization (Currey, 1988, 1990; Martin and Ishida, 1989; Schaffler and Burr, 1988). More than 80% of the variation in the elastic modulus of cortical bone can be explained by a power-law relationship with matrix mineralization and porosity as explanatory variables (Currey, 1988, 1990). Some studies show that, with increasing age, the mineralization of the matrix increases, leading to stiffer, but more brittle material behavior (Currey, 1969; Grynpas, 1993), although other studies indicate no age-related changes in the degree of mineralization (Roschger *et al.*, 2003, 2008).

The elastic modulus and strength of trabecular bone are also determined to a great extent by bone density (Keaveny *et al.*, 2001). Power-law relationships with bone density as the explanatory variable explain 60% to 90% of the variation the modulus and strength of trabecular bone (Carter and Hayes, 1976, 1977b; Gibson and Ashby, 1988; Hernandez *et al.*, 2001; Keaveny and Hayes, 1993; Morgan and Keaveny, 2001; Mosekilde *et al.*, 1987; Rice *et al.*, 1988) (Fig. 4). These power-law relationships indicate that small changes in apparent density can lead to dramatic changes in mechanical behavior. For instance, a 25% decrease in apparent density, approximately equivalent to 15 to 20 years of age-related bone loss (Riggs *et al.*, 2004), would be predicted to lead to a 44% decrease in the

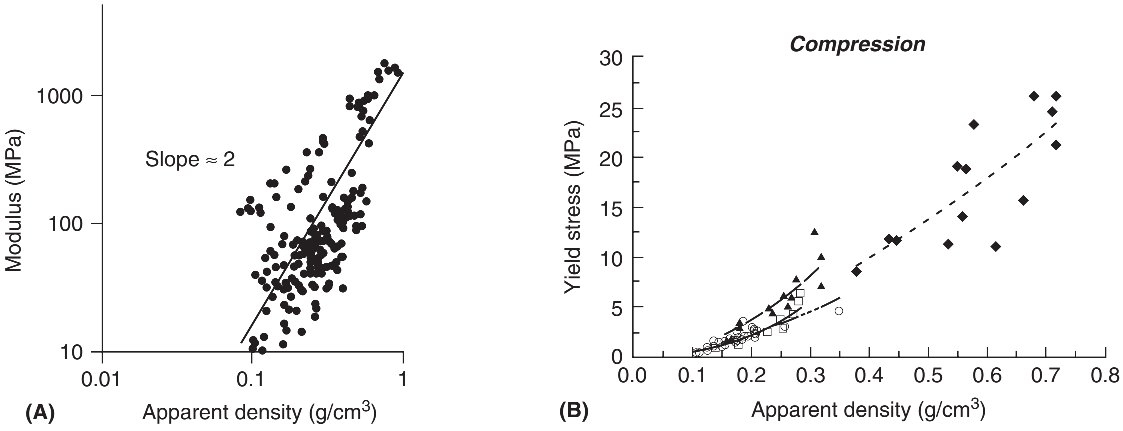


FIGURE 4 (**A**) Elastic modulus as a function of apparent density for trabecular bone specimens from a wide variety of species and anatomic sites. In general, the modulus varies as a power-law function of density, with an exponent of approximately two. (From Keaveny and Hayes, 1993, with permission.) (**B**) Compressive yield stress as a function of apparent density for human trabecular bone specimens from multiple anatomic sites. In general, the dependence of yield stress on density is different for different anatomic sites, although the exponents of the power-law relationships are approximately two. (From Morgan and Keaveny, 2001, with permission).

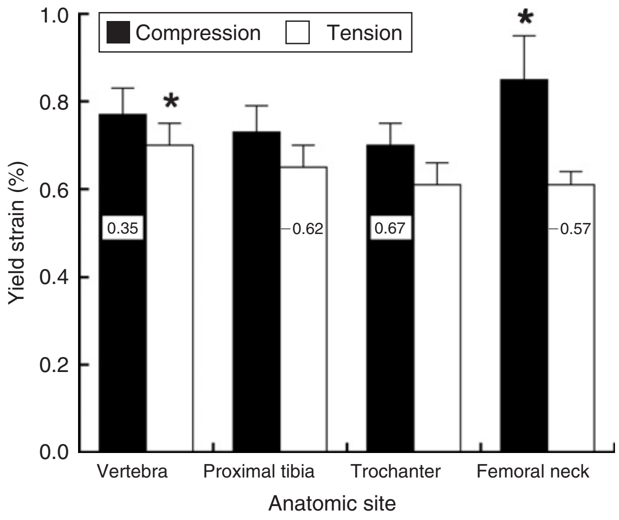


FIGURE 5 Mean compressive and tensile yield strains of human trabecular bone from multiple anatomic sites. Error bars indicate 1 SD. Significant relationships with apparent density are indicated by the Pearson correlation coefficient, *r*, within the column. An asterisk (*) denotes a significant difference in the mean with respect to all other sites in the same loading mode. (From Morgan and Keaveny, 2001, with permission).

strength of trabecular bone. Notably, however, the yield and ultimate strains of trabecular bone are only weakly, if at all, dependent on density (Kopperdahl and Keaveny, 1998; Morgan and Keaveny, 2001; Turner, 1989) and are relatively constant for a given anatomic site (Morgan and Keaveny, 2001) (Fig. 5).

Both cortical and trabecular bone are anisotropic materials, meaning that their mechanical properties depend on the loading direction. The anisotropic nature of bone reflects its function as a load-bearing structure, because it is generally stiffest and strongest in the primary loading direction. For example, cortical bone from the femoral diaphysis has a higher elastic modulus and strength when loaded in the longitudinal direction than when loaded in the transverse direction (Reilly and Burstein, 1975; Reilly *et al.*, 1974) (Fig. 6). Trabecular bone from the vertebral body is much stiffer and stronger in the vertical direction than in the transverse direction (Arnold *et al.*, 1966; Mosekilde and Mosekilde, 1986; Mosekilde *et al.*, 1985). Yet, trabecular bone properties from the iliac crest and central femoral head are nearly isotropic (Ciarelli *et al.*, 1991; Mosekilde and Mosekilde, 1988), indicating that the degree of anisotropy varies with anatomical site and functional loading (Amling *et al.*, 1996; Ulrich *et al.*, 1999). Although the anisotropy of bone tissue affords greater resistance to loads applied in the primary loading direction for a given amount of tissue, it necessarily

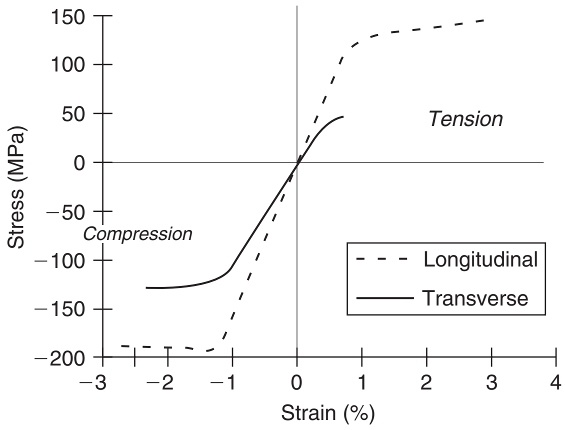


FIGURE 6 Typical stress versus strain diagram for longitudinally (L) and transversely (T) oriented specimens of cortical bone from the diaphysis. For specimens tested in compression, load and displacement are converted to stress and strain by dividing by the cross-sectional area and original length of the specimen, respectively. The figure shows the inherent anisotropy in bone, as specimens testing in the longitudinal direction are significantly stronger than those tested in the transverse direction.

results in a lesser load-bearing capacity in directions "off-axis," or oblique, to the principal loading direction. The off-axis mechanical behavior of trabecular bone may be particularly relevant during impact after a fall, which results in applied loads of different magnitudes and directions than those arising from habitual activities. Moreover, the anisotropy of bone complicates efforts to determine the mechanical behavior of bone tissue under multiaxial stress states (a combination of normal and/or shear stresses acting along multiple directions). Multiaxial stresses can arise during trauma (Lotz *et al.*, 1991) and in regions surrounding bone implants (Cheal *et al.*, 1985). Multiaxial yield criteria for trabecular (Bayraktar *et al.*, 2004a; Keaveny *et al.*, 1999; Zysset *et al.*, 1999) and cortical bone (Cezayirlioglu *et al.*, 1985) have been developed; however, little is known about the multiaxial, postyield behavior of either type of bone tissue.

Given the anisotropic nature of trabecular bone and the variation in predicted modulus for a given density (Britton and Davie, 1990; Keaveny *et al.*, 2001), it is clear that density alone cannot explain all of the variability in the mechanical behavior of trabecular bone. Empirical observations and theoretical analyses indicate that trabecular architecture plays an important role in determining the mechanical properties of trabecular bone. Trabecular architecture can be characterized by the thickness, number, and separation of the individual trabecular elements, as well as the extent to which these elements are interconnected. Advances in nondestructive, high-resolution imaging techniques have provided new insights into the relative influence of architecture and density on age-related changes in the mechanical behavior of trabecular bone (Goulet *et al.*, 1994; Ulrich *et al.*, 1999).

However, defining the precise role of microarchitecture in prediction of the mechanical behavior of bone and its influence on fracture risk is complicated by the fact that microarchitecture characteristics are strongly correlated to each other and to bone density. Trabecular number, trabecular thickness, and connectivity all decline with decreasing density, whereas trabecular separation and anisotropy increase (Compston, 1994; Goulet *et al.*, 1994; Mosekilde, 1988, 1989; Snyder *et al.*, 1993; Ulrich *et al.*, 1998). Previous studies using architectural features derived from a model that assumes that trabecular bone architecture is "plate-like" suggested that architectural features provided only modest improvements in the prediction of mechanical properties over those provided by bone density alone (Goulet *et al.*, 1994; Snyder and Hayes, 1990). However, these previous findings should be interpreted with caution, because more recent data indicate significant differences in structural indices derived from the traditional plate-model compared with those computed directly from high-resolution three-dimensional images (Hildebrand *et al.*, 1999), and indices of trabecular structure determined directly from three-dimensional microcomputed architecture have been shown to significantly improve the prediction of the mechanical behavior of trabecular bone specimens from several skeletal sites (Majumdar *et al.*, 1998; Matsuura *et al.*, 2008; Newitt *et al.*, 2002; Ulrich *et al.*, 1999) and prediction of the strength of whole vertebrae (Hulme *et al.*, 2007).

Several studies have indicated that trabecular architecture differs in fracture subjects compared with those who have not suffered a fracture (Aaron *et al.*, 2000; Legrand *et al.*, 2000; Link *et al.*, 1999). However, few of these studies have controlled for the confounding influence of differences in bone density between the two groups, and few have investigated microarchitecture at the sites of fracture. Ciarelli and colleagues (2000) measured microarchitecture of trabecular bone specimens from the femoral neck in subjects with hip fracture compared with unfractured autopsy subjects. Whereas there were no differences in trabecular thickness, number, separatio, or connectivity among samples that were matched for equal bone density, the degree of anisotropy differed between the two groups even after controlling for density differences. These data suggest a role for trabecular architecture in the etiology of fractures that may be independent of changes in bone density. Recent *in vivo* studies also suggest that independent of bone mineral density (BMD) status, trabecular architecture is deteriorated in those with fragility fractures (Boutroy *et al.*, 2005; Link *et al.*, 2002; Sornay-Rendu *et al.*, 2007). Clearly, this is an area of great interest, and additional studies are required to define the role of *in vivo* assessments of trabecular architecture in the prediction of fracture risk.

As mentioned previously, because changes in trabecular architecture are strongly intercorrelated, it is difficult to discern the relative effect on bone strength of reductions in trabecular number versus trabecular thickness for both vertically and horizontally oriented trabecular struts. To address this issue Silva and Gibson (1997) developed a two-dimensional model of vertebral trabecular bone to simulate the effects of age-related changes in trabecular microstructure. They found that reductions in the number of trabeculae decreased vertebral bone strength two to five times more than reductions in trabecular thickness that resulted in an identical decrease in bone density (Fig. 7). For instance, removing longitudinally oriented trabecular elements to create a 10% reduction in density resulted in a 70% reduction in bone strength. In contrast, reducing trabecular thickness to achieve a 10% reduction in density resulted in only a 20% reduction in strength. This study implies that it is important to maintain trabecular number in order to preserve bone strength with aging. Consequently, therapies designed to counter age-related declines in bone strength should strive to maintain or restore the number of trabeculae, rather than just increasing the thickness of existing trabecular struts. A final aspect of trabecular architecture that may have been underappreciated initially is the potential detrimental effect of increased variability in trabecular thickness and number within a

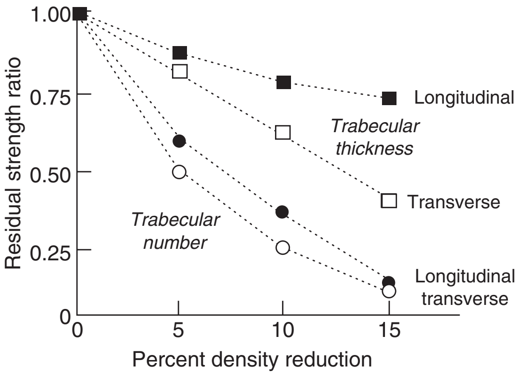


FIGURE 7 A plot of the predicted effect of bone density reductions, either by a reduction in trabecular thickness or loss of trabecular elements, on the strength of vertebral trabecular bone. (From Silva and Gibson, 1997). Strength reductions were at least twice as sensitive to changes in the number of trabeculae as to changes in the thickness of trabeculae. Findings were similar for loading in the transverse direction.

given trabecular bone specimen (Kothari *et al.*, 1999; Yeh and Keaveny, 1999).

Other microstructural and compositional features that influence the mechanical behavior of bone include the histological structure (primary versus osteonal bone), the collagen content and orientation of collagen fibers, the number and composition of cement lines, and the presence of fatigue microdamage. In reviewing the potential role of skeletal microdamage in age-related fractures, Burr and colleagues (1997) suggest that microdamage caused by repetitive loading of bone likely initiates at the level of the collagen fiber or below, and may include collagen fiber-matrix debonding, disruption of the mineral-collagen aggregate, and failure of the collagen fiber itself. They hypothesize that the accumulation and coalescence of these small defects eventually leads to microcracks that are visible under light microscopy. Laboratory studies have shown that damage accumulation in devitalized bone leads to a decrease in bone strength (Burr *et al.*, 1998; Carter and Hayes, 1977a; Hoshaw *et al.*, 1997; Wachtel and Keaveny, 1997). Thus, it has been hypothesized that the accumulation of microdamage *in vivo* may contribute to the increased fragility of the aging skeleton (Burr, 2003).

Microcracks occur naturally in human specimens from several anatomic locations, including trabecular bone from the femoral head, iliac crest, and vertebral body, as well as cortical bone from the femoral and tibial diaphyses (Chapurlat *et al.*, 2007; Courtney *et al.*, 1996; Fazzalari *et al.*, 1998; Mori *et al.*, 1997; Norman and Wang, 1997; Schaffler *et al.*, 1994, 1995; Stepan *et al.*, 2007; Wenzel *et al.*, 1996). It appears that the incidence of microcracks increases with age, probably in an exponential fashion, and that after age 40, microdamage may accumulate faster in women than in men (Norman and Wang, 1997; Schaffler *et al.*, 1995). Studies have also shown that the density of microcracks in the femoral head of older women is more than double that seen in younger women (Mori *et al.*, 1997), and that microdamage morphology differs between bone tissue from young versus old donors, with "diffuse damage" more prevalent in the young tissue and linear microcracks more prevalent in old tissue (Diab *et al.*, 2006; Diab and Vashishth, 2007). In addition, results of several studies indicate that microcracks accumulate more rapidly as bone mass decreases (Mori *et al.*, 1997; Stepan *et al.*, 2007; Wenzel *et al.*, 1996).

The effects of *in vivo* microdamage on bone mechanical properties have proved difficult to establish. In a series of studies investigating the consequences of suppressed bone remodeling by bisphosphonate treatment in dogs, Burr and colleagues have determined that one year of treatment leads to increased microdamage accumulation and reduced bone toughness in the rib (Mashiba *et al.*, 2000). In this study, a significant linear relationship between increased microdamage and decreased toughness was found. However, whereas bisphosphonate treatment was also found to reduce the toughness of vertebral trabecular bone, the treatment had no effect on work-to-failure and led to an increase in ultimate load for the vertebra (Mashiba *et al.*, 2001). Moreover, a longer-term follow-up study demonstrated that microdamage did not continue to accumulate between one and three years of treatment, yet toughness declined further over this period of time (Allen and Burr, 2007). These data indicate that the observed decrease in toughness may result from factors other than microdamage. In the context of evaluating the safety of long-term bisphosphonate treatment, it is important to note that these studies have used normal (i.e., gonadally sufficient) dogs and employed bisphosphonate doses equivalent to five to six times those used for treatment postmenopausal osteoporosis. Indeed, an investigation using doses comparable to those used clinically has indicated that, despite increases in microdamage accumulation with treatment, no adverse effect on bone mechanical properties results (Allen *et al.*, 2006).

Age-Related Changes in the Material Properties of Bone

The elastic modulus and ultimate strength of cortical (Burstein *et al.*, 1976; Currey, 1969; Lindahl and Lindgren, 1967; McCalden *et al.*, 1993; Smith and Smith, 1976; Zioupos and Currey, 1998) and trabecular (Bell *et al.*, 1967; Ding *et al.*, 1997; Mosekilde and Mosekilde, 1986, 1990; Mosekilde *et al.*, 1985, 1987) bone decrease with increasing age in both men and women. In human cortical bone from the femoral mid-diaphysis, the tensile and compressive strengths and elastic modulus decrease approximately 2% per decade after age 20 (Burstein *et al.*, 1976). In addition, the deformation incurred and energy absorbed before fracture decrease approximately 5% to 12% per decade,

TABLE I Age-Related Changes in Vertically Oriented Trabecular Bone Specimens that were Compressed in Either the Vertical or Horizontal Direction

	Vertical loading		Horizontal loading	
	% per decade	Correlation with age (*r*)	% per decade	Correlation with age (*r*)
Ash density	−8.7	−0.85 *	−8.7	not reported
Ultimate stress	−12.8	−0.79 **	−15.5	−0.87**
Elastic modulus	−13.5	−0.83 **	−15.9	−0.83**
Energy to failure	−14	−0.75 **	−15.2	−0.88**
Ultimate strain	+4	0.45 *	+3.1	0.30***

The mean percent change per decade and the linear correlation with age are presented. Specimens were taken from 42 persons, aged 15 to 87. (Data from Mosekilde *et al.*, 1987.)$^{*}P < 0.01$; $^{**}P < 0.001$; $^{***}0.05 < P < 0.06$

indicating that cortical bone becomes more brittle and less tough with increasing age (Burstein *et al.*, 1976; McCalden *et al.*, 1993; Zioupos and Currey, 1998). Indeed, the fracture toughness decreases by approximately 4% per decade (Zioupos and Currey, 1998), and the energy required to fracture a cortical bone specimen under impact loading decreases threefold between the ages of 3 and 90 (Currey, 1979). These changes in the elastic and ultimate properties of cortical bone are caused, in large part, by the increase in porosity with age (McCalden *et al.*, 1993). However, additional possible causes of the age-related decline in cortical bone properties include increased matrix mineralization and/or altered collagen cross-linking (Vashishth *et al.*, 2001).

Human trabecular bone exhibits a similar age-related decline in material properties (Ding *et al.*, 1997; Mosekilde and Mosekilde, 1986, 1990; Mosekilde *et al.*, 1985, 1987), primarily as a consequence of the decline in apparent density with age. For example, the density of vertebral trabecular bone declines approximately 50% from ages 20 to 80, and the material properties (compressive elastic modulus, ultimate stress, and energy to failure) decrease approximately 75% to 90% (Mosekilde *et al.*, 1987) (Table I). In trabecular bone of the proximal tibia, an age-related decline in apparent density of 25% is accompanied by a 30% to 40% reduction in compressive strength and energy absorption properties (Ding *et al.*, 1997). In addition, the strength anisotropy of trabecular bone from human lumbar vertebrae increases with age, as the ratio of compressive strengths of vertically and horizontally loaded specimens increases from about 2 at age 20 to 3.5 at age 80 (Arnold *et al.*, 1966; Mosekilde *et al.*, 1985). This observation may reflect age-related changes in the trabecular architecture of vertebral bodies, whereby horizontally oriented trabeculae thin and disappear to a greater extent than vertically oriented trabeculae (Mosekilde, 1988; Mosekilde *et al.*, 1987; Parfitt, 1984; Twomey *et al.*, 1983).

It is important to note that changes in density do not fully describe the age-related decline in trabecular bone mechanical properties. In trabecular bone specimens from the iliac crest that were matched pairwise for density, yield stress was approximately 40% lower in specimens from older donors (older than 60 years) compared with younger donors (younger than 40 years) (Britton and Davie, 1990). This difference between young and old bone may reflect differences in trabecular architecture and in the mechanical properties of trabecular tissue (the material comprising individual trabeculae). Although few data are available on the effects of age on trabecular tissue, results of a recent study indicate that the elastic modulus, yield stress, and yield strain of trabecular tissue are higher in osteoporotic versus normal trabecular bone (McNamara *et al.*, 2006). Overall, in general, it is believed that the elastic modulus and yield strain of trabecular tissue are slightly lower than those of cortical bone (Bayraktar *et al.*, 2004b; Guo and Goldstein, 1997).

MECHANICS OF WHOLE BONES

Diaphyseal fracture patterns

An important goal of producing fractures in the laboratory is to simulate the mechanisms of accidents that typically result in fractures and to produce types of fractures that are similar to those observed clinically. Numerous classifications of fractures exist and the classification strongly depends on the anatomical localization of the bone involved. For long bones, which are frequently involved in biomechanical testing, fractures can be classified based on morphological criteria related to the mechanism of the injury into transverse, oblique, spiral, or butterfly fractures (Fig. 8). Transverse fractures, which can be produced by tensile loading or pure bending, are characterized by a cortex that is broken once and a fracture line that is normal or slightly oblique with the longitudinal axis of the bone. During bending the transverse fracture line presents on the opposite site of the fulcrum, the bone fails in tension. In oblique fractures the fracture line ascends at an angle of approximately 30° or greater. Oblique fractures are most likely produced by pure compressive forces, but may occur under bending loads if bending is applied unevenly or with an additional compressive component. Spiral fractures have two distinct types of fracture lines visible on a projectional x-ray: a line spiraling around the entire bone and a longitudinal line linking the proximal and distal portions of the spiral. The angle of ascent may vary between 20° and 90° but is never transverse. Spiral fractures are produced by torsional loads or by a combination of torsional and axial loads. Butterfly fractures are characterized by at least one fragment in which one cortex is broken once and the other cortices are broken several times. They are typically produced by bending or axial compression with bending. Heavily comminuted fractures with undefined direction of the fracture line result from crush fractures in which the bone is compressed until failure perpendicular to its longitudinal axis.

The types of loading associated with clinically observed fractures at anatomic sites such as the distal radius, spine, and proximal femur are not as clear. For example, wedge fractures in the vertebra likely result from combined axial compression and anterior bending, whereas biconcave fractures may be caused by the relatively high compressive forces transferred from the nucleus pulposis to the center of the endplates. To some extent, these hypotheses are supported by results of *in vitro* experiments that determine what applied loading conditions produce gross fracture patterns comparable to those occurring *in vivo*. Similarly, laboratory tests on the proximal femur simulate gait or fall-loading conditions and are able to reproduce clinically observed fracture patterns.

Age-Related Changes in Bone Geometry

Age-related changes in the material properties of bone tissue are frequently accompanied by a redistribution of the cortical and trabecular bone material. It is likely that the structural rearrangement of bone tissue is driven both by “preprogrammed” behavior of the endosteal and periosteal bone cells as well as the local mechanical loading environment and biochemical signals. Hence, the adaptation pattern depends on age, gender, skeletal site, physical activity patterns, and expression (local and systemic) of cytokines and growth factors.

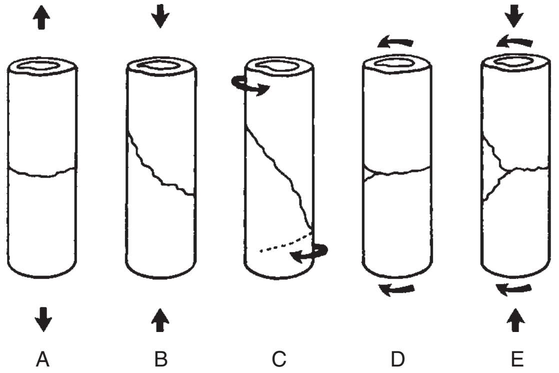


FIGURE 8 Fracture patterns in the diaphysis and associated loading conditions (Morgan *et al.*, 2008).

The general pattern of adaptation in the appendicular skeleton includes endosteal resorption and periosteal apposition of bone tissue (Fig. 9). Thus, the diameter of the bone increases, but the thickness of the cortex decreases. This redistribution of bone tissue away from the center of the bone allows the bone to better resist bending and torsional loads. Resistance to bending and torsional loading is particularly important, because the highest stresses in the appendicular skeleton are because of these loading modes (Martin, 1993). The most efficient design for resisting bending and torsional loads involves distributing the material far from the neutral axis of bending or torsion (generally the center of the bone). The distribution of mass about the center of a structural element is quantitatively described by the area moment of inertia. For example, consider three circular bars, each of the same length and composed of the same material (Fig. 10). The resistance of each bar to tensile and compressive loads is directly proportional to the cross-sectional area. However, the resistance to bending and torsional loads is influenced not only by how much bone (i.e., the cross-sectional area), but also by how it is distributed. Therefore, the bending and torsional rigidities of Bar C are twice that of Bar A owing to its greater moment of inertia.

Some studies indicate that both men and women exhibit endosteal resorption accompanied by periosteal expansion (Bouxsein *et al.*, 1994; Garn *et al.*, 1967; Ruff and Hayes, 1982, 1983; Smith and Walker, 1964), whereas others report that, in comparison with men, women exhibit lesser (Beck *et al.*, 1993; Martin and Atkinson, 1977; Meema, 1963; Seeman, 2001, 2002, 2003). Smith and Walker (1964) studied femoral radiographs of 2030 women aged 45 to 90 and reported that periosteal diameter and cortical cross-sectional area (assuming a circular cross-section) both increased approximately 11% in 35 years. Furthermore, the section modulus (an indicator of the resistance to bending loads) increased 32% in the same period. In contrast, direct assessment of cadaveric femurs and tibiae from 75 Caucasian adults showed that although

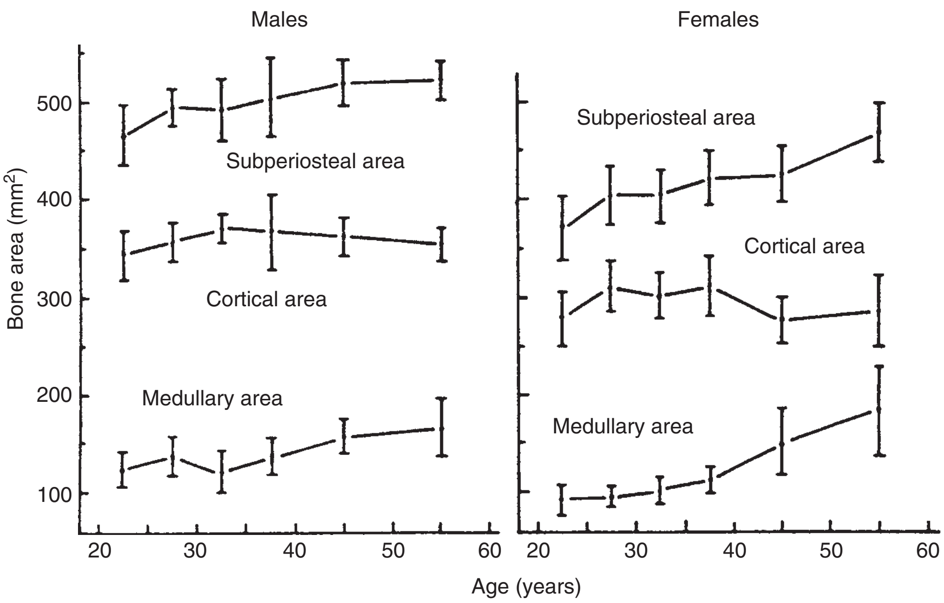
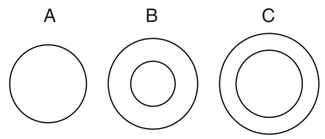


FIGURE 9 Age-related changes in the femoral midshaft demonstrating periosteal expansion and endosteal resorption. The data represent the mean ±2 standard errors. (From Ruff and Hayes, 1982, with permission).



	A	B	C
Area	1	1	0.87
Resistance to tensile and compressive loads	100%	100%	87%
Resistance to bending and torsional loads	100%	164%	208%

FIGURE 10 Illustration of the influence of cross-sectional geometry on the structural strength of circular structures.

both men and women undergo endosteal resorption and medullary expansion with age, only men show subperiosteal expansion and bone apposition at the femoral diaphysis (Ruff and Hayes, 1988). In men, cortical area is nearly constant and moments of inertia increase slightly with age. In women, however, both cortical area and moments of inertia decrease with age. The authors conclude, therefore, that in this sample from modern humans, only men exhibit bone-remodeling patterns that would compensate for the age-related decline in bone material properties in long bones (Ruff and Hayes, 1988).

In general, it is thought that men undergo the pattern of favorable geometric adaptation mentioned earlier to a greater extent than women, and that this may contribute to lower fracture rates in elderly men than women (Beck *et al.*, 1993; Duan *et al.*, 2001b; Kaptoge *et al.*, 2003; Ruff and Hayes, 1982, 1988; Seeman, 1997; Smith and Walker, 1964); however, recent data employing three-dimensional quantitative computed tomography challenge this paradigm (Riggs *et al.*, 2004; Sigurdsson *et al.*, 2006). Riggs *et al.* examined 373 women and 323 men aged 20 to 97 years and showed that, whereas women have greater declines in volumetric bone density, both men and women show similar age-related increases in bone area and moments of inertia at the femoral neck and vertebral body, demonstrating that the extent to which bone geometry exhibits favorable geometric changes with aging is similar in men and women (Riggs *et al.*, 2004). It is clear that carefully conducted longitudinal studies are needed to sort out this issue. However, because age-related expansion of bone size is subtle in the adult skeleton, a definitive study will require highly accurate measurements over several years duration. Indeed, a recent longitudinal study with measurements over approximately seven years showed that in postmenopausal women periosteal expansion does not compensate fully for endosteal resorption at the one-third radius site, and furthermore that women with the highest bone remodeling exhibited the greatest declines in cortical geometry (Szulc *et al.*, 2006).

As seen from the results of the previous studies, the sex-specific nature of age-related changes in skeletal structure remains controversial. The discrepancies in findings related to sex-specific bone adaptation patterns may be attributed to several factors. Most importantly, most of these studies use a cross-sectional design, thereby possibly introducing secular changes that confound the data and eliminating the possibility of a causal relationship with age. In addition, differences in methodology (direct versus *in vivo* measurements), subject populations (archaeological versus modern human specimens), and measurement site (metacarpal versus femoral shaft versus femoral neck) likely contribute to the conflicting findings. However, the extent to which age-related changes in bone geometry contribute to the increased fracture risk with increasing age is unknown.

Contributions of Cortical and Trabecular Bone

One of the foremost goals of research on the material properties of cortical and trabecular bone is to apply knowledge gained about these properties to the study of the biomechanics of whole bones. In this respect, it is of interest to determine the respective contributions of cortical and trabecular bone to whole-bone strength and stiffness. For the vertebra and proximal femur, the strength of the whole bone is predicted well simply by the density of the trabecular bone and measures of bone cross-sectional area (Brinckmann *et al.*, 1989; Cody *et al.*, 1991; Lotz *et al.*, 1990; McBroom *et al.*, 1985). However, research to date also suggests that the role of the cortical shell in the vertebra is complex and varies with age. No strong consensus exists as to how much the cortical shell contributes to the stiffness and strength of the vertebra (Eswaran *et al.*, 2006; McBroom *et al.*, 1985; Rockoff *et al.*, 1969; Yodanandan *et al.*, 1988). A recent micro-finite-element study estimated that the fraction of the applied compressive load borne by the shell varies from 0.38 to 0.54 across vertebrae (Eswaran *et al.*, 2006). Several studies have also indicated that the shell load fraction is maximal at the narrowest transverse cross-section of the vertebra (Cao *et al.*, 2001; Eswaran *et al.*, 2006; Homminga *et al.*, 2001; Silva *et al.*, 1997) and increases as the density and modulus of the trabecular bone decrease (Cao *et al.*, 2001; Faulkner *et al.*, 1991). This latter finding implies that the load-bearing capacity of the shell becomes increasingly important with age.

The respective contributions of trabecular and cortical bone in the proximal femur are similarly multifaceted. Finite-element analyses have indicated that for both gait and sideways fall-loading conditions, the cortical bone bears approximately 30% of the load in the subcapital region, 50% at the midpoint of the femoral neck, 96% at the base of the femoral neck, and 80% in the intertrochanteric region (Lotz *et al.*, 1995). Comparisons of stress and strain distributions in healthy versus osteoporotic femora indicate that although the distributions are similar, peak stresses and strains are elevated in the osteoporotic bones (Lotz *et al.*, 1995; Van Rietbergen *et al.*, 2003). Although the locations of the peak stresses and strains are not exclusively in the trabecular compartment, results of other finite-element studies indicate that, for a variety of loading conditions that simulate various types of falls, failure of the trabecular bone begins prior to or simultaneously with failure of the cortical shell (Ford *et al.*, 1996; Keyak *et al.*, 1998; Lotz *et al.*, 1991).

BIOMECHANICS OF AGE-RELATED FRACTURES

Applied Load versus Bone Strength: The Factor of Risk Concept

Strategies designed to prevent fractures must be based on a sound understanding of their etiology. From an engineering viewpoint, fractures of any type are caused by a structural failure of the bone. This failure occurs when the forces and/or moments applied to the bone exceed its load-bearing capacity. The load-bearing capacity of a bone depends primarily on the material that comprises the bone (and its corresponding mechanical behavior), the geometry of the bone (its size, shape, and distribution of bone mass), and the specific loading conditions. Thus, it is clear that factors related both to the forces applied to the bone, as well as to its load-bearing capacity are important determinants of fracture risk (Fig. 11). In support of this concept, clinical studies have repeatedly shown that factors related both to skeletal fragility as well as to the loads applied to the skeleton are important determinants of fracture risk (Bouxsein *et al.*, 2006b; Cumming and Klineberg, 1994; Greenspan *et al.*, 1994, 1998; Grisso *et al.*, 1991; Hayes *et al.*, 1993; Melton *et al.*, 2007; Nevitt and Cummings, 1993; Riggs *et al.*, 2006).

Insight into the relative contributions of skeletal fragility versus skeletal loading may be gained by using a standard engineering approach for evaluating the risk of structural failure, whereby the loads applied to the structure during its normal usage are compared with the loads known to cause failure. This comparison of applied load versus failure load gives an estimate of how "safely" the structure is designed. To apply these concepts in the study of the etiology of fractures, one can compute a "factor of risk," Φ, which is defined as the ratio of the load delivered to a bone (applied load) to the load-bearing capacity of that bone (failure load) (Bouxsein, 2007; Hayes, 1991):

$$\Phi = \text{applied load/failure load}$$

Theoretically, when the factor of risk is less than one, the forces applied to the bone are lower than those required to fracture it, and the bone is not at risk for fracture. However, when the factor of risk exceeds one (i.e., applied loads exceed bone failure load), fracture is predicted to occur. A high factor of risk can occur either when the bone is very weak and its load-bearing capacity is compromised, or when very high loads, such as those resulting from trauma, are applied to the bone. In elderly individuals, it is likely that the coupling of a weak bone with an increased incidence of traumatic loading leads to the dramatic rise in fracture incidence with age (Bouxsein *et al.*, 2006b; Riggs *et al.*, 2006).

To apply the factor-of-risk concept in studies of hip and vertebral fracture, the loads applied to the bone of interest and the corresponding load required to fracture the bone must

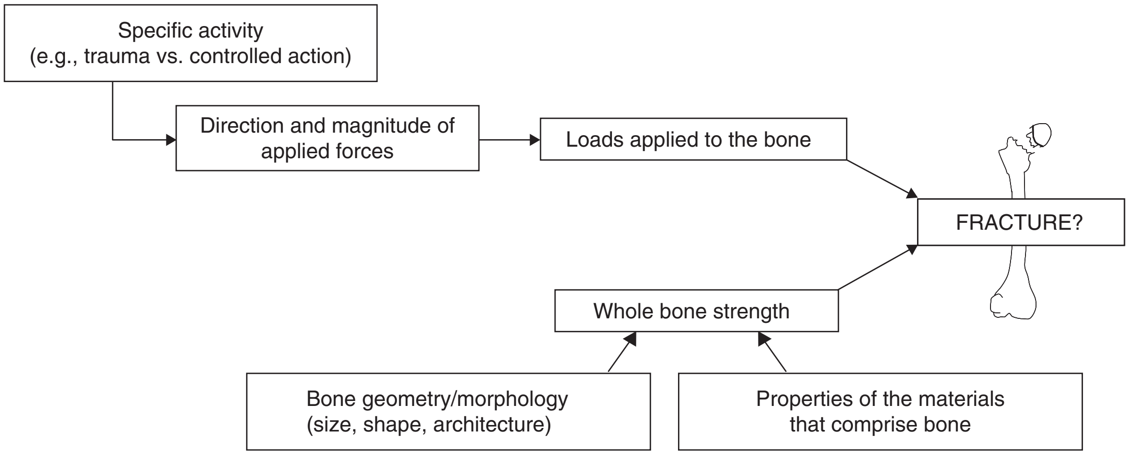

Specific activity
(e.g., trauma vs. controlled action)
Direction and magnitude of applied forces
Loads applied to the bone
FRACTURE?
Whole bone strength
Bone geometry/morphology (size, shape, architecture)
Properties of the materials that comprise bone

FIGURE 11 Etiology of fractures from biomechanical perspective. Fractures occur when the loads (or forces) applied to the bone exceed its strength (or structural capacity).

be identified. For example, the majority of hip fractures are associated with a fall. Therefore, to compute the factor of risk for hip fracture owing to a fall, information about the loads applied to the femur during a fall and about the load-bearing capacity of the femur in a fall configuration is required. Although this approach is relatively easy to conceive, in practice it is difficult to apply. There are surprisingly few data describing the magnitude and direction of loads applied to the skeleton during activities of daily living and even fewer data describing the loads engendered during traumatic events, such as a trip, slip, or fall. Moreover, because of the complex morphology of the skeleton and associated muscle and tendon attachments, it is difficult to design a laboratory study that mimics the loading environment encountered by the bone *in vivo*. Therefore, it is challenging to determine the load-bearing capacity of skeletal elements under realistic loading conditions. Moreover, because these are "biological structures," both the applied loads and structural capacity can change with aging, pharmacological intervention, and disease. Nevertheless, despite these uncertainties and limitations, rough estimates of the factor of risk for hip and vertebral fracture can be derived to provide insights into the complex roles of loading severity and skeletal fragility in the etiology of age-related fractures (Bouxsein *et al.*, 2006a, 2006b; Duan *et al.*, 2001a; Melton *et al.*, 2007; Riggs *et al.*, 2006).

Biomechanics of Hip Fractures

Recall that the "biomechanics" view of fractures states that a fracture occurs when the loads applied to the bone exceed its load-bearing capacity. Therefore, to study the etiology of hip fractures it is important first to identify what event(s) are associated with hip fractures, and then to determine the loads that are applied to the bone during that event, and what the load-bearing capacity of the femur is during that loading situation. It is estimated that more than 90% of hip fractures in the elderly are associated with a fall (Cummings *et al.*, 1990; Grisso *et al.*, 1991). Yet, fewer than 2% of falls in the elderly result in a hip fracture (Michelson *et al.*, 1995; Nevitt *et al.*, 1991; Tinetti, 1987), leading to the question of what is a "high-risk" fall with regard to hip fracture?

Several surveillance studies have been conducted to more fully characterize falls as they relate to hip fracture (Cumming and Klineberg, 1994; Greenspan *et al.*, 1994, 1998 ; Hayes *et al.*, 1993; Nevitt and Cummings, 1993; Nevitt *et al.*, 1991). For example, among nursing home residents, falling to the side and impacting the hip or side of the leg increased the risk of hip fracture approximately 20-fold relative to falling in any other direction (Hayes *et al.*, 1993). An increase in the potential energy content of the fall, computed from fall height and body mass, was also associated with an increased risk for fracture. Several studies have confirmed that falling to the side, versus falling in any other direction, is a risk factor for hip fracture (Greenspan *et al.*, 1994, 1998; Kannus *et al.*, 2006; Nevitt and Cummings, 1993).

Factors that Influence the Load Applied to the Femur in a Sideways Fall

Laboratory investigations have been conducted to explore the characteristics of sideways falls. In a study of the descent phase of sideways falls, young, healthy adults (age 19 to 30) were asked to fall sideways, as naturally as possible, onto a thick gymnastics mattress and impact velocities and energies that occur during falls from standing height were studied (van den Kroonenberg *et al.*, 1996). The impact velocity was 7% lower in "relaxed" than in "muscle-active" falls, and despite instructions to break the fall with an outstretched arm, only two of six subjects were able to do so (Fig. 12). In the remaining subjects hip impact occurred first, followed by impact of the arm or hand. Finally, in these young adults, approximately 70% of the total energy available was dissipated during the descent

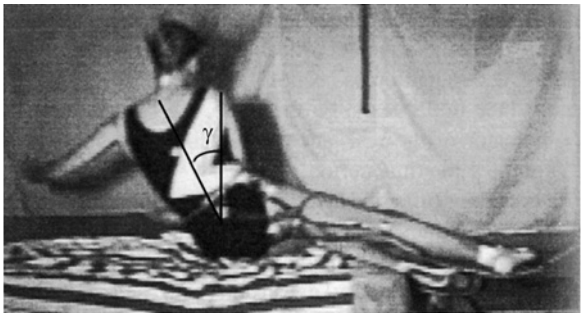


FIGURE 12 Example of a sideways fall onto a thick gymnastics mattress. Despite instructions to break the fall with the hand, only two of six subjects were able to do so. In the other subjects, hip impact occurred first, thus providing insight into the high-risk nature of sideways falls. (From van den Kroonenberg *et al.*, 1996, with permission).

phase of a sideways fall owing to muscle activity and the stiffness and damping characteristics of the hip and knee joints. It is likely that with age, the ability to dissipate energy during a fall and/or to activate protective responses will decrease, thus explaining why elderly individuals suffer more severe falls than young adults.

The forces applied to the proximal femur during a sideways fall depend not only on the dynamics of the descent phase of the fall, but also on characteristics of the impact phase of the fall. Using a system that allows impact forces from falls to be predicted with reasonable accuracy from the body's response to safe, simulated collisions, Robinovitch and colleagues (1991, 1995, 1997a, 1997b) found that during a sideways fall with impact to the greater trochanter, only about 15% of the total impact force is distributed to structures peripheral to the hip, whereas the remainder of the force is delivered along a load path directly in line with the hip (Robinovitch *et al.*, 1997a). The forces applied to the hip are reduced by increasing thickness of trochanteric soft tissues, but the force attenuation owing to trochanteric soft tissues alone is likely insufficient to prevent hip fracture in a fall where an elderly person lands directly on the hip (Robinovitch *et al.*, 1995). Biomechanical models developed to estimate peak impact forces delivered to the proximal femur during a sideways fall from standing height indicate that peak impact forces applied to the greater trochanter ranged from 2900 to 4260 N (≈650 to 960 lbs) for the 5th to 95th percentile woman, based on weight and height (van den Kroonenberg *et al.*, 1995).

Factors that Influence the Strength of the Proximal Femur

As mentioned previously, several factors contribute to the load-bearing capacity of the proximal femur, including its intrinsic material properties as well as the total amount (size) and spatial distribution (shape) of the bone tissue. Because the mechanical properties of both cortical and trabecular bone are strongly related to bone density, age-related bone loss has been argued to be a primary contributor to the steep increase in hip fracture incidence with age. Indeed, there is strong evidence from prospective clinical studies that low BMD is a risk factor for hip fracture (Cummings *et al.*, 2002; Marshall *et al.*, 1996).

Several laboratory studies have evaluated the load-bearing capacity of the proximal femur by using a configuration designed to simulate the single-leg stance phase of gait (Alho *et al.*, 1988; Bousson *et al.*, 2006; Cody *et al.*, 1999; Dalén *et al.*, 1976; Keyak *et al.*, 1998; Leichter *et al.*, 1982). The loads required to fracture the femur in this configuration range from approximately 1000 N to 13,000 N (225 to 3000 lbs). These studies demonstrated a strong relationship between the load required to fracture the femur in this stance configuration and noninvasive measurements of bone geometry and bone mineral density or content.

Other studies have evaluated the load-bearing capacity of the proximal femur in a configuration designed to simulate a sideways fall with impact to the greater trochanter (Bauer *et al.*, 2006; Bouxsein *et al.*, 1995a, 1995b; Cheng *et al.*, 1997a; Courtney *et al.*, 1994, 1995; Keyak *et al.*, 1998; Lochmuller *et al.*, 2002; Pinilla *et al.*, 1996). Tested at impact-loading rates, femurs from young individuals (age 17 to 51) are approximately 80% stronger than those from the older individuals (age 59 to 83) (Courtney *et al.*, 1995). Loading direction dramatically influences femoral failure loads. For instance, 1.5- to 2-fold greater loads are required to fracture femurs testing in a single-leg stance configuration than in a sideways fall configuration, further supporting the high risk of sideways fall in terms of hip fracture risk (Keyak *et al.*, 1998). Moreover, subtle differences in the direction of a sideways fall can influence femoral strength as much as 25 years of age-related bone loss (Pinilla *et al.*, 1996).

In addition to age, loading rate, and loading direction, femoral geometry also influences the load-bearing capacity of the proximal femur. The relationship between femoral geometry and load-bearing capacity is not unexpected. Because the load-bearing capacity is a structural property, it is influenced by the size of the specimen, and thus larger femurs have a greater load-bearing capacity. Femoral neck area, neck width, and neck axis length are all positively correlated with femoral failure loads (Bouxsein *et al.*, 1995b; Cheng *et al.*, 1997a; Courtney *et al.*, 1995; Pinilla *et al.*, 1996). However, additional laboratory studies are required to understand the complex relationship between hip geometry and femoral failure load.

Although it is important to understand what factors influence femoral strength in the laboratory environment, it is also critical to develop techniques that can be used clinically to predict femoral strength. Several studies have confirmed that noninvasive assessments of bone mineral density and geometry using dual-energy x-ray absorptiometry (DXA) or quantitative computed tomography (QCT) are strongly correlated to the strength of human cadaveric femurs. In particular, femoral bone mineral content and density explain between 40% and 80% of the variation in load-bearing capacity of the proximal femur (Bauer *et al.*, 2006; Bouxsein *et al.*, 1995b, 1999; Cheng *et al.*, 1997a; Cody *et al.*, 1999; Lochmuller *et al.*, 1998b). Application of QCT-based finite-element analysis may improve these predictions.

Interactions between Fall Severity and Femoral Strength: The Factor of Risk for Hip Fracture

Case-control studies have demonstrated the importance of both fall severity and bone mineral density as risk factors for hip fracture (Greenspan *et al.*, 1994, 1998; Nevitt and Cummings, 1993). Further insight may be achieved by considering a factor of risk for hip fracture. The previous two sections have described how laboratory techniques can be used to develop and validate methods for estimating the loads applied to the femur and the load-bearing capacity of the femur from data that can be acquired in a clinical setting. Thus, these findings can be used to estimate the factor of risk for hip fracture owing to a sideways fall from standing height.

A few studies have applied the factor-of-risk concept in a case-control study of individuals with hip fracture compared with those that are fracture free. The numerator of the factor of risk, the applied load, was estimated from previous studies of the descent and impact phases of a sideways fall with impact to the lateral aspect of the hip. Each individual's body height and weight was used as input parameters for the model to estimate the impact force delivered to the proximal femur during a sideways fall from standing height. The denominator of the factor of risk, or load-bearing capacity of the proximal femur, was determined from linear regressions between noninvasive bone densitometry and femoral failure loads in a fall configuration (Bouxsein *et al.*, 1999). Myers and coworkers (1994b) reported a strong association between the factor of risk and hip fracture in elderly fallers, with the odds of hip fracture increasing by 5.1 for a 1 SD increase in the factor of risk (95% confidence interval: 2.9, 9.2), whereas the odds ratio for a 1 SD decrease in femoral BMD was 2.0 (95% confidence interval: 1.4, 2.6). In another study, trochanteric soft tissue thickness, femoral BMD, and the ratio of fall force to femoral strength was compared in 21 postmenopausal women with incident hip fracture versus 42 age-matched controls (Bouxsein *et al.*, 2006a). Reduced trochanteric soft tissue thickness and low femoral BMD were associated with increased risk of hip fracture. The factor of risk for hip fracture was significantly higher (i.e., worse) in cases than controls (0.92 ± 0.44 vs. 0.6 5± 0.50, respectively, $P = 0.04$).

Biomechanics of Vertebral Fractures

Investigations of the etiology and biomechanics of vertebral fractures are particularly difficult, because the precise definition of a vertebral fracture remains controversial (Ferrar *et al.*, 2005). Second, a minority of radiographically evident vertebral deformities come to clinical attention (Cooper *et al.*, 1992; Delmas *et al.*, 2005; Nevitt *et al.*, 1998), although they are associated with significant morbidity and are strong predictors of future fracture risk (Delmas *et al.*, 2003; Klotzbuecher *et al.*, 2000; Nevitt *et al.*, 1998). Furthermore, few vertebral deformities are of acute onset; rather, they are believed to develop slowly over time, and therefore the activities associated with vertebral fracture are poorly understood.

In contrast to the growing recognition of the importance of bone fragility and fall severity in the etiology of hip fractures, the role of spinal loading in the etiology of age-related vertebral fractures has received relatively little attention. Because loads are applied to the spine during nearly every activity of daily living, it is crucial to distinguish which activities (and the resulting loads on the spine) are associated with vertebral fractures to try to understand the loading environment that leads to structural failure of the vertebrae.

Factors that Influence the Loads Applied to the Spine

Although no clinical study has yet examined the relative roles of bone fragility and load severity as risk factors for vertebral fracture, several investigators have reviewed medical records or interviewed patients to assess the "degree of trauma" associated with vertebral fractures (Bengnér *et al.*, 1988; Cooper *et al.*, 1992; Patel *et al.*, 1991; Santavirta *et al.*, 1992). Cooper *et al.* (1992) reviewed medical records from a 5-year period to determine the circumstances associated with "clinically diagnosed" vertebral fractures in a population-based sample of 341 Rochester, Minnesota, residents. In their study, a specific loading event was reported for approximately 50% of the total fractures (Table II). In contrast to the commonly held belief that lifting plays a major role in the development of vertebral fractures, relatively few of the fractures were associated with lifting. Excluding fractures that were diagnosed incidentally, only 10% of fractures were associated with "lifting a heavy object," whereas nearly 40% were associated with falling. In a hospital-based study, nearly 50% of acute, symptomatic vertebral fractures in individuals over age 60 were associated with a fall, whereas 20% were associated with "controlled" activities, such as bending, lifting, and reaching (Myers *et al.*, 1996). Therefore, determining the forces on the spine during controlled activities and falls may improve our understanding of the biomechanics of vertebral fractures.

Although it is impossible to measure the loads on the vertebral bodies *in vivo*, investigators have used kinematic

TABLE II Circumstances Associated with Clinically Diagnosed Vertebral Fractures

Reported activity/ circumstance	No. of persons	% of symptomatic fractures	% of total fractures
Pathological fracture	12	4	3.5
Traffic accident	20	7	6
Fall from greater than standing height	27	9	8
Fall from standing height or less	86	30	25
Lifting a heavy object	29	10	8.5
"Spontaneous"	113	39	33
Diagnosed incidentally (asymptomatic)	54	NA	16

Data from Cooper et al. (1992).

analysis, electromyographic measurements, and biomechanical modeling to estimate the loads on the lumbar spine during various activities (Adams and Dolan, 2005). These models were originally developed to study the potential origins and mechanisms of low-back pain and injury in working adults. Therefore, they are generally based on anthropometric data from young, healthy adults and are limited to estimating the vertebral forces in the lumbar region. However, Wilson (1994) extended these models to include the mid- and lower thoracic spine and incorporated geometric properties of the trunk by using QCT scans of older individuals. Using this model, the compressive forces applied to the T8, T11, and L2 vertebrae during various activities for a woman who weighed 65 kg and was 1.6 m tall (mean values from a cohort of 120 women aged 65 years or older) were computed (Table III). The estimated forces applied to the spine ranged from approximately 400 to 2100 N for typical activities. For example, rising from a chair without the use of one's hands results in compressive forces equal to 60% and 173% of body weight on the T11 and L2 vertebrae, respectively. Standing straight and holding an 8-kg weight with the arms slightly extended creates a compressive load on L2 equal to 230% of body weight, whereas flexing the trunk forward 30° and holding the same weight generates a compressive force on L2 of 320% of body weight. From these estimates, it is clear that everyday activities, such as rising from a chair or bending over and picking up a full grocery bag, can generate high forces on the spine. Although it may be highly relevant, there are no estimates of the load applied to the spine during falls.

Factors that Influence Vertebral Strength

The use of noninvasive assessments of skeletal status to predict vertebral strength *in vivo* is based on the assumption that much of the variability in the strength of whole vertebrae can be explained by variations in bone mineral density and/or geometry. As in other skeletal structures, the load-bearing capacity of a whole vertebra is determined by its intrinsic material properties, as well as its overall geometry and shape. In the spine, compressive loads are transferred from the intervertebral discs to adjacent vertebral bodies. Therefore, age-related changes in the properties of the intervertebral disc, the vertebral centrum, and the vertebral shell can each influence the load-bearing capacity of the vertebrae. For instance, the thickness of the shell decreases from approximately 400–500 μm at age 20 to 40, to 200–300 μm at age 70 to 80, and to 120–150 μm in osteoporotic individuals; whereas bone density of the vertebral centrum declines from 15% to 20% at age 20 to 40 to 4% to 8% in osteoporotic individuals (Mosekilde, 1998).

A number of laboratory studies have investigated the relationships among the strength of human lumbar and thoracic vertebrae and age, bone density, and vertebral geometry (Bell *et al.*, 1967; Biggemann *et al.*, 1988, 1991; Cheng *et al.*, 1997b; Cody *et al.*, 1991; Crawford *et al.*, 2004; Lochmuller *et al.*, 1998a; McBroom *et al.*, 1985; Moro *et al.*, 1995; Mosekilde *et al.*, 1985; Myers *et al.*, 1994a; Vesterby *et al.*, 1991). These studies indicate that the strength of thoracolumbar vertebrae is reduced from a value of 8000–10,000 N at age 20 to 30 to 1000–2000 N by age 70 to 80 (Biggeman and Brinckman, 1995; Mosekilde, 1998), and in severely osteoporotic individuals, the load-bearing capacity may be even less (Moro *et al.*, 1995).

The strength of human vertebrae is strongly correlated with noninvasive estimates of vertebral bone density and geometry, with approximately 50% to 80% of the variance in load-bearing capacity explained by parameters measured noninvasively. For example, strong correlations have been

TABLE III Predicted Compressive Loads on the L2 and T11 Vertebrae during Various Activities

Activity	Predicted load on T11		Predicted load on L2	
	N	% of body weight	N	% of body weight
Relaxed standing	240	41	290	51
Rising from a chair, without use of hands	340	60	980	173
Standing, holding 8-kg weight close to body	320	57	420	74
Standing, holding 8-kg weight with arms extended	660	117	1302	230
Standing, trunk flexed 30°, arms extended	370	65	830	146
Standing, trunk flexed 30°, holding 8-kg weight with arms extended	760	135	1830	323
Lift 15-kg weight from floor, knees bent, arms straight down	593	104	1810	319

The loads were computed from the model developed by Wilson (1994) *for a woman who weighs* 58 *kg and is* 162 *cm tall.*

reported between vertebral failure loads and (1) bone density and vertebral cross-sectional area assessed by QCT (Brinckmann *et al.*, 1989; Buckley *et al.*, 2007; Cody *et al.*, 1991), (2) bone mineral density assessed by DXA (Cheng *et al.*, 1997b; Lochmuller *et al.*, 1998a; Moro *et al.*, 1995; Myers *et al.*, 1994a; Tabensky *et al.*, 1996), and (3) estimated vertebral strength by QCT-based finite-element analysis (Buckley *et al.*, 2007; Crawford *et al.*, 2004). Thus, it appears that noninvasive assessments of bone mass and bone mineral density provide a reasonable estimate of the failure loads of cadaveric vertebrae subjected to controlled compression tests in the laboratory. It remains to be seen whether BMD or other bone density parameters can predict the strength of vertebrae subjected to loading conditions that more closely resemble those occurring *in vivo*, such as falling or compression combined with forward flexion or compression combined with lateral bending.

Interactions between Spinal Loading and Vertebral Strength: The Factor of Risk for Vertebral Fracture

Although it has not been clearly demonstrated by clinical surveillance studies, it seems reasonable to suggest that, similar to hip fractures, both bone fragility and skeletal loading are important factors in the etiology of vertebral fractures. To explore this, Myers and Wilson (1997b) examined relationships between spinal loading and vertebral fragility by computing the factor of risk for vertebral fractures, Φ, defined as the ratio of applied forces to failure load, for a various activities of daily living. Their analyses predicted that for very low BMD values, a woman would be at high risk for vertebral fracture during many routine activities of daily living (Fig. 13). Individuals with extremely low bone mineral density may be at risk for vertebral fracture during simple activities such as tying one's shoes or opening a window. Individuals with low bone mineral density (but still in the osteopenic range) may be at risk for vertebral fracture when lifting groceries out of the car or picking up a toddler. These examples illustrate the need for strategies to prevent vertebral fractures, such as reducing spinal loading by avoiding certain "high-risk" activities.

Application of the factor of risk for vertebral fracture in a population-based sample of 697 women and men aged 20 to 97 years (Bouxsein *et al.*, 2006b) showed that men had a higher predicted vertebral strength at all ages, largely owing to their greater vertebral cross-sectional area. Whereas both sexes exhibited a marked decline in lumbar vertebral compressive strength with age ($P < 0.001$), the decline was greater in women than men (-49% versus -31%, $P < 0.001$). The factor of risk for vertebral fracture increased with age in both sexes, but significantly more so in women than in men, such that for bending forward and lifting, the factor of risk exceeded the fracture "threshold" of one in 31% of women and 12% of men who were 50 years and older, values that are similar to the reported prevalence of vertebral fracture (Fig. 14). Moreover, the estimated factor of risk for vertebral fracture

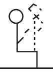
BMD (g/cm^2)		0.3	0.4	0.5	0.6	0.7	0.8	0.9
Get up from sitting		1.5	0.6	0.4	0.3	0.2	0.2	0.2
Lift 15 kg knees straight		2.6	1.1	0.7	0.5	0.4	0.3	0.3
Lift 15 kg w/ deep knee bend		2.1	0.9	0.6	0.4	0.3	0.3	0.2
Lift 30 kg knees straight		3.7	1.5	1.0	0.7	0.6	0.5	0.4
Lift 30 kg w/ deep knee bend		3.0	1.3	0.8	0.6	0.5	0.4	0.3
Open window w/ 50 N of force		1.1	0.5	0.3	0.2	0.2	0.1	0.1
Open window w/ 100 N of force		1.4	0.6	0.4	0.3	0.2	0.2	0.2
Tie shoes sitting down		1.4	0.6	0.4	0.3	0.2	0.2	0.2

FIGURE 13 Factor of risk for vertebral fracture for eight common activities as a function of lumbar bone mineral density. The numerator of the factor of risk was determined from models of spine loading at L2 for an elderly woman of average height and weight. The denominator was determined on the basis of regression analysis between lateral lumbar BMD and the load-bearing capacity of the L2 vertebrae. The values for lateral BMD cover a wide range, including very low values. The *t*-score (number of standard deviations from the mean value for BMD in young women) is approximately +1 for a BMD = 0.9 g/cm^2 and is −5 for BMD = 0.4 g/cm^2. The factor of risk is predicted to be greater than or close to 1 for low BMD values (i.e., fracture predicted to occur, shaded area). (From Myers and Wilson, 1997a, with permission).

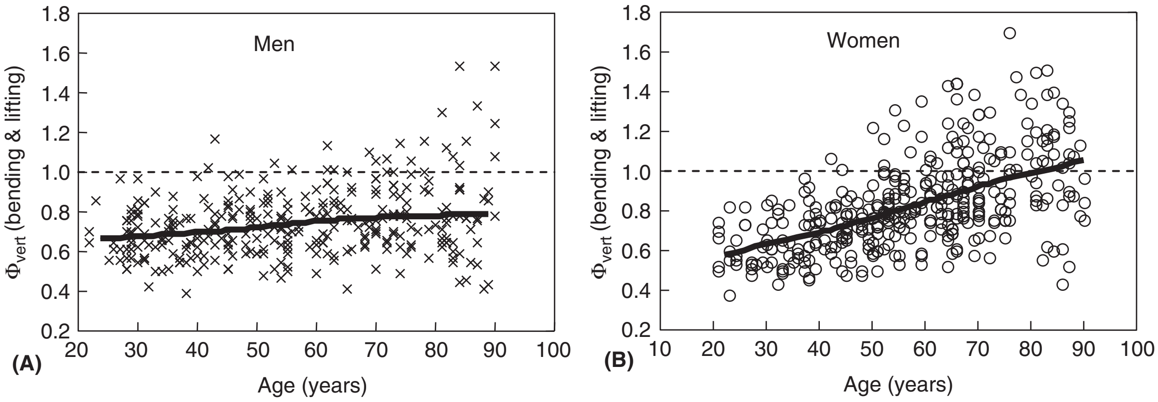


FIGURE 14 Factor of risk for vertebral fracture in men **(A)** and women **(B)** aged 20 to 97 for the activity of bending forward and lifting 10 kg. Note the marked age-related increase in the factor of risk in women. (Data from Bouxsein *et al.*, 2006b, with permission).

was significantly higher in subjects with prevalent vertebral fractures than in those with no fractures (Melton *et al.*, 2007).

SUMMARY AND CLINICAL IMPLICATIONS

When studying the mechanical behavior of whole bones, one must consider the material properties of bone tissue in addition to whole-bone geometry. Both of these factors contribute to the structural behavior of the whole bone, and both can change with age. Basic descriptions of bone material properties include measures of the elastic, yield, and postyield behavior. Both cortical and trabecular bone are anisotropic, and although these tissues are frequently tested in the primary loading direction, the material properties in other directions can be highly relevant in scenarios such as falls, when the applied loads are oblique to the primary loading direction. In addition, stresses and strains that occur in bone tissue *in vivo* can be multiaxial, and comparatively little is known about bone material behavior under general multiaxial stress and strain states once the elastic region has been exceeded.

Many aspects of the composition and microstructure profoundly affect bone material properties. Variations among bone specimens in characteristics such as porosity, mineralization, and trabecular architecture can explain much of the heterogeneity in bone material properties and also much of the age-related decline in these properties. However, the effects of composition and microstructure on the postyield behavior of cortical bone and trabecular tissue are less well understood. In addition, the mechanical consequences of microdamage accumulation *in vivo* are still unclear.

Age-related changes in bone material properties are accompanied by subtle but significant changes in how cortical and trabecular bone are distributed throughout the whole bone. This redistribution can alter the way in which the applied loads are shared between the cortical shell and trabecular compartment in bones such as the vertebra and proximal femur. Evidence to date suggests that this redistribution also results in changes in bone geometry that compensate for, or at least mitigate, the decline in the mechanical competence of bone tissue with age. The sex-specific nature of this compensatory mechanism remains controversial, and noninvasive methods for accurate quantification of changes in bone geometry would tremendously benefit longitudinal studies of this type of bone adaptation.

In this chapter, we have emphasized the concept that age-related fractures represent a structural failure whereby the forces applied to the bone exceed its load-bearing capacity. Viewing fractures in this manner, it is clear that studies of their etiology must include both factors that influence skeletal fragility, as well as those that influence the forces that are applied to the skeleton. Biomechanically based estimates of fracture risk that incorporate both skeletal loading and bone strength may improve identification of those at greatest risk, and also enhance interpretation of therapeutic interventions.

To date investigators have focused primarily on methods to prevent bone loss and to restore bone to the osteopenic skeleton. However, alternative approaches for fracture prevention that are directed at reducing the loads applied to the skeleton may prove to be both effective and cost-efficient. Ultimately, fracture prevention may be best achieved by an educational program designed to limit high-risk activities in conjunction with interventions targeted at increasing bone mass and reducing loads applied to the skeleton during traumatic events.

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Chapter 3

Embryonic Development of Bone and Regulation of Intramembranous and Endochondral Bone Formation

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INTRODUCTION

The skeletal system is multifunctional in that it provides the rigid framework and support that gives shape to the body, serves to protect delicate internal organs, endows the body with the capability of movement, acts as the primary storage site for mineral salts, and functions in hematopoiesis. The vertebrate skeleton comprises two main subdivisions, the axial and appendicular components. The axial skeleton encompasses the skull, spine, sternum, and ribs, whereas the appendicular skeleton defines the bones of the extremities. The skull, in turn, is best regarded as consisting of two units: the chondrocranium, whose elements first develop in cartilage and include the cranial base and capsules surrounding the inner ears and nasal organs, and the cranial vault and most of the upper facial skeleton, which arise from the direct conversion of undifferentiated mesenchymal cells into bone.

Skeletal cells are derived from three distinct embryonic cell lineages: neural crest cells contribute to the craniofacial skeleton; sclerotome cells from somites give rise to the axial skeleton; and lateral plate mesoderm cells form the appendicular component. Bone formation arising from a cartilaginous template is referred to as endochondral ossification. This is a complex, multistep process requiring the sequential formation and degradation of cartilaginous structures that serve as templates for the developing bones. Formation of calcified bone on a cartilage scaffold, however, occurs not only during skeletogenesis but is also an integral part of postnatal growth, bone remodeling, and fracture repair. Intramembranous bone differs from the endochondral component in that it is formed in the absence of cartilaginous anlagen. Rather, it arises directly from mesenchymal cells condensing at ossification centers and being transformed directly into osteoblasts.

The organization and morphology of the developing skeleton are established through a series of inductive interactions. The functional elements in these inductive and morphogenetic processes are not individual cells but rather interacting populations that elaborate an extensive extracellular matrix that, in turn, feeds back onto these matrix-producing cells and controls their differentiation potential. Over the past 15 years, considerable insight has been gained into the molecular mechanisms that control these developmental programs. Genetic and biochemical analyses of human heritable skeletal disorders in concert with the generation of transgenic and knockout mice have provided useful tools for identifying key molecular players in mammalian skeletogenesis. It is the nature and interplay of these signaling cascades controlling skeletal patterning and cellular differentiation that will be the focus of this chapter.

THE AXIAL SKELETON

Somitogenesis

A defining feature of the vertebrate body plan is metameric segmentation of the musculoskeletal and neuromuscular systems. The origin of this basic anatomic plan during embryogenesis is segmentation of the paraxial mesoderm (for reviews, see Burke, 2000; Christ *et al.*, 1998). Upon gastrulation, paraxial mesoderm cells segregate from axial and lateral mesoderm to form two identical strips of unsegmented tissue (referred to as presomitic mesoderm in the mouse embryo or segmental plate in the avian embryo) on

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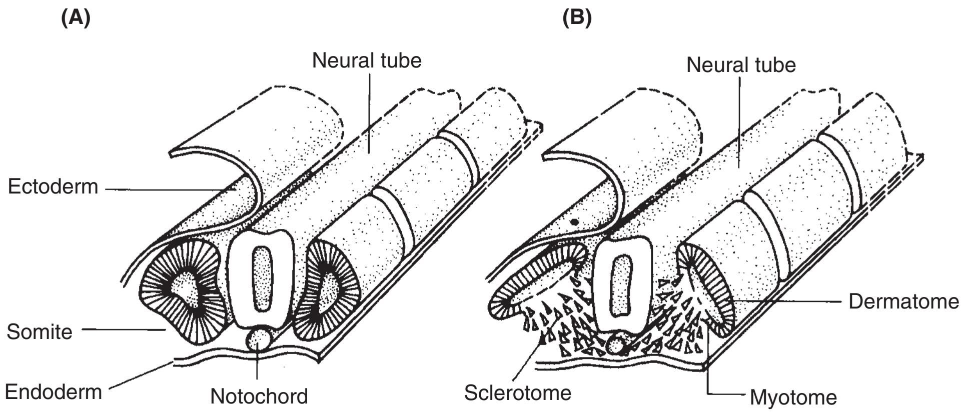

FIGURE 1 Diagrammatic representation of sclerotome formation. (**A**) Organization and differentiation of somites in the trunk region of the mouse embryo. (**B**) Ventral medial somite cells (those further away from the back and closer to the neural tube) undergo mitosis, migrate ventrally, lose their epithelial characteristics, and become mesenchymal cells that give rise to the sclerotome. They ultimately become the vertebral chondrocytes that are responsible for constructing the axial skeleton (vertebrae and ribs). The notochord provides the inductive signal by secreting SHH. After formation of the vertebral bodies, the notochordal cells die, except in between the vertebrae where they form the intervertebral discs. Adapted from Hogan *et al.* (1994), Fig. 26, p. 77).

either side of the neural tube. Paraxial mesoderm in vertebrates gives rise to the axial skeleton as well as all trunk and limb skeletal muscles and portions of the trunk dermis and vasculature. Through a series of molecular and morphogenetic changes this unsegmented tissue is converted into a string of paired tissue blocks on either side of the axial organs, called somites (Fig. 1A). The process, referred to as somitogenesis, occurs sequentially by the addition of new somites in a strict craniocaudal (head-to-tail) direction along the body axis with a periodicity that reflects the segmental organization of the embryo (see later). The recruitment of new presometic tissue from the primitive streak into the posterior end of the presometic mesoderm, as well as cell division within it, permits the presomite mesoderm to maintain its longitudinal dimension as somite budding is taking place anteriorly. Somite formation is preceded by epithelialization of the presometic mesoderm so that a new pair of somites is formed when cells are organized into an epithelial sphere of columnar cells enveloping mesenchymal cells within the central cavity, the somitocoel. This epithelial structure, however, is not maintained because somite maturation is accompanied by a commitment of its cells to different lineages in response to signals that arise from adjacent tissues. Cells on the ventral margin undergo an epitheliomesenchymal transition as they disperse and move toward the notochord, giving rise to the sclerotome, which serves as the precursor of the vertebrae and ribs (see Fig. 1B). The dorsal epithelial structure of the somite is maintained in the dermomyotome which eventually gives rise to the epaxial muscles of the vertebrae and back (medial myotome), the hypaxial muscles of the body wall and limbs (lateral myotome), and the dermis of the skin of the trunk (dermatome).

Somitogenesis has long been known to be driven by mechanisms intrinsic to the presomitic mesoderm. Although many of the elements of the network have been identified, their interaction and their influence on segmentation remain poorly understood. Although mathematical models have been proposed to explain the dynamics of subsets of the network (Baker *et al.*, 2008; Cinquin, 2007), the mechanistic bases remain controversial. In general, two distinct molecular pathways have been implicated in vertebrate segmentation. The first is referred to as the oscillator or "segmentation clock" (McGrew *et al.*, 1998; Palmeirim *et al.*, 1997; Pourquie, 1999). This clock corresponds to a molecular oscillator identified on the basis of rhythmic production of mRNAs for the basic helix-loop-helix (bHLH) Notch target genes *mHes1*, *mHes7*, the glycosyltransferase Notch modifier *Lunatic fringe* (*mLfng*), the Notch ligand *Delta-like 1* (*Dll1*), as well as genes in the Wnt signaling pathways (Aulehla *et al.*, 2008), and also the fibroblast growth factor (FGF) pathway (Dequeant *et al.*, 2006). It is the coordinated regulation of these three pathways that underlies the clock oscillator although it is not known, in any species, which set of genes "drives" the oscillations. Nevertheless, the expression of these genes appears as a wave that arises caudally and progressively sweeps anteriorly across the presomitic mesoderm (Fig. 2(A). Although a new wave is initiated once during the formation of each somite, the duration of the progression of each wave equals the time to form two somites. This wave does not result from cell displacement or from signal propagation in the presomitic mesoderm but rather reflects intrinsically coordinated pulses of *Lunatic fringe* expression.

The second pathway implicated in somitogenesis is *Notch/Delta-like 1* signaling, an essential regulator of paraxial mesoderm segmentation (see Fig. 2B). It centers on a large transmembrane receptor called Notch, which is able to recognize the transmembrane ligand, Delta-like 1. Upon ligand binding, Notch undergoes a proteolytic cleavage at

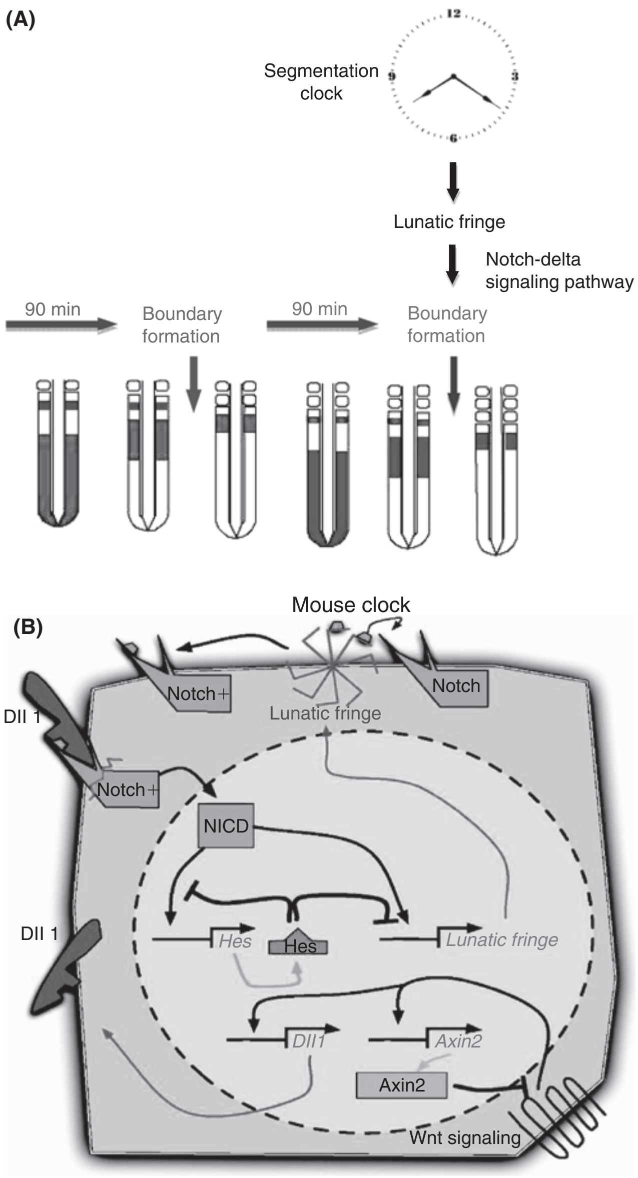

FIGURE 2 Proposed role of the segmentation clock in somite boundary formation. **(A)** Waves of *c-hairy* and Lunatic *Fringe* expression arise caudally and get narrower as they move anteriorly. A wave completes this movement in the time required to form two somites. Boundary formation occurs when the wave has reached its most rostral domain of expression where it is associated with rhythmic activation of the Notch signaling pathway that endows cells with setting-of-boundary properties. Adapted and modified from Pourquié (1999, Fig. 1, p. 560). **(B)** The mouse clock. The molecular mechanisms that have been proposed to drive the oscillation in mouse clocks. Mouse *hairy, hey*, and *hes* genes are summarized as Hes. NICD, Notch intracellular domain. See text for details. Adapted from Cinquin (2007) Fig. 2, p. 504.

the membrane level leading to the translocation of its intracytoplasmic domain into the nucleus. In the mouse, this leads to activate expression of downstream genes such as *Hes1/Hes7*, transcriptional repressors of the bHLH family downstream of Notch signaling. Many of the genes in this pathway are expressed strongly in the presomitic mesoderm and mutation studies in the mouse have established their role in the proper formation of rostral-caudal compartment boundaries within somites, pointing to a key role for a Notch signaling pathway in the initiation of patterning of vertebrate paraxial mesoderm (Barrantes *et al.*, 1999; Conlon *et al.*, 1995; Kusumi *et al.*, 1998; Yoon and Wold, 2000). It is now recognized that *Lunatic fringe* is the link between the *Notch* signaling pathway and the segmentation clock as its promoter is directly upregulated by Notch signaling and, in turn, Lunatic fringe modifies the Notch receptor post-translationally, making it more sensitive to activation by its ligand Delta-like 1.

Wnt/β-catenin signaling is cyclic in mouse presomitic mesoderm (Aulehla *et al.*, 2003), and induces cyclic transcription of the Wnt signaling inhibitors *Axin2*, *Nkd1*, *Dact1*, and *Dkk1*. *Axin2* is functionally equivalent to *Axin*, a critical component of the Wnt signaling pathway that acts as a scaffold for the β-catenin destruction complex. It has been proposed that *Axin2* and Wnt signaling form a negative feedback loop responsible for driving the mouse somitogenesis clock. Wnt signaling would drive transcription of *Axin2* mRNA, and Axin2 protein would inhibit Wnt signaling. However, *Axin2* mutants have no somitogenesis phenotype (Yu *et al.*, 2005), which suggests that *Axin2* may function in the clock redundantly with other genes. Recently it was shown that a Wnt signaling gradient is established through a nuclear β-catenin protein gradient in the posterior presomitic mesoderm. This gradient of nuclear β-catenin defines the size of the oscillatory field and controls key aspects of presometic mesoderm maturation and segment formation, emphasizing the central role of Wnt signaling in this process (Aulehla *et al.*, 2008).

As discussed previously, it is *Lunatic fringe* that delimits domains of Notch activity, thereby allowing for the formation of the intersomitic boundaries characterized morphologically by the epithelization event (for review, see Cinquin, 2007; Pourquie, 1999). Epithelization requires the expression of the gene *paraxis*. Paraxis is a bHLH transcription factor expressed in paraxial mesoderm and somites. In mice homozygous for a paraxis-null mutation, cells from the paraxial mesoderm are unable to form epithelia and so somite formation is disrupted (Burgess *et al.*, 1996). In the absence of normal somites, the axial skeleton and skeletal muscle form but are improperly patterned. Sosic *et al.* (1997) have shown that *Paraxis* is a target for inductive signals that arise from the surface ectoderm. The nature of this signal was recently reported to involve the β-catenin pathway. Wnt6 in the ectoderm overlaying the somites is responsible for the maintenance of the epithelial structure of the dorsal compartment of the somite: the dermomyotome (Linker *et al.*, 2005). The Wnt receptor molecule Frizzled7 transduces the Wnt6 signal. Intracellularly, this leads to the activation of the β-catenin/LEF1-dependent pathway. Hence, β-catenin activity, initiated by Wnt6 and mediated by paraxis, is required for the maintenance of the epithelial structure of somites.

Specifying the Anterior-Posterior Axis

Initially, somites at different axial levels are almost indistinguishable morphologically and eventually give rise to the same cell types such as muscle, bone, and dermis. A great deal of research has therefore focused on identifying factors that dictate the ultimate differentiation fate of somitic cells as well as the overall patterning of the body plan. Burke (2000) has proposed that correct pattern requires two levels of information. At one level, short-range, local signals could dictate to a cell to differentiate into a chondroblast instead of a myoblast. These signals, however, would not contain all of the information required to also bestow regional identity to this chondrocyte and ensure that it would, for example, contribute to the development of the appropriate vertebral body, be that cervical, thoracic, or lumbar. In fact, additional information would be needed to provide global landmarks and ensure correct pattern formation.

Patterning of somites extends beyond the formation of distinct epithelial blocks. Early on they acquire cues that dictate anteroposterior as well as dorsoventral position. What signals are important for regional specification of segments along the anterior-posterior dimensions into occipital, cervical, thoracic, lumbar, and sacral domains? This regionalization is, in part, achieved by specific patterns of *Hox* gene expression (for reviews, see Mark *et al.*, 1997; McGinnis and Krumlauf, 1992; Veraksa *et al.*, 2000; Wellik, 2007). All bilateral animals, including humans, have multiple *Hox* genes, but in contrast to the single *Hox* cluster in *Drosophila* and other invertebrates, four clusters of *Hox* genes, *HOXA*, *HOXB*, *HOXC*, and *HOXD* have been identified in vertebrates. The mammalian *HOX* genes are numbered from 1 to 13, starting from the 3′ end of the complex. The equivalent genes in each complex (*HOXA-1*, *HOXB-1*, and *HOXD-1*) are referred to as a paralogous group. Comprising a total of 39 genes in humans, these clusters are arranged such that all the genes in each cluster are oriented in the same 5′ to 3′ direction. Moreover, genes located at the 3′ end of the cluster are expressed prior to and extend more anteriorly in the developing embryo than those at the 5′ end. The high degree of evolutionary conservation of homeotic gene organization and transcriptional expression pattern of these genes in flies and mammals argues strongly for a common scheme in anteroposterior axis formation.

How do *Hox* genes dictate pattern formation? It appears that there is a code of *Hox* gene expression that determines the type of vertebrae along the anterior-posterior axis. For example, in the mouse, the transition between cervical and thoracic vertebrae is between vertebrae 7 and 8, whereas in the chick, it is between vertebrae 13 and 14. In either case, the *Hox-5* paralogues are seen in the last cervical vertebra and the *Hox-6* paralogues extend up to the first thoracic vertebra, their anterior boundary. Changes in the *Hox* code lead to shifting in the regional borders and axial identities, otherwise known as homeotic transformations. Therefore, *Hox* loss of function results in the affected body structures resembling more anterior ones, whereas gain-of-function mutant phenotypes owing to ectopic expression of more posterior *Hox* genes cancel the function of more anterior ones and specify extra posterior structures. Persistent expression of *Hox* genes in discrete zones on the anteroposterior axis is required to remind cells of their position identity along the axis. Hox proteins are all transcription factors that contain a 60-amino-acid motif referred to as the homeodomain and exert their effect through activation and repression of numerous target genes. In mammals, little is known about the upstream mechanisms that initiate *Hox* gene expression (Manzanares *et al.*, 1997; Marshall *et al.*, 1996). More is known about factors involved in the maintenance of *Hox* expression in both flies and mice (see Veraksa *et al.*, 2000, and references therein). Studies with the Trithorax and Polycomb protein groups indicate that the former functions as transcriptional activators and the latter functions as transcriptional repressors of the *Hox* genes. In loss-of-function mutants for *Polycomb* genes *Bmi1* and *eed*, the domain of expression of the *Hox* gene is expanded causing homeotic transformation and, conversely, loss of the *Trithorax*-group gene *Mll* results in diminished levels of expression of the *Hox* gene with the phenotype resembling the mutants of the *Hox* gene themselves. Interestingly, the axial-skeletal transformations and altered *Hox* expression patterns of *Bmi1*-deficient and *Mll*-deficient mice are normalized when both *Bmi1* and *Mll* are deleted, demonstrating their antagonistic role in determining segmental identity (Hanson *et al.*, 1999). In summary, repeated identical units formed by the action of segmentation genes become different owing to *Hox* gene action.

Specifying the Dorsal-Ventral Axis

The newly formed somites comprise a sphere of columnar epithelial cells and a central cavity, the somatocoel, containing mesenchymal cells. Early somites are characterized by the expression of the *Pax3* gene. Following somite formation, however, expression of the gene is downregulated in the ventral half of the somite epithelium and in the somatocoel cells, whereas it persists in the dorsal half of the somite. The ventral medial cells of the somite subsequently undergo mitosis, lose their epithelial characteristics, and become mesenchymal cells again. This epitheliomesenchymal transition of the ventral part of the somite is preceded by the expression of *Pax1* in the somitic ventral wall and somatocoel cells as it signals the beginning of sclerotome formation. Mutations in *Pax1* affect sclerotome differentiation, as reported with different mutations in the *undulated* (*un*) locus (Balling *et al.*, 1988). Successful dorsoventral compartmentalization of somites ultimately leads to the development of the sclerotome ventrally and the dorsally located dermomyotome.

In the fourth week of human development, cells from the somites migrate to the most ventral region of the somite in an area surrounding the notochord forming the ventral sclerotome (see Fig. 1B). These mesenchymal cells will differentiate to prechondrocytes and ultimately form the template of vertebral bodies and ribs. Initiation of sclerotome formation is under control of the notochord. Sonic hedgehog (Shh), a secreted signaling molecule known to play a role in the patterning of the central nervous system and the limb in vertebrates, is expressed in the notochord at that time and has been implicated as the key inductive signal in patterning the ventral neural tube and initiation of sclerotome formation (Fan and Tessier-Lavigne, 1994; Johnson *et al.*, 1994). Mutations in the gene encoding human SHH are associated with holoprosencephaly 3, an autosomal dominant disorder characterized by single brain ventricle, cyclopia, ocular hypotelorism, proboscis, and midface hypoplasia (Roessler *et al.*, 1996). In humans, loss of one *SHH* allele is insufficient to cause ventralization defects of sclerotomes. In the mouse, loss of both *Shh* alleles leads to brain abnormalities and a skeletal phenotype typified by complete absence of the vertebral column and posterior portion of the ribs (Chiang *et al.*, 1996). Formation of the sclerotome, however, does take place although the sclerotomes are smaller and *Pax1* expression is markedly decreased, suggesting that Shh does not initiate but rather maintains the sclerome program. McMahon and associates (1998) reported that *Noggin*, which encodes a bone morphogenetic protein (BMP) antagonist expressed in the node, notochord, and dorsal somite, is required for normal Shh-dependent ventral cell fate. In *Noggin*-null mice, somite differentiation is deficient in both muscle and sclerotomal precursors and *Pax1* expression is delayed, whereas addition of Noggin is sufficient to induce *Pax1*. These findings suggest that different pathways mediate induction and that *Noggin* and *Shh* induce *Pax1* synergistically. Inhibition of BMP signaling by axially secreted Noggin, therefore, is an important requirement for normal induction of the sclerotome.

In contrast to the sclerotome, it is dorsal signals that promote the development of the dermomyotome. These are members of the Wnt family of proteins emanating from the dorsal neural tube and the surface ectoderm necessary for the induction of myogenic precursor cells in the dermomyotome (Wagner *et al.*, 2000). Ectopic *Wnt* expression (*Wnt1*, *3a*-, and *4*) is able to override the influence of ventralizing signals arising from notochord and floor plate. This shift of the border between the two compartments is identified by an increase in the domain of *Pax3* expression and a complete loss of *Pax1* expression in somites close to the ectopic *Wnt* signal. Therefore, Wnts disturb the normal balance of signaling molecules within the somite, resulting in an enhanced recruitment of somitic cells into the myogenic lineage. In contrast, Shh reduces *Wnt* activity in the somitic mesoderm, at least in part, by upregulating *Secreted frizzled-related protein 2* (*Sfrp2*), which encodes a potential *Wnt* antagonist (Lee *et al.*, 2000).

In summary, dorsoventral polarity of the somitic mesoderm is established by competitive signals originating from adjacent tissues. Studies suggest that dorsoventral patterning of somites involves the coordinate action of multiple dorsalizing and ventralizing signals. The ventrally located notochord provides the ventralizing signals to specify the sclerotome, whereas the dorsally located surface ectoderm and dorsal neural tube provide the dorsalizing signals to specify the dermomyotome.

Sclerotome Differentiation

Pax1-expressing cells that arise from the ventromedial end of the sclerotome invade and colonize the perinotochordal space. These cells, expressing additional sclerotome markers such as twist and scleraxis, proliferate under the influence of *Shh* signaling from the notochord and form the perinotochordal tube from which vertebral bodies and intervertebral discs will develop. Segmentation begins by condensation of sclerotome cells that represent the intervertebral discs, thereby defining the boundaries of the future vertebral bodies. Notochordal cells die if surrounded by sclerotome cells that form a vertebral body, whereas those that become part of the intervertebral disc form the nucleus pulposus. The ribs, pedicle, and lamina of the neural arch arise from *Pax1*-expressing cells in the lateral sclerotome.

Not all of the sclerotome cells are under the influence of Shh and Noggin emanating from the notochord and consequently express *Pax1*. Cells located in the ventrolateral and dorsomedial angles of the sclerome escape the ventralizing signals. Whereas other sclerotomal cells migrate ventrally to surround the notochord where they form the vertebral body, these cells move dorsomedially to form the dorsal mesenchyme, which is the precursor of the dorsal part of the neural arch and the spinous process. These sclerotome cells express homeobox genes (*Msx1* and *Msx2*) as they are subjected to a different microenvironment, specifically to signals arising from the roof plate of the neural tube and surface ectoderm (Monsoro-Burq *et al.*, 1994). BMP4 is transiently expressed in these structures and likely exerts a positive effect on the induction of dorsalizing gene expression in sclerotome cells (Monsoro-Burq *et al.*, 1996).

The Cranial Vault and Upper Facial Skeleton

In contrast to the obvious segmentation of the axial skeleton, craniofacial development is a poorly understood process. The craniofacial skeleton forms primarily from neural crest cells that migrate from hindbrain rhombomeres into the branchial arches. Neural crest cells are multipotential stem cells that contribute extensively to vertebrate development and give rise to various cell and tissue types, including mammalian craniofacial development. Migrating from the rhombomeric neuroectoderm to the pharyngeal arches, these cephalic neural crest cells proliferate as the ectomesenchyme within the arches, form mesenchymal condensations, and differentiate into cartilage and bone of endochondral and membranous skull, respectively. Little is known about the molecular basis underlying their migration but it appears that interactions with tissues encountered during migration strongly influence this segmental migratory pattern. Neural crest cells possess integrin receptors that are essential for interacting with extracellular matrix molecules in their surroundings. Aberrant migration of cephalic neural crest cells leads to craniofacial defects, as demonstrated in platelet-derived growth factor-α receptor-(Soriano, 1997) and Shh-deficient mice embryos (Ahlgren and Bronner-Fraser, 1999). Homozygotes die during embryonic development and exhibit incomplete cephalic development. Increased apoptosis is observed on pathways followed by migrating neural crest cells, indicating that these signaling molecules affect their survival.

Interactions between the neural crest-derived ectomesenchymal cells and surrounding cells are critical because defects in this process can also lead to craniofacial malformation. The development of the facial primordia is, in part, mediated by transcription factors that are programmed by an intricate array of intercellular signaling between the ectomysenchymal neural crest-derived cells and the epithelial and mesodermal cell populations within the arches (Francis-West *et al.*, 1998). *Hox* gene products, including *Hoxa1*, *Hoxa2*, and *Hoxa3*, play a role in the development of craniofacial structures derived from the second and third branchial arches, but they are not involved in the patterning of first arch derivatives. Other homeodomain proteins are expressed in cranial neural crest cells that migrate into the first branchial arch, including goosecoid (*Gsc*), *MHox*, and members of the *Dlx* and *Msx* families. A pivotal role in this process has been ascribed to components of the endothelin pathway. The G protein-coupled endothelin-A receptor (ET_A) is expressed in the ectomesenchyme, whereas the cognate ligand for ET_A, endothelin-1 (ET-1), is expressed in arch epithelium and the paraxial mesoderm-derived arch core. Absence of either ET-1 (Kurihara *et al.*, 1994) or ET_A (Clouthier *et al.*, 1998) results in numerous craniofacial defects. While neural crest cell migration in the head of ET_A-null embryos appears normal, expression of transcription factors (*Gsc*, *Dlx-2*, *Dlx-3*, *dHAND*, *eHAND*, and *Barx1*) important in the differentiation of cephalic crest cells in the arches during epithelial-mesenchymal interactions is either absent or significantly reduced in the ectomesenchymal cells (Clouthier *et al.*, 2000). Because *Dlx-1*, *Hoxa-2*, and *MHox* are normally expressed in these mutants it would argue that additional pathways work in conjunction with the ET_A pathway in patterning the facial primordia from buds of undifferentiated mesenchyme into the intricate series of bones and cartilage structures that, together with muscle and other tissues, form the adult face.

LIMB INITIATION AND DEVELOPMENT

Overview of Limb Development

Not all of the mesoderm is organized into somites. Adjacent to the somitic mesoderm is the intermediate mesodermal region, which gives rise to the kidney, and genital ducts and further laterally on either side is the lateral plate mesoderm. In the second month of human development, proliferation of mesenchymal cells from the lateral plate mesoderm give rise to the formation of limb buds (Fig. 3A). *Hox* genes expressed within the lateral plate mesoderm specify the positions at which forelimbs and hindlimbs will be developing (for review, see Ruvinsky and Gibson-Brown, 2000). T-box genes, which encode a family of transcription factors that share a conserved domain with the classical mouse *Brachyury* (*T*) gene, function as activators or repressors of transcription of downstream target genes involved in the regulation of vertebrate limb development. Specifically, transcripts of two of these genes, *Tbx5* and *Tbx4*, are activated as a result of a “read-out” of the *Hox* code for the pectoral and pelvic appendages, respectively (Takeuchi *et al.*, 2003; Zakany and Duboule, 2007). The positional information then leads to limb development within the perspective fields. This concept, however, is not supported by an elegant experiment leading to expression swapping between *Tbx5* and *Tbx4* in the forelimb (Minguillon *et al.*, 2005). Under these conditions, *Tbx4*, which is proposed to specify hindlimb identity, supports formation of a perfectly normal forelimb. In contrast, *Ptx1*, a gene normally expressed specifically in the hindlimb territory, can partially transform the forelimb into hindlimb when ectopically expressed (Lanctot *et al.*, 1999), and remains the best candidate for posterior limb identity specification.

The vertebrate limb is an extremely complex organ in that its patterning takes place in three distinct axes (for review, see Schwabe *et al.*, 1998). First is the proximal-distal axis (the line connecting the shoulder and the finger tip) which is defined by the apical ectodermal ridge (AER), a single layer of epidermal cells that caps the limb bud and promotes the proliferation of mesenchymal cells underneath. As the limb elongates, mesenchymal cells condense to form the cartilage anlagen of the limb bones. Second is the posterior-anterior axis (as in the line between the little finger and the thumb) which is specified by the zone of polarizing activity (ZPA), a block of mesodermal tissue near the posterior junction of the limb bud and the body wall. Third, is the dorsal-ventral axis (as in the line

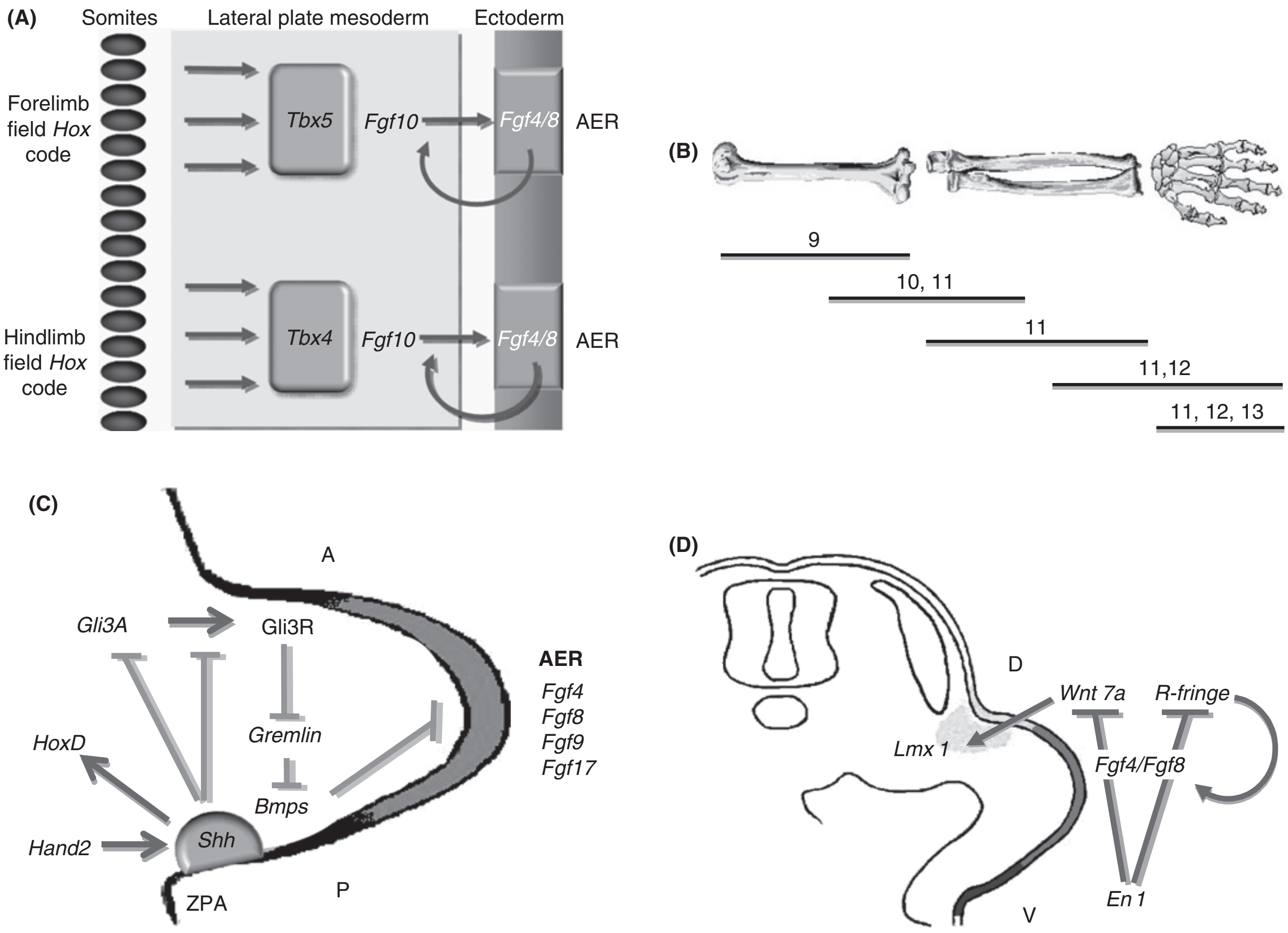

FIGURE 3 Molecular regulation of limb patterning. (**A**) Schematic model for specification of limb position. *Hox* genes expressed in the lateral plate mesoderm define the positions where limbs will develop. *Tbx4* and *Tbx5* expression activates the perspective fields and sets up the *Fgf10-Fgf4/8* positive-feedback loop implicated in induction of the AER in the overlying ectoderm and initiation of limb bud outgrowth. Adapted and modified from Ruvinsky and Gibson-Brown (2000), Fig. 3, p. 5237. (**B**) *HoxA* and *HoxD* genes involved in limb specification. Group 9 paralogous genes organize the proximal part of the limb, group 11 genes organize the distal part of the limb, and group 13 genes pattern the digits. Adapted and modified form Zakany and Duboule (1999), Fig. 1, p. 20. (**C**) Specification of the anterior-posterior axis. *Shh* expressed in the ZPA and *Fgfs* in AER participate in a positive feedback loop to provide the polarizing signal for anterior-posterior patterning of the limb. Adapted and modified from Robert and Lallemand (2006), Fig. 2, p. 2339. (**D**) Specification of the dorsal-ventral axis. *Wnt7a* and *Lmx1* expression correlate with dorsal fate whereas *En1* expression dictates ventral fate by repressing *Wnt7a* and *R-fhg*, a secreted molecule that directs the formation of the AER in the boundary between cells that express it in the dorsal ectoderm and cells that do not express it in the ventral ectoderm. D, dorsal; V, ventral. Adapted and modified from Niswander (1997), Fig. 1, p. 531.

between the upper and lower surfaces of the hand) which is defined by the dorsal epithelium.

The Proximal-Distal Axis

A variety of growth factors, patterning morphogens, transcription factors, and adhesion molecules participate in a highly orchestrated system that dictates the blueprint of the developing mammalian limb. The first step, initiation of the site where the presumptive limb will develop, is critically dependent on fibroblast growth factor (FGF) signaling mediated by high affinity FGF receptors (FGFRs). Expressed in the lateral plate mesoderm, *Fgf10* binds and activates the IIIb splice form of the FGF receptor 2 (FGFR2) in the AER in the overlying ectoderm. This signaling is absolutely crucial for limb bud initiation as evidenced by the complete absence of limb development in mice homozygous for a null *Fgf10* (Min *et al.*, 1998; Sekine *et al.*, 1999) or *Fgfr2* (Xu *et al.*, 1998) allele. In turn, *Fgf4* and *Fgf8* expressed in the AER act on the underlying mesoderm to maintain *Fgf10* expression, thereby promoting the elongation of the limb. As the limb grows, cells directly underneath the AER, in a region termed the progress zone (PZ), maintain their characteristics of undifferentiated mesenchyme while they continue to proliferate. In contrast, the more proximal mesenchymal cells begin to condense and differentiate into the cartilage anlage of the limb. In this scheme, *Hox* gene expression pattern activates downstream target genes according to the position along the axis. These key signaling pathways control various aspects of limb development, including establishment of the early limb field, determination of limb identity, elongation of the limb bud, specification of digit pattern, and sculpting of the digits. Accumulating evidence indicates that *Hoxa* and *Hoxd* genes are involved in limb specification (for review, see Zakany and Duboule, 1999), as was demonstrated in mice lacking all Hoxa and Hoxd functions in their forelimbs (Kmita *et al.*, 2005). Such limbs were arrested early in their developmental patterning and displayed severe truncations of distal elements, partly owing to the absence of Shh expression. In contrast, *Hoxb* and *Hoxc* genes do not participate in limb patterning. Targeted mutations for each gene and compound mutants produced in mice have indicated that genes belonging to groups 9 and 10 determine the length of the upper arm, groups 10, 11, and 12 pattern the lower arm, and groups 11, 12, and mostly 13 organize the digits (see Fig. 3B).

The Anterior-Posterior Axis

It is now established that it is the mutual antagonism between Hand2 and Gli3, two transcriptional factors expressed in the posterior and anterior limb bud, respectively, that determines the anterior-posterior axis (reviewed in Robert and Lallemand, 2006) (see Fig. 3.3C). Hand2 induces in the ZPA expression of *Sonic hedgehog* (*Shh*), the major molecular determinant in the anterior-posterior patterning of the limb (Riddle *et al.*, 1993). Shh is a member of the vertebrate homologues of the *Drosophila* segment polarity gene, hedgehog (*hh*). Although only one *hh* gene has been identified in *Drosophila*, several *hh* genes are present in vertebrates. The mouse Hedgehog (Hh) gene family consists of *Sonic* (*Shh*), *Desert* (*Dhh*), and *Indian* (*Ihh*) hedgehog, all encoding secreted proteins implicated in cell–cell interactions. Signaling to target cells is mediated by a receptor that consists of two subunits, Patched (Ptc), a twelve-transmembrane protein that is the binding subunit (Marigo *et al.*, 1996; Stone *et al.*, 1996), and Smoothened (Smo), a seven-transmembrane protein that is the signaling subunit. In the absence of Hh, Ptc associates with Smo and inhibits its activities. In contrast, binding of Hh to Ptc relieves the Ptc-dependent inhibition of Smo (Nusse, 1996). Signaling then ensues and includes downstream components such as the Gli family of transcriptional factors. The three cloned *Gli* genes (*Gli1*, *Gli2*, and *Gli3*) encode a family of DNA-binding zinc finger proteins with diverse target specificities.

Expression of Shh in the polarizing region regulates the level of Gli3 by repressing the transcription of *Gli3* and preventing transformation of the protein (Gli3A) into its truncated form (Gli3R). *Shh* also acts to induce *HoxD* gene expression in the posterior limb bud. The unequivocal requirement for Shh signaling in limb development has been demonstrated by the *Shh* loss-of-function mutation resulting in the complete absence of distal limb structures (Chiang *et al.*, 1996). The profound truncation of the limbs indicates the existence of an interaction between the ZPA and the AER. The presence of *Gremlin* (*Gre*) in the initial mesenchymal response to *Shh* is required to relay this signal to the AER (Zeller *et al.*, 1999; Zuniga *et al.*, 1999). Gremlin expression is inhibited by the Gli3R and therefore concentrates in the posterior limb bud where it represses Bmp signaling. In turn, this prevents downregulation of *Fgf4* and *Fgf8* in the AER. This Shh/Fgf4 model is supported by genetic evidence showing that *Fgf4* expression is not maintained in *Shh*-null mouse limbs. Thus, Shh expression provides a molecular mechanism for coordinating the activities of these two signaling centers (Niswander *et al.*, 1994).

The Dorsal-Ventral Axis

Dorsoventral patterning is the least understood of the three axes of pattern formation in the limb (for review, see Niswander, 1997; Chen and Johnson, 2002). Molecular studies indicate that the signaling molecule *Wnt7a*, a secreted molecule encoded by *Radical fringe* (*R-fng*), and the transcription factor *Engrailed-1* (*En1*) are intimately involved in this process (see Fig. 3.3D). *Wnt7a* is expressed in the dorsal ectoderm and regulates the expression of a LIM homeodomain gene, *Lmx1*, in the dorsal mesenchyme, important for maintaining dorsal structure identity. *R-fng* expression is also localized in the dorsal ectoderm and dictates the location of AER as defined by the boundary between cells that do and cells that do not express *R-fng* (Laufer *et al.*, 1997). On the other hand, *En1* expression restricts *R-fng*, *Wnt7a*, and *Lmx1* to the dorsal ectoderm and mesenchyme and correlates with ventral fate.

The Skeletal Dysplasias

It is implicit from the foregoing discussion that mutations in the genes involved in limb patterning would tend to have profound effects on the final outcome of human limb design. For example, expansion of a polyalanine stretch in the amino-terminal end the protein product of *HOXD13* is the cause of synpolydactyly, type II, an autosomal dominant disorder characterized by variable syndactyly and insertion of an extra digit between digits III and IV (Muragaki *et al.*, 1996). In homozygous individuals, homeotic transformation of metacarpal and metatarsal bones occurs so that they resemble carpal and tarsal anlages rather than long bones.

Mutations in *GLI3*, the transcription factor involved in the transduction of hedgehog signaling, have been described in four human autosomal dominant disorders: Greig cephalopolysyndactyly syndrome, characterized by peculiar skull shape, frontal bossing, high forehead, and the presence of (poly)syndactyly (Vortkamp *et al.*, 1991); Pallister–Hall syndrome, a neonatally lethal disorder characterized by hypopituitarism, renal agenesis, cardiac defects, cleft palate, short nose, flat nasal bridge, and short limbs (Kang *et al.*, 1997); postaxial polydactyly type A, a trait typified by the presence of a rather well formed extra digit that articulates with the fifth or an extra metacarpal (Radhakrishna *et al.*, 1997); and preaxial polydactyly IV, distinguished by mild thumb duplication, syndactyly of fingers III and IV, first or second toe duplication, and syndactyly of all toes (Radhakrishna *et al.*, 1999).

Heterozygous mutations in the *LMX1B* gene have been described in patients with the nail-patella syndrome, an autosomal dominant disorder encompassing nail dysplasia, hypoplastic patella, decreased pronation and supination, iliac horns, and proteinuria (Dreyer *et al.*, 1998). Functional studies indicate that these mutations either disrupt sequence-specific DNA binding or result in premature termination of translation. These are the first described mutations in a LIM-homeodomain protein that account for an inherited form of abnormal skeletal patterning.

MESENCHYMAL CONDENSATION AND SKELETAL PATTERNING

Mesenchymal Condensation

Mesenchymal condensations originate in areas where cartilage is to appear and where bone is to form by intramembranous ossification. These condensations define not only the position of the skeletal elements they represent but also their basic shape. Therefore, if a condensation were in the wrong place or of the wrong shape and size, it would be expected to produce a skeletal element that is similarly misplaced or misshapen.

Condensations can be easily visualized *in vivo* as they express cell surface molecules that bind peanut agglutinin lectin (Stringa and Tuan, 1996). Their formation takes place when previously dispersed mesenchymal cells form aggregations and, once again, it is *Shh*, *Bmp* (*Bmp2*-5, Bmp7), *Fgf*, and *Hox* genes that determine the fundamental attributes such as the timing, position, and shape that they will assume. Mesenchymal condensation can be envisioned as a multistep process involving initiation, setting of boundary, proliferation, adherence, growth, and finally, differentiation (for review, see Hall and Miyake, 2000) (Fig. 4A). Initiation arises as a result of epithelial–mesenchymal interaction upregulating the expression of a number of molecules associated with prechondrogenic and preosteogenic condensations, such as tenascin, fibronectin, N-CAM, and N-cadherin. Transforming growth factor-beta (TGFβ), and other members of the TGFβ superfamily that regulate many aspects of growth and differentiation, plays a pivotal role in this process (reviewed in Moses and Serra, 1996). This family of signaling molecules, which includes several TGFβ isoforms, the activin and inhibins, growth and differentiation factors (GDFs), and the BMPs, potentiate condensation by promoting the establishment of cell–cell and cell–extracellular matrix interactions (Chimal-Monroy and Diaz de Leon, 1999; Hall and Miyake, 1995). Cell surface adhesion and extracellular matrix proteins contribute to the formation of condensations as they participate in cell attachment, growth, differentiation, and survival. The integrin family of cell surface receptors serves to mediate cell–matrix interactions thereby providing the link between the extracellular matrix and intracellular signaling that can affect gene expression. Integrins that act as receptors for fibronectin (α5β1), types II and VI collagen (α1β1, α2β1, α10β1), laminin (α6β1), and fitronectin and osteopontin (α5β3) are expressed early in the condensation process (Loeser, 2000). Further work is required, however,

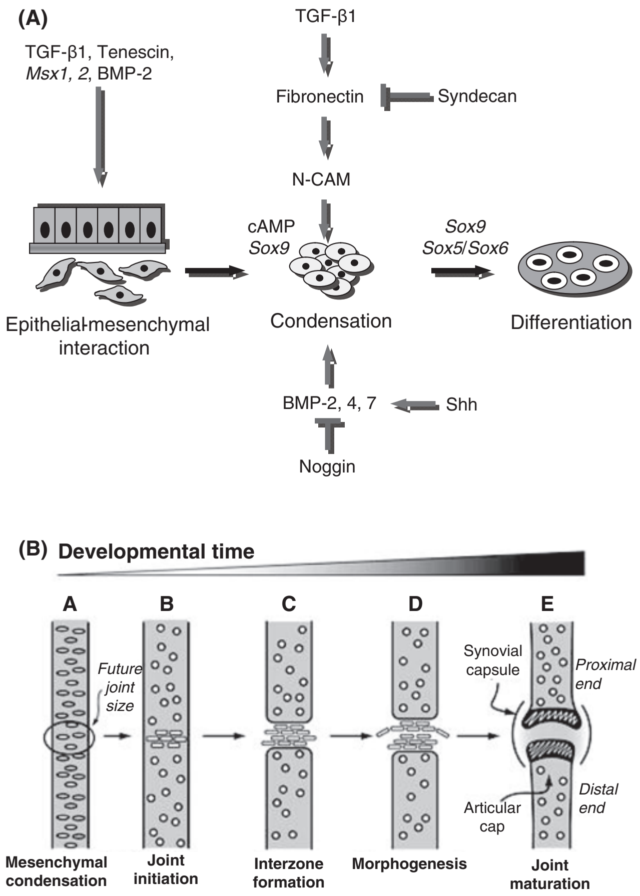

FIGURE 4 Regulation of mesenchymal condensation and joint formation. (**A**) Diagrammatic representation of the network of signaling factors involved in the formation of mesenchymal condensations and their subsequent transition to overtly differentiated cartilage. Adapted and modified from Hall and Miyake (2000), Fig. 3, p. 140). (**B**) Schematic representation of the major steps of synovial joint formation. Adapted from Pacifici *et al.* (2006), Fig. 1, p. 76). See text for details.

to precisely define the role of these molecules in the developmental program of the process.

Mesenchymal cells within the condensations can differentiate either into chondrocytes or osteoblasts. Whereas the former takes place in areas of endochondral ossification, the latter takes place in areas of intramembranous ossification. The choice in cell fate is regulated by the canonical Wnt signaling which is itself induced in part by sonic hedgehog (Hu *et al.*, 2005). In the absence of Wnt signaling, the mesenchymal cells differentiate into chondrocytes. Levels of intracellular cAMP increase during prechondrogenic condensation and, along with the concomitant cell–cell interactions, are thought to mediate upregulation of chondrogenic genes. The transcription factor Sox9 [SRY (sex-determining region Y)-related HMG box gene 9] is a potent inducer of genes required for cartilage formation, such as collagen II (*Col2a1*) and aggrecan (*Agc*), and its phosphorylation by protein kinase A (PKA) increases its DNA binding and transcriptional activity (Huang *et al.*, 2000). Sox9 expression starts in mesenchymal chondroprogenitor cells and reaches a high level of expression in differentiated chondrocytes. Cells deficient in Sox9 are excluded from all cartilage but are present as a juxtaposed mesenchyme that does not express the chondrocyte-specific markers (Bi *et al.*, 1999). This exclusion occurs at the condensing mesenchyme stage of chondrogenesis, suggesting that Sox9 controls expression of cell surface proteins needed for mesenchymal condensation, thereby identifying Sox9 as the first transcription factor that is essential for chondrocyte differentiation and cartilage formation.

Sox5 and Sox6, which have a high degree of sequence identity with each other, belong to a different subgroup of Sox proteins and present no sequence homology with Sox9 except for the HMG box. Sox5 and Sox6 are coexpressed with Sox9 during chondrogenic differentiation. Physiological roles of Sox5 and Sox6 in chondrogenic differentiation were demonstrated by genetically manipulated mice. Whereas *Sox5* and *Sox6* single null mice are born with mild skeletal abnormalities, *Sox5*/*Sox6* double null fetuses die *in utero* with virtual absence of cartilage (Smits *et al.*, 2001). In these double mutants, mesenchymal condensations are formed, but there is no overt chondrocyte differentiation. Although levels of *Sox9* mRNA are comparable to those in wild-type cartilage, inactivation of the *Sox9* gene before chondrogenic mesenchymal condensations results in loss of *Sox5* and *Sox6* expression and severe generalized chondrodysplasia similar to that seen in $Sox5^{-/-}/Sox6^{-/-}$ mice. These data demonstrate important and redundant roles of Sox5 and Sox6 that lie downstream of Sox9 in chondrogenic differentiation after mesenchymal condensations (reviewed in Ikeda *et al.*, 2005). Once condensed mesenchymal cells differentiate into chondroblasts they begin to produce a matrix rich in collagen II, the molecule that best defines the chondroblast/chondrocyte phenotype, as well as collagens IX and XI and mucopolysaccharides.

Mesenchymal prechondrogenic condensations appearing in the early limb bud show no indication of future sites for synovial joint formation and are thus uninterrupted (reviewed in Pacifici *et al.*, 2006). Unknown upstream mechanisms possibly involving *Hox* genes determine the exact location for joint initiation (see Fig. 3.4B). The first overt morphological sign of joint formation is a further gathering and condensation of cells that are connected by gap junctions known as the interzone. It is readily recognizable as a thicker and quite compact structure oriented perpendicularly to the long axis of the long bone model and is composed of mesenchymal cells that never express matrilin-1 (Hyde *et al.*, 2007). Rather, these cells in the interzone express *Gdf5* and give rise to articular cartilage, synovial lining and other joint tissues, but contribute little if any to underlying growth plate cartilage and shaft (Koyama *et al.*, 2008). A number of genes, including *Noggin*, *Wnt14*, *Hif1a*, *Gdf5*, *Gdf6*, *Gli3*, and *Crux1*, have been implicated in a variety of cellular and developmental processes during joint formation and when mutated or misexpressed, can cause pathologies (Brunet *et al.*, 1998; Hartmann and Tabin, 2001; Hopyan *et al.*, 2005; Lizarraga *et al.*, 2002; Provot *et al.*, 2007; Settle *et al.*, 2003; Storm and Kingsley, 1996, 1999).

The Skeletal Dysplasias

A variety of human skeletal disorders arise as a consequence of gain-of-function and loss-of-function mutations in signaling pathways involved in mesenchymal condensation. GDF5 belongs to the TGFβ superfamily and is predominantly expressed throughout mesenchymal condensations in the developing skeleton. Mutations in *GDF5* are the cause of acromesomelic chondrodysplasia, Hunter–Thompson type (Thomas *et al.*, 1996), an autosomal recessive disorder characterized by short forearms, hands, and feet, and very short metacarpals, metatarsals, and phalanges. Mutations in *GDF5* have also been reported in patients with Grebe-type chondrodysplasia, an autosomal recessive disorder characterized by severe limb shortening and dysmorphogenesis with a proximal–distal gradient of severity (Thomas *et al.*, 1997). It is proposed that the mutant GDF5 protein is not secreted and is inactive *in vitro*. It produces a dominant negative effect by preventing the secretion of other, related BMPs, likely through the formation of heterodimers.

Defects in *SOX9* are the cause of campomelic dysplasia, a rare, dominantly inherited chondrodysplasia, characterized by craniofacial defects, bowing and angulation of long bones, hypoplastic scapulae, platyspondyly, kyphoscoliosis, eleven pairs of ribs, small thorax, and tracheobronchial hypoplasia (Foster *et al.*, 1994). It is often lethal, soon after birth, owing to respiratory distress attributed to the hypoplasia of the tracheobronchial cartilage and restrictive thoracic cage.

INTRAMEMBRANOUS BONE FORMATION

Overview

Intramembranous bone formation is achieved by direct transformation of mesenchymal cells into osteoblasts, the skeletal cells involved in bone formation. It is the process responsible for the development of the flat bones of the cranial vault, including the cranial suture lines, some facial bones, and parts of the mandible and clavicle. Although the addition of bone within the periosteum on the outer surface of long bones is also described as arising from intramembranous bone formation, current studies suggest that, in fact, it may be developmentally distinct (see "Endochondrial Ossification: Overview"). With respect to the molecular mechanisms leading to osteoblast differentiation, it can be said that they are rather sketchy. Like cartilage, bone cells are induced initially by specific epithelia (Hall and Miyake, 2000). Here, the cranial sutures will be discussed as intramembranous bone growth sites and this will be followed by a brief description of transcription factors, growth factors, and their receptors associated with normal and abnormal suture development. Intramembranous ossification in the periosteum will be described later on, in conjunction with endochondral bone formation.

Cessation of condensation growth in areas of membranous ossification leads to differentiation characterized by expression of the runt-related transcription factor 2, Runx2 (also known as Cbfa1 for core-binding factor, alpha subunit 1, or Osf2 for osteoblast-specific *cis*-acting element-2) in osteogenic cells, whereas it is downregulated in chondrogenic lineages (Ducy and Karsenty, 1998). Runx2 expression depends on Wnt signaling which results in high levels of β-catenin in mesenchymal cells. In turn, Runx2 induces the expression of another transcription factor called Osterix (Osx) (Nakashima *et al.*, 2002). These two factors are critical for differentiation of mesenchymal cells to osteoblasts. Ontogenetic cells begin to produce, most notably, collagen I in conjunction with a variety of noncollagenous, extracellular matrix proteins that are deposited along with an inorganic mineral phase. The mineral is in the form of hydroxyapatite, a crystalline lattice composed primarily of calcium and phosphate ions.

The Cranial Sutures

Cranial vault sutures identify the fibrous tissues uniting bones of the skull and are the major site of bone growth, especially during the rapid growth of the neurocranium. Sutures need to maintain patency while allowing rapid bone formation at the edges of the bone fronts in order to accommodate the rapid, expansile growth of the neurocranium (for review, see Morriss-Kay and Wilkie, 2005; Opperman, 2000). The closure of sutures is tightly regulated by growth factors and transcription factors (BMPs, FGFs and FGFR1-3, *EFNB1*, TWIST1, and MSXs) involved in epitheliomesenchymal signaling between the sutural mesenchyme, the underlying dura, and the approaching bone fronts. It is proposed that the approximating bone fronts set up gradients of growth factor signaling between them, which initiate suture formation. For example, a gradient of FGF ligand, from high levels in the differentiated region to low levels in the environment of the osteogenic stem cells, modulates differential expression of *FGFR1* and *FGFR2*. Signaling through FGFR2 regulates stem cell proliferation whereas signaling through FGFR1 promotes osteogenic differentiation (Iseki *et al.*, 1999). As sutures fuse, factors involved in pattern formation (*SHH*, *MSX1*) are all downregulated whereas at the same time *RUNX2* and collagen I (*COL1A1*) expression is seen at the bone fronts. In the end, the completely fused suture is indistinguishable from bone.

Recent research has advanced significantly our understanding of the molecular nature of interactions between FGF receptors, TWIST1, RUNX2 and downstream osteogenic differentiation genes in normal and abnormal skull growth (for review, see Opperman, 2000). Future work will undoubtedly provide additional insights into how these factors interact with MSX2 and other genes, thereby providing a more complete picture of the network of gene and protein interactions that control development and growth of the skull vault in health and disease.

The Craniofacial Disorders

As can be inferred from the preceding discussion, the composite structure of the mammalian skull requires precise pre- and postnatal growth regulation of individual calvarial elements. Disturbances of this process frequently cause severe clinical manifestations in humans. The homeobox genes *MSX1* and *MSX2* are of particular interest in that mutated forms are associated with human craniofacial disorders (for reviews, see Cohen, 2000, 2006). An autosomal dominant form of hypodontia is caused by a mutation in *MSX1* (Vastardis *et al.*, 1996) whereas heterozygous mutations in *MSX2* cause parietal foramina (oval-shaped defects on either side of the sagittal suture arising from deficient ossification around the parietal notch, normally obliterated during the fifth fetal month) (Wilkie *et al.*, 2000). These mutations, which lead to decreased parietal ossification by haploinsufficiency, are in marked contrast to the reported gain-of-function mutation (Pro148His) in *MSX2* associated with premature osseous obliteration of the cranial sutures or craniosynostosis Boston-type (Jabs *et al.*, 1993). It is likely that *MSX2* normally prevents differentiation and stimulates proliferation of preosteoblasic cells at the extreme ends of the osteogenic fronts of the calvariae, facilitating expansion of the skull and closure of the suture. Its haploinsufficiency decreases proliferation and accelerates differentiation of calvarial preosteoblast cells resulting in delayed suture closure whereas its "overexpression" results in enhanced proliferation, favoring early suture closure (Dodig *et al.*, 1999).

Osteogenic cell differentiation is influenced by the transcription factor, RUNX2. The function of RUNX2 during skeletal development has been elucidated by the generation of mice in which the *Runx2* locus was targeted (Otto *et al.*, 1997). Heterozygous loss of function leads to a phenotype very similar to human cleidocranial dysplasia, an autosomal dominant inherited disorder characterized by hypoplasia of the clavicles and patent fontanelles that arises from mutations in *RUNX2* (Mundlos *et al.*, 1997). Loss of both alleles leads to a complete absence of bone owing to a lack of osteoblast differentiation. *RUNX2*, therefore, controls differentiation of precursor cells into osteoblasts and is essential for membranous as well as endochondral bone.

Fibroblast growth factor receptors are major players in cranial skeletogenesis and, activating mutations of the human *FGFR1*, *FGFR2*, and *FGFR3* genes, cause craniosynostosis. Most mutations are found in *FGFR2*, although there are several in *FGFR3* and in *FGFR1*. A C→G transversion in exon 5 of *FGFR1*, resulting in proline to arginine substitution (P252R) in the extracellular domain of the receptor has been reported in affected members of five unrelated families with Pfeiffer syndrome, an autosomal dominant disorder, characterized by mild craniosynostosis, flat facies, shallow orbits, hypertelorism, acrocephaly, broad thumb, broad great toe, polysyndactyly and interphalangeal ankylosis (Muenke *et al.*, 1994).

Mutations in *FGFR2* (C342Y, C342R, C342S, and a C342W) have been described in patients with Crouzon syndrome (Reardon *et al.*, 1994; Steinberger *et al.*, 1995). This disorder, encompassing craniosynostosis, hypertelorism, hypoplastic maxilla, and mandibular prognathism, is easily distinguishable from Pfeiffer syndrome by the absence of hand abnormalities. Interestingly, the C342Y mutation is also reported in patients with Pfeiffer syndrome and in individuals with Jackson–Weiss syndrome (Tartaglia *et al.*, 1997), an autosomal dominant disorder characterized by midfacial hypoplasia, craniosynostosis, and cutaneous syndactyly, indicating that the same mutation can give rise to one of several phenotypes. Another conserved cysteine at position 278 is similarly predisposed to missense mutations leading to the same craniosynostotic conditions. On the other hand, mutations in S252 and P253 residues have been reported in most cases of Apert syndrome, a condition characterized by craniosynostosis and severe syndactyly (cutaneous and bony fusion of the digits). Although the mechanism whereby the same mutation can give rise to distinct phenotypes remains to be clarified, sequence polymorphisms in other parts of the mutant gene may affect its phenotypic expression (Rutland *et al.*, 1995). Finally, mutations within *FGFR2* have also been reported in other rare craniosynostotic conditions (for reviews, see Cohen 2006; Passos-Bueno *et al.*, 1999).

In contrast to the propensity of mutations in FGFR1 and FGFR2 affecting craniofacial development, only rarely do mutations in FGFR3 cause craniosynostoses (Muenke syndrome). For the most part, mutations in FGFR3 are associated with dwarfism, suggesting that the primary function of FGFR3 is in endochondral rather than intramembranous ossification. The vast majority of FGFR mutations identified to date are dominantly inherited and result in increased signaling by the mutant receptor (Naski and Ornitz, 1998). Altered cellular proliferation and/or differentiation are believed to underlie their pathogenetic effects.

As already discussed, other gene mutations for craniosynostosis are also known (outlined fully in Cohen, 2006, and references therein). *TWIST* mutations have been reported in Saethre–Chotzen syndrome. Most mutations truncate the *TWIST* protein, resulting in haploinsufficiency. Craniofrontonasal syndrome is caused by heterozygous loss-of-function mutations in *EFNB1* whereas *EFNA4* mutations are the cause of nonsyndromal coronal synostosis.

ENDOCHONDRAL OSSIFICATION

Overview

The axial and appendicular skeletons develop from cartilaginous model, the growth of which arises in a variety of ways (Johnson, 1986). Cartilage is unique among skeletal tissues in that it has the capacity to grow interstitially, i.e., by division of its chondrocytes. This property is what allows cartilage to grow very rapidly. Moreover, cartilage utilizes apposition of cells on its surface, matrix deposition, and enlargement of the cartilage cells as additional means of achieving maximal growth. Appositional growth is the principal function of the perichondrium, which envelops the epiphyses and the cartilaginous diaphysis, serving as the primary source of chondroblasts. With time, these cells differentiate to chondrocytes that secrete collagen II, aggrecan, and a variety of other matrix molecules that constitute the extracellular matrix of the hyaline cartilage (Fig. 5A). As development proceeds, a predetermined program of chondrocyte differentiation ensues in the central diaphysis, leading to chondrocyte hypertrophy. The hypertrophic chondrocytes express collagen X and direct the mineralization of the surrounding matrix, while signaling to adjacent perichondrial cells to direct their differentiation into osteoblasts, and also to stimulate the invasion of blood vessels. Capillaries invade the perichondrium surrounding the future diaphysis and transform it into the periosteum, whereas osteoblastic cells differentiate, mature, and secrete collagen I and other bone-specific molecules, including alkaline phosphatase. This will ultimately mineralize by intramembranous ossification and give rise to the bony collar, the cortical bone.

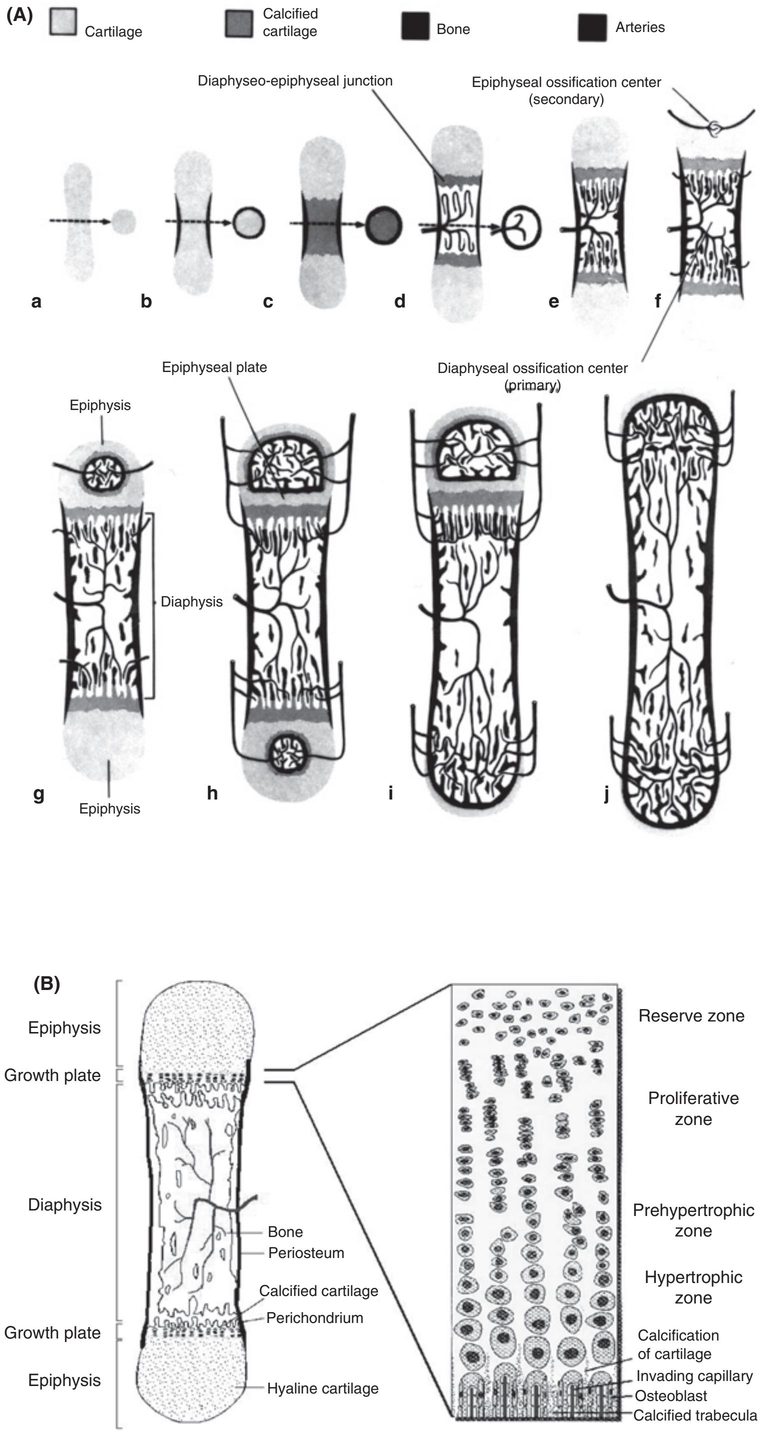
(A)
Cartilage
Calcified cartilage
Bone
Arteries
Diaphyseo-epiphyseal junction
Epiphyseal ossification center (secondary)
a
b
c
d
e
f
Epiphyseal plate
Diaphyseal ossification center (primary)
Epiphysis
Diaphysis
g
Epiphysis
h
i
j
(B)
Epiphysis
Growth plate
Diaphysis
Growth plate
Epiphysis
Bone
Periosteum
Calcified cartilage
Perichondrium
Hyaline cartilage
Reserve zone
Proliferative zone
Prehypertrophic zone
Hypertrophic zone
Calcification of cartilage
Invading capillary
Osteoblast
Calcified trabecula

FIGURE 5 Formation and growth of long bones by endochondral ossification. (**A**) Mesenchymal condensation leads to the development of a cartilage model (a). Capillaries invade the perichondrium surrounding the future diaphysis and transform it into the periosteum (b). Chondrocyte differentiation ensues, just underneath the bone collar, leading to chondrocyte hypertrophy and apoptotic death associated with mineralization of the cartilage matrix (c). Vascular invasion from vessels allows for the migration of osteoblast precursor cells that deposit bone on the degraded matrix scaffold. Chondrogenesis at the ends of the long bone establishes the formation of growth plates (d, e). Secondary centers of ossification begin in late fetal life (f). Growth plates serve as a continuous source of cartilage conversion to bone thereby promoting linear growth (g, h). Long bones cease growing at the end of puberty, when the growth plates are replaced by bone but articular cartilage persists (i, j). Adapted from Recker (1992), Fig. 6, p. 223. (**B**) The mammalian growth plate. Schematic representation of the organization of the mammalian growth plate. Adapted from Wallis (1996), Fig. 1, p. 1578. See text for details.

Matrix mineralization, in turn, is followed by vascular invasion from vessels originating in the periosteal collar that allows for the migration of osteoblast precursor cells into the cartilaginous model (primary ossification center). These cells transform into mature osteoblasts and initiate new bone formation on the degraded matrix scaffolding. Osteoclasts, cells derived from the hematopoietic lineage, also enter the cartilage mold and digest the matrix that had been synthesized by the hypertrophic chondrocytes. The primary growth plates are then established and serve as a continual source of cartilage conversion to bone and linear growth of the long bone during development and postnatally. In late fetal life and early childhood, secondary centers of ossification appear within the cartilaginous epiphyses by a mechanism very similar to that used in the formation of the primary center. Cartilage is retained at the growth plate, extending the full width of the bone and separating epiphysis from diaphysis. Cessation of growth occurs at the end of puberty, when growth plates are replaced by bone.

The Growth Plate

The organization of the mammalian epiphyseal growth plate is represented diagrammatically in Fig. 3.5B. The growth plate conforms to a general basic plan that consists of four zones that, although distinct, encompass a merging continuum (for reviews, see Johnson, 1986; Stevens and Williams, 1999). In the reserve zone, chondrocytes are nearly spherical in cross section and appear to be randomly arranged, separated by large amounts of matrix consisting largely of collagen II and proteoglycans. Although parts of this zone are mitotically inert, others function as stem cell sources. The cells from this zone eventually become discoid and are arranged into rather regular columns forming the zone of proliferation. Column formation is in part owing to the characteristic division of chondrocytes in that their mitotic axis is perpendicular to the long axis of the bone. Two daughter cells become flattened and are separated by a thin septum of cartilage matrix. Elongation of the anlagen occurs mainly at its ends and arises primarily from the division of chondrocytes because it is here that cell proliferation is maximal. Eventually, the chondrocytes from this zone enlarge and lose their characteristic discoidal shape as they enter the zone of maturation (prehypertrophic chondrocytes). Growth here ceases to be caused by cell division and continues by increases in the size of the cells. In the midsection, the chondrocytes mature, enlarge in size (hypertrophy), and secrete a matrix rich in collagen X. These cells continue to enlarge to the point that their vertical height has increased nearly five times. Once glycogen stores have been depleted, they undergo programmed cell death or apoptosis (Farnum and Wilsman, 1987), leaving behind longitudinal lacunae separated by septae of cartilaginous matrix that become selectively calcified as well as largely uncalcified transverse septae. In response to these changes, vascular invasion ensues as new blood vessels enter the lower hypertrophic zone from the primary spongiosum and penetrate the transverse septae whereas calcified cartilage is removed by chondroclasts that accompany this erosive angiogenic process. The remaining longitudinal septae that now extend into the diaphysis are used by osteoblasts derived from bone marrow stromal cells to settle on and lay down extracellular matrix (osteoid) that calcifies into woven bone. With time, osteoclasts, cells derived from the hematopoietic lineage, resorb the woven bone and osteoblasts replace it with mature trabecular bone, thereby completing the process of endochondral ossification.

Mediators of Growth Plate Chondrocyte Proliferation and Differentiation

Proper skeletal formation, growth, and repair are critically dependent on the accurate orchestration of all the processes participating in the formation of endochondral bone at the growth plate. It is only recently, however, that fundamental insight has emerged into the molecular pathways regulating these processes. The major systemic and local influences on growth plate chondrocyte proliferation and differentiation and associated developmental abnormalities arising from their failure to function in the appropriate fashion will now be discussed. In specific situations, the distinction between systemic as opposed to local mediators may not be so apparent, and this will be pointed out.

Systemic Mediators

A variety of systemic hormones such as the growth hormone-insulin-like growth factor 1 (GH-IGF1) signaling system, thyroid hormone, estrogens, glucocorticoids, and vitamin D partake in the regulation of linear growth pre- and postnatally. The importance of these hormones in linear skeletal growth has been highlighted by both genetic studies in animals and by “experiments of nature” in humans.

Growth hormone and insulin-like growth factor 1

GH plays an important role in longitudinal bone growth. Children with elevated GH levels owing to pituitary adenomas exhibit gigantism. Alternatively, bone growth is impaired both in GH-deficient humans (Laron *et al.*, 1966; Rosenfeld *et al.*, 1994) and in the GH receptor-null mouse (Sjogren *et al.*, 2000; Zhou *et al.*, 1997). Homozygous-null mice display severe postnatal growth retardation, disproportionate dwarfism, and markedly decreased bone mineral content. Reduced bone length in GH receptor-negative mice is associated with premature growth-plate contraction and reduced chondrocyte proliferation that is not detectable until 3 weeks of age; before this, bone growth proceeded normally, indicating that GH is not required for normal murine prenatal development or early postnatal growth.

Although cortical and longitudinal bone growth and bone turnover are all reduced in GH receptor deficiency, many of these effects can be reversed by IGF1 treatment (Sims *et al.*, 2000), suggesting that the main defect relates to reduced IGF1 levels in the absence of GH receptor.

In the original "somatomedin hypothesis," it was proposed that GH's primary effect was to promote IGF1 production by the liver, with circulating IGF1 then stimulating the longitudinal expansion of growth plates in an endocrine fashion. Because longitudinal bone growth is not affected in the liver-specific *Igf1*-knockout mouse (Yakar *et al.*, 1999), locally produced IGF1 and/or direct effects of GH may substitute for deficient systemic IGF1. More recent work suggests that GH acts directly at the growth plate to amplify the production of chondrocytes from germinal zone precursors and then to induce local IGF1 synthesis (Nilsson *et al.*, 2005), proposed to stimulate the clonal expansion of chondrocyte columns in an autocrine/paracrine manner (Ohlsson *et al.*, 1998). Although the actions of GH on a range of cell types is mediated by Stat5 signaling, interestingly, the bone phenotypes in GH receptor- and *Stat5*-knockout animals (Teglund *et al.*, 1998) are different, suggesting that the effects of GH on bone, whether direct or through IGF1, are not mediated by the Stat5 transcription factors but by other cytokine or signaling cascades.

Recent data suggest that IGF-I protein in the growth plate is not produced primarily by the chondrocytes themselves. Instead, it derives from surrounding perichondrium and bone (Parker *et al.*, 2007). IGF1 plays a pivotal role in longitudinal bone growth, because *Igf1* gene deletion results in dwarfism in mice (Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993) and extreme short stature in humans (Woods *et al.*, 1996). A study of longitudinal bone growth in the *Igf1*-null mouse indicates that growth plate chondrocyte proliferation and cell numbers are preserved despite a 35% reduction in the rate of long bone growth (Wang *et al.*, 1999). The growth defect owing to *Igf1* deletion has been traced to an attenuation of chondrocyte hypertrophy, which is associated with Glut4 glucose transporter expression, glycogen synthesis (GSK3β serine phosphorylation), and ribosomal RNA levels being significantly diminished in *Igf1*-null hypertrophic chondrocytes, resulting in reduced glycogen in these cells. Glycogen stores are normally accumulated by proliferative and early hypertrophic chondrocytes and depleted during maturation of the hypertrophic chondrocytes. Hypertrophic chondrocytes are highly active metabolically and are dependent on glycolysis to fuel their expansive biosynthetic activity. The decrease in ribosomal RNA in *Igf1*-null hypertrophic chondrocytes may reflect cellular "starvation" for fuel and building blocks for protein synthesis.

Thyroid hormones

Thyroid hormone deprivation has deleterious effects on bone growth (reviewed in Shao *et al.*, 2006). The observed delay in bone development is mediated by a direct effect of thyroid hormone on cartilage and an indirect effect of the hormone on GH and IGF1 action (O'Shea *et al.*, 2005; Weiss and Refetoff, 1996). Thyrotoxicosis, on the other hand, accelerates growth rate and advances bone age. In euthyroid human (Williams *et al.*, 1998) and rat cartilage (Stevens *et al.*, 2000), thyroid hormone receptor alpha1 (TRα1), TRα2, and TRβ1 proteins are localized to reserve zone progenitor cells and proliferating chondrocytes. When animals are rendered hypothyroid, growth plates become grossly disorganized, and hypertrophic chondrocyte differentiation fails to progress (Fraichard *et al.*, 1997; Stevens *et al.*, 2000). In thyrotoxic growth plates, histology is essentially normal but mRNA for parathyroid hormone-related protein (Pthrp) and its receptor is undetectable (Stevens *et al.*, 2000). PTHrP signaling exerts potent inhibitory effects on hypertrophic chondrocyte differentiation (see later) suggesting that dysregulation of local mediators of endochondral ossification may be a key mechanism that underlies growth disorders in childhood thyroid disease. Although thyroid hormone may also act directly on osteoblasts (Abu *et al.*, 2000), these effects have received much less attention.

Estrogens

The biosynthesis of estrogens from testosterone in the ovary, adipose tissue, skeletal muscle, skin, hair follicles, and bone is catalyzed by the enzyme aromatase, the product of the *CYP19* gene. In recent years a number of patients, two men and five women, have been described as suffering from aromatase deficiency owing to mutations in *CYP19*, resulting in the synthesis of a nonfunctional gene product and failure to synthesize estrogens (reviewed in Faustini-Fustini *et al.*, 1999). Males with this condition have sustained linear growth into adulthood as a consequence of failed epiphyseal closure. Reduced bone mineral density and bone age are also characteristic. The women show absence of a growth spurt and delayed bone age at puberty as well as unfused epiphyses later on, despite evidence of virilization.

Although not all effects of 17β-estradiol are due to direct control of gene expression (Pedram *et al.*, 2006), most its biological actions are mediated by two estrogen receptors (ER), ERα and ERβ, which regulate transcription through direct interaction with specific binding sites on DNA in promoter regions of target genes (for review, see Pettersson and Gustafsson, 2001). Smith and associates (1994) have described a man with a bi-allelic inactivating mutation of the ERα gene. This patient had normal genitalia but suffered from osteoporosis and was still growing at the age of 28 because the epiphyseal plates were unfused.

These two "experiments of nature" (aromatase and ERα deficiency), supported by the recent identification of ERα and ERβ expression in chondrocytes (Ushiyama *et al.*, 1999) and osteogenic cells of trabecular and cortical bone (Rickard *et al.*, 1999), have firmly established that estrogens exert direct effects on the growth plate and are crucial for peripubertal growth and epiphyseal growth plate fusion at the end of puberty in both women and men. Moreover, they have revealed a greater appreciation for the importance of estrogens in bone mass maintenance in both sexes.

Glucocorticoids

Glucocorticoids have well-documented effects on the skeleton, as pharmocological doses cause stunted growth in children (Canalis, 1996). The skeletal actions of glucocorticoids are mediated via specific receptors, which are widely distributed at sites of endochondral bone formation. Studies now indicate that glucocorticoids are involved in chondrocyte proliferation, maturation, and differentiation earlier in life, whereas at puberty they are implicated primarily in chondrocyte differentiation and hypertrophy (reviewed in Nilsson *et al.*, 2005). Further investigation is required, however, to clarify the physiological actions of glucocorticoids on cartilage.

Glucocorticoid receptors are also highly expressed in rodent and human osteoblastic cells both on the bone-forming surface and at modeling sites (Abu *et al.*, 2000). Pharmacological doses of glucocorticoids in mice inhibit osteoblastogenesis and promote apoptosis in osteoblasts and osteocytes, thereby providing a mechanistic explanation for the profound osteoporotic changes arising from their chronic administration (Weinstein *et al.*, 1998).

Vitamin D

Vitamin D deficiency is the major cause of rickets in children and osteomalacia in adults. Inactivating mutations in the coding sequences of 25-hydroxyvitamin D_3 1α-hydroxylase (*CYP27B1*) (Fu *et al.*, 1997) and VDR genes are associated with rickets characterized by expansion of the hypertrophic zone of the growth plate coupled with impaired extracellular matrix calcification and angiogenesis (Donohue and Demay, 2002). However, a mineral-enriched diet can normalize the growth plates of vitamin D-resistant animals (Amling *et al.*, 1999) suggesting that the skeletal consequences of VDR ablation are a result of impaired intestinal mineral absorption. A direct role of vitamin D on bone is also suggested because VDR is expressed in osteoblasts and osteoclast precursors (Johnson *et al.*, 1996; Mee *et al.*, 1996).

The 25-hydroxyvitamin D-24-hydroxylase enzyme (24-OHase; *CYP24*) is responsible for the catabolic breakdown of $1,25(OH)_2$ vitamin D_3. The enzyme can also act on the 25-(OH)vitamin D_3 substrate to generate $24,25\text{-}(OH)_2$ vitaminD_3, a metabolite whose physiological importance remains unclear. Although earlier studies in *Cyp24*-knockout mice had suggested that the 24-hydroxylated metabolite of vitamin D, $24R,25(OH)_2$ vitamin D_3 exerts distinct effects on intramembranous bone mineralization (St-Arnaud, 1999), more recent work has concluded that this metabolite is dispensable during bone development (St-Arnaud *et al.*, 2000).

Local Mediators

TGFβ

TGFβ1, *-2*, and *-3* mRNAs are synthesized in the mouse perichondrium and periosteum from 13.5 days post coitus until after birth (Millan *et al.*, 1991). As already discussed, TGFβ promotes chondrogenesis in early undifferentiated mesenchyme (Leonard *et al.*, 1991), but in high-density chondrocyte pellet cultures or organ cultures it inhibits terminal chondrocyte differentiation (Ballock *et al.*, 1993) and this effect appears to be partly mediated by induction of PTHrP expression (see later) (Serra *et al.*, 1999). TGFβs signal through heteromeric type I and type II receptor serine/threonine kinases. To delineate the role of TGFβs in the development and maintenance of the skeleton *in vivo*, Serra *et al.* (1997) generated transgenic mice that express a cytoplasmically truncated, functionally inactive TGF-type II receptor under the control of a metallothionein-like promoter, which can compete with the endogenous receptors for complex formation, thereby acting as a dominant-negative mutant. Loss of responsiveness to TGFβ promoted chondrocyte hypertrophy, suggesting an *in vivo* role for TGFβ in limiting terminal differentiation. In mouse embryonic metatarsal bone rudiments grown in organ culture, TGFβ inhibits several stages of endochondral bone formation, including chondrocyte proliferation, hypertrophic differentiation, and matrix mineralization (Serra *et al.*, 1999). Moreover, activation of Ihh signaling pathway fails to induce PTHrP or inhibit chondrocyte hypertrophy in embryonic metatarsal organ cultures from *TGFβ2*-null mice, indicating that TGFβ2 is at least one of the mediators of these responses to Ihh (see later) (Alvarez *et al.*, 2002).

Parathyroid hormone-related protein (PTHrP) and Indian hedgehog (Ihh)

PTHrP is a major determinant of chondrocyte biology and endochondral bone formation. It was discovered as the mediator of hypercalcemia associated with malignancy but is now known to be expressed by a large number of normal fetal and adult tissues (Philbrick *et al.*, 1996; Wysolmerski and Stewart, 1998). The amino-terminal region of PTHrP reveals limited but significant homology with parathyroid hormone (PTH), resulting in the interaction of the first 34 to 36 residues of either protein with a single seven-transmembrane spanning G protein-linked receptor termed the PTH/PTHrP receptor or the PTH receptor type 1 (PTHR1). Both PTH and PTHrP, through their interaction with this receptor, activate cAMP and calcium second messenger signaling pathways by stimulating adenylate cyclase and/or phospholipase C activity, respectively (Abou-Samra *et al.*, 1992; Juppner *et al.*, 1991). Targeted inactivation of *Pthrp* and *Pthr1* has established a fundamental role for this signaling pathway in chondrocyte proliferation, differentiation, and apoptotic death (Amizuka *et al.*, 1994, 1996; Karaplis *et al.*, 1994; Lanske *et al.*, 1996; Lee *et al.*, 1996). Mice homozygous for the *Pthrp*- or *Pthr1*-null alleles display a chondrodysplastic phenotype characterized by reduced chondrocyte proliferation, and premature and inappropriate hypertrophic differentiation resulting in advanced endochondral ossification. Conversely, targeted expression of PTHrP (Weir *et al.*, 1996) or a constitutively active form of PTHR1 (Schipani *et al.*, 1997) to the growth plate leads to delayed mineralization, decelerated conversion of proliferative chondrocytes into hypertrophic cells, and prolonged presence of hypertrophic chondrocytes with delay of vascular invasion. Moreover, in chimeric mice with some chondrocytes missing the PTHR1, the mutant chondrocytes become hypertrophic much sooner than their wild-type counterparts.

Correlation of these findings to human chondrodysplasias arose initially from studies in patients with Jansen-type metaphyseal dysplasia. This autosomal dominant disorder is characterized by short stature, abnormal growth plate maturation, and laboratory findings indistinguishable from primary hyperparathyroidism despite low normal or undetectable levels of PTH and PTHrP. Schipani and associates (1995, 1996, 1999) reported heterozygous missense mutations in *PTHR1* that promote ligand-independent cAMP accumulation but with no detectable effect on basal inositol phosphate accumulation. These activating mutations have therefore provided an explanation for the observed biochemical abnormalities and the abnormal endochondral ossification characteristic of Jansen metaphyseal chondrodysplasia. In contrast to *PTHR1*-activating mutations in this disorder, Blomstrand chondrodysplasia arises from the absence of a functional PTHR1 (Jobert *et al.*, 1998; Karaplis *et al.*, 1998; Karperien *et al.*, 1999; Zhang *et al.*, 1998). This is a rare autosomal recessive chondrodysplasia characterized by skeletal abnormalities that bear a remarkable resemblance to the phenotypic alterations observed in the *Pthr1*-knockout mice. Mutations in *PTHR1* are also reported in patients with echondromatosis (Hopyan *et al.*, 2002) or with Eiken syndrome (Duchatelet *et al.*, 2005).

Binding of PTHrP to the PTHR1 stimulates multiple heterotrimeric G proteins, including G_s, G_q family, and $G_{12,13}$ (Singh *et al.*, 2005). Activation of each G protein triggers a series of downstream events that culminate in biological consequences whose physiological actions vary. Using genetic tools, the roles of activation of the G_s and G_q pathways by PTHrP in the fetal growth plate has been dissected. Signaling via G_q slows the proliferation and hastens the differentiation of chondrocytes, actions that oppose the effects of G_s signaling pathways which include delayed ossification and increased chondrocyte proliferation (Guo *et al.*, 2002). This seemingly counterproductive opposition may in fact help to smooth the functional gradient of PTHrP action across the growth plate (Kronenberg, 2006).

The pivotal role of PTHrP signaling in the growth plate has served as the impetus for subsequent studies aimed to identify and characterize upstream and downstream molecular components that regulate chondrocyte proliferation and differentiation.

Ihh is expressed in prehypertrophic chondrocytes of the mouse embryo. Earlier studies using overexpression and misexpression of Ihh in the developing cartilage demonstrated that Ihh delays the hypertrophic differentiation of growth plate chondrocytes (Vortkamp *et al.*, 1996). A number of *in vitro* as well as *in vivo* studies now indicate that the capacity of Ihh to slow chondrocyte differentiation is mediated by PTHrP. Ihh upregulates *Pthrp* expression in the growth plate. This expression, however, is abolished by targeted disruption of *Ihh* and leads to premature chondrocyte differentiation (St-Jacques *et al.*, 1999), thereby implicating PTHrP as the mediator of Ihh actions on chondrocyte hypertrophy. These observations, among others, have led to the proposal that an Ihh/PTHrP feedback loop regulates the pace of chondrocyte differentiation in the growth plate (Fig. 6) (Chung and Kronenberg, 2000; Kronenberg, 2006). PTHrP is synthesized by chondrocytes and perichondrial cells at the ends of the developing bones and acts on chondrocytes bearing PTHR1 to keep them proliferating and delaying their differentiation. Chondrocytes sufficiently far away from the source of PTHrP, however, stop proliferating and only then synthesize Ihh. Then, directly or indirectly, Ihh signals back

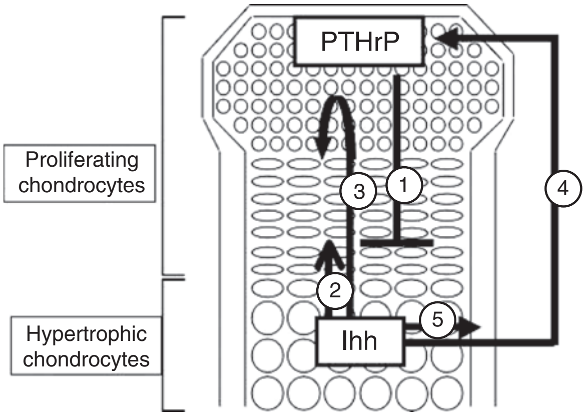

FIGURE 6 Ihh and PTHrP interaction in the growth plate. Ihh and PTHrP participate in a negative feedback loop to regulate the rate of chondrocyte proliferation and differentiation. PTHrP is expressed and secreted from perichondrial cells and chondrocytes at the ends of long bones and acts on proliferating chondrocytes to keep them proliferating and delay their differentiation into prehypertrophic and hypertrophic chondrocytes (1). When the source of PTHrP production is sufficiently distant, then chondrocytes stop proliferating and synthesize Ihh. In turn, Ihh increases the proliferation of adjacent chondrocytes (2), accelerates the differentiation of round proliferative chondrocytes into flat proliferating chondrocytes (3), stimulates the production of PTHrP at the ends of bones (4), and acts on perichondrial cells to convert them into osteoblasts of the bone collar (5). Adapted and modified from Kronenberg (2006, Fig. 2, p. 4).

to the end of the growth plate to stimulate the synthesis of more PTHrP. This feedback system thus determines the site at which chondrocytes stop proliferating and start making Ihh. In so doing, this negative feedback loop serves to regulate the rate of chondrocyte differentiation and hence the length of the zone of chondrocyte proliferation as well as to contribute to the striking uniformity of chondrocyte morphology across the growth plate. Ihh has a number of actions that are independent of PTHrP. Ihh stimulates the proliferation of adjacent chondrocytes, accelerates the differentiation of round proliferative chondrocytes into flat proliferating chondrocytes, and directs adjacent perichondrial cells to become osteoblasts (Long *et al.*, 2004; Long, 2001). PTHrP and Ihh together, therefore, determine both the entry and the exit of chondrocytes from the pool of flat proliferating chondrocytes. Further, by determining the site of Ihh production, PTHrP thus also determines the site at which perichondrial cells first become true osteoblasts in the bone collar adjacent to the growth plate.

What is the mechanism by which Ihh stimulates *Pthrp* expression? Although it is possible that Ihh interacts directly with *Pthrp*-expressing cells in the growth plate, it is more likely, given the number of restrictions imposed on Ihh diffusion (Chuang and McMahon, 1999), that the action is indirect. BMPs have been proposed to serve as secondary signals downstream of Ihh. For example, viral expression of a constitutively active form of the BMP receptor IA increased *Pthrp* mRNA expression in embryonic chicken limbs and blocked chondrocyte differentiation in a similar manner as misexpression of Ihh without inducing Ihh expression (Zou *et al.*, 1997). Further studies have indicated that BMP2 and BMP4 are the likely secondary signals, which act through the BMP receptor-IA to mediate the induction of Pthrp expression (Pathi *et al.*, 1999). It is of interest to note that TGFβ also stimulates *Pthrp* expression in mouse embryonic metatarsal bone rudiments grown in organ culture (Serra *et al.*, 1999). Furthermore, terminal differentiation is not inhibited by TGFβ in metatarsal rudiments from *Pthrp*-null embryos, supporting the model that TGFβ acts upstream of PTHrP to regulate the rate of hypertrophic differentiation. Whether, it is TGFβ or other members of the BMP family of proteins that serve as the intermediary relay that links the Ihh and PTHrP signaling pathways remains to be determined. Other studies, however, have failed to support a role for BMPs in this process (Haaijman *et al.*, 1999). Therefore, for now it would be prudent to conclude that the mechanism transmitting Ihh signaling to PTHrP-expressing chondrocytes remains, for the most part, uncertain. It is known, however, that when the gene encoding the transcription factor Gli3 is ablated along with *Ihh* in mice, then the double-null animals synthesize PTHrP in perichondrial cells but not in chondrocytes (Koziel *et al.*, 2005). The finding that ablation of Gli3 reverses some of the suppression of PTHrP synthesis in *Ihh*-null mice suggests that Ihh normally regulates perichondrial PTHrP expression, at least in part, by suppressing the expression of Gli3.

What are the downstream molecular mechanisms that convey PTHrP's inhibitory action on chondrocyte differentiation? Several transgenic studies have examined this question by assessing the significance of the cyclic AMP/PKA and phospholipase C/PKC signal transduction pathways on the cartilage differentiation program. In the first scenario, a PTHR1 with normal phospholipase C signaling, but deficient $G_s\alpha$ signaling was expressed in chimeric mice (Chung and Kronenberg, 2000). Cells with deficient $G_s\alpha$ signaling underwent premature maturation in the growth plate, whereas wild-type cells had a normal rate of differentiation. In the second scenario, mice expressing a mutant PTHR1 with normal $G_s\alpha$ signaling, but deficient phospholipase C signaling, exhibited abnormalities in embryonic endochondral bone development, including delayed ossification and increased chondrocyte proliferation (Guo *et al.*, 2002). These genetic experiments support the contention that PLC signaling via PTHR1 normally slows the proliferation and hastens the differentiation of chondrocytes, actions that oppose the dominant effects of PTHR1 and that involve cAMP-dependent signaling pathways. Thus, activation of G_s and G_q by activation of PTHR1 in chondrocytes leads to actions that oppose each other. The usefulness of this seemingly wasteful opposition of the two pathways is not certain. As in many other settings in which a stimulus activates opposing pathways, this complicated pattern may allow regulatory interactions with other pathways that are useful (Kronenberg, 2006).

Given that PKA-phosphorylated-SOX9 is present in the prehypertrophic zone of the growth plate, the same location where the gene for PTHR1 is expressed, then SOX9 is a likely target for PTHrP signaling (Fig. 7). What is the evidence to support this conclusion? SOX9 phosphorylated at serine 181 (S181), one of two consensus PKA phosphorylation sites, is detected almost exclusively in chondrocytes

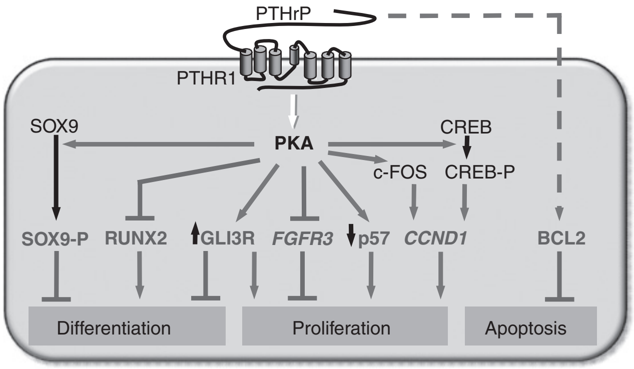

FIGURE 7 PTHrP and chondrocyte biology. Signaling pathways proposed to mediate the effects of PTHrP on differentiation, proliferation, and apoptotic death of chondrocytes (see text for details). The dashed line depicts putative PTHrP action that may not be mediated by PTHR1 (intracrine effect).

of the prehypertrophic zone in wild-type mouse embryos (Huang *et al.*, 2000). Moreover, no phosphorylation of SOX9 is observed in prehypertrophic chondrocytes of the growth plate or any chondrocytes of *Pthr1*-null mutants. Phosphorylation of SOX9 by PKA enhances its transcriptional and DNA-binding activity (Huang *et al.*, 2000). PTHrP greatly potentates the phosphorylation of SOX9 (S181), and increases the SOX9-dependent activity of chondrocyte-specific enhancers in *Col2a1*, the gene for collagen II. These findings indicate that SOX9 is a target of PTHrP signaling in prehypertrophic chondrocytes in the growth plate and that the PTHrP-dependent increased transcriptional activity of SOX9 helps maintain the chondrocyte phenotype of cells in the prehypertrophic zone, thereby delaying their maturation to the hypertrophic state. Recent experiments demonstrate that PTHrP also decreases the production of the transcription factor, Runx2, in chondrocytes (but not in osteoblasts) in explants of fetal long bones (Guo *et al.*, 2006). In such explants, PTH administration rapidly leads to a fall in Runx2 mRNA and protein in chondrocytes. Runx2 is required for the hypertrophic differentiation of chondrocytes and directly binds to the promoter region of the *Ihh* gene and strongly induces its expression (Yoshida *et al.*, 2004). This suggests that Runx2 is essential for chondrocyte maturation and regulates limb growth by organizing chondrocyte maturation and proliferation through the induction of *Ihh* expression. The action of PTHrP to suppress Runx2 production probably contributes in part to the delay in the differentiation of chondrocytes caused by PTHrP. The activity of Runx2 may also be repressed by Sox9 through a direct interaction between the two transcription factors (Zhou *et al.*, 2006).

Although the effects of PTHrP on chondrocyte differentiation are generally considered only in terms of its interaction with the cell surface receptor (PTHR1), studies *in vitro* as well as *in vivo*, now indicate that the capacity of PTHrP to influence this process, must also be assessed in relation to its intracrine actions at the level of the nucleus/nucleolus (Fiaschi-Taesch *et al.*, 2006; Henderson *et al.*, 1995). Nuclear localization of the protein has been associated with inhibition of differentiation, stimulation of proliferation, and delay in the apoptotic death of chondrocytes (Henderson *et al.*, 1996), likely by increasing *Bcl2* gene expression in these cells (Amling *et al.*, 1997). However, the events that determine the timing and degree of PTHrP nucleolar translocation, or the role that it may serve in the *in vivo* biology of chondrocytes, remain for the most part undefined.

Mechanisms underlying the molecular regulation of chondrocyte proliferation have been investigated, notably using transgenic mice that carry either gain- or loss-of-function mutations. From these studies, it has become evident that one of the most potent inducers of chondrocyte proliferation is Ihh. In support of this contention is the observation that the most striking feature of the *Ihh*-null endochondral skeleton is a profound decrease in limb length arising as a consequence of severe reduction in growth plate chondrocyte proliferation (St-Jacques *et al.*, 1999). Although this effect, unlike that on differentiation, is for the most part independent of PTHrP (Karp *et al.*, 2000), it is evident that PTHrP exerts its own unique influence on proliferation (Amizuka *et al.*, 1994; Karp *et al.*, 2000), even in the absence of Ihh (Koziel *et al.*, 2005).

Several signaling pathways have been implicated in mediating PTHrP's action on chondrocyte proliferation. G_s activation leads to production of cyclic AMP and activation of protein kinase A (PKA). It appears that it is the actions of cyclic AMP and PKA that not only delay differentiation but also lead chondrocytes to continue proliferating. Many of the transcriptional effects of cAMP are mediated by the cAMP response element (CRE) binding protein CREB, which binds to the CRE element in the upstream region of a variety of genes. CREB is a member of the CREB/activating transcription factor (ATF) family of transcription factors and is phosphorylated by PKA following increases in intracellular cAMP levels. Phosphorylation permits its interaction with p300/CBP and other nuclear coactivators, leading to gene transcription (Montminy, 1997). A role for CREB in skeletal development was not suggested initially by the phenotype of the *Creb*-knockout mice perhaps in part because of functional compensation by other CREB family members (Rudolph *et al.*, 1998). In keeping with this supposition is the observation that targeted overexpression of a potent dominant negative inhibitor for all CREB family members to the murine growth plate causes a profound decrease in chondrocyte proliferation resulting in short-limbed dwarfism and perinatal lethality owing to respiratory compromise (Long *et al.*, 2001). Similarly, disruption of the gene encoding the transcription factor ATF2, another member of the CREB/ATF family, also profoundly inhibits proliferation of chondrocytes (Reimold *et al.*, 1996).

The cyclin D1 gene (*Ccnd1*) is a key regulator of progression through the G_1 phase of the cycle (see later) and has been identified as a target for the transcription factor ATF2 (Beier *et al.*, 1999). ATF2 is present in nuclear extracts from chondrogenic cell lines and binds, as a complex with a CRE-binding protein (CREB)/CRE modulator protein, to the cAMP response element (CRE) in the cyclin D1 promoter. Moreover, site-directed mutagenesis of the cyclin D1 CRE causes a reduction in the activity of the promoter in chondrocytes, whereas overexpression of ATF2 in chondrocytes enhances activity of the cyclin D1 promoter. Inhibition of endogenous ATF2 or CREB by expression of dominant-negative inhibitors of CREB and ATF2 significantly reduce the activity of the promoter in chondrocytes through the CRE. Finally, levels of cyclin D1 protein are drastically reduced in chondrocytes of ATF2-negative mice. These data identify the cyclin D1 gene as a direct target of ATF2 in chondrocytes and suggest that reduced expression of cyclin D1 contributes to the defective cartilage development of these mice. Homozygous deletion of *Ccnd1* in mice results primarily in reduced postnatal growth (Sicinski *et al.*, 1995). It is likely that alterations in the proliferation of chondrocytes may have contributed to this phenotype. However, the skeletal defects of these mice are clearly less severe than those of the ATF2-null mice, possibly because of the presence of intact cyclins D2 and D3. This advocates that additional target genes of ATF2 are involved in the reduction of chondrocyte proliferation in ATF2-deficient mice. In particular, it will be of interest to determine whether other D-type cyclin genes (cyclin D2 and D3) are regulated by ATF2 in chondrocytes. Finally, it remains to be seen whether PTHrP induces cyclin D1 expression through activation of CREB and how this impacts on chondrocyte proliferation. Alternatively, such a response may be mediated by the transcription factor AP-1, which is also central to PTHrP's action in chondrocytes (Ionescu *et al.*, 2001). Signaling by PTHR1 activates AP-1, a complex formed through interactions between c-Fos and c-Jun family members, by inducing the expression of c-Fos in chondrocytes. The protein complex binds to the phorbol 12-myristate 13 acetate (PMA) response element (TRE), a specific *cis*-acting DNA consensus sequence in the promoter region of target genes, like cyclin D1.

Because the genes of the cell cycle machinery execute the intracellular control of proliferation, it is likely that these genes play a pivotal role during endochondral ossification. A large body of experimental evidence now indicates that the major regulatory decisions controlling cell cycle progression, and hence proliferation of mammalian cells, takes place during the G_1/S phase checkpoint (reviewed in Sherr, 1993; Sherr and Roberts, 1995). Because cell cycle genes play an important role in proliferation, it is reasonable to speculate that they might be involved in the biological responses of chondrocytes to PTHrP. This view is supported by targeted disruption in mice of the pRb-related p107 and p130 genes (Cobrinik *et al.*, 1996) and the CDK inhibitor $p57^{Kip2}$ (p57) (Yan *et al.*, 1997). In both cases, bones from mutant mice show delayed ossification, elevated chondrocyte proliferation rate, and increased chondrocyte cell density, leading to severe skeletal defects. The absence of PTHrP or p57 has opposite effects on endochondral bone development, suggesting that PTHrP's proliferative actions in chondrocytes might be mediated by opposing p57. PTHrP was subsequently shown to decrease expression of *p57* in chondrocytes both in bone explants and *in vivo*, as deduced from measuring levels of *p57* mRNA and protein in mice missing PTHrP (MacLean *et al.*, 2004). Moreover, in double-knockout mice missing both *PTHrP* and *p57*, many growth plates exhibit patterns of proliferation much closer to normal than in the *PTHrP*-null growth plates. This outcome suggests that suppression of p57 synthesis is a major mechanism used by PTHrP to keep chondrocytes proliferating.

Last, it was shown recently that PTHrP promotes growth plate chondrocyte proliferation, in part, by potentiating the levels of the repressor form of Gli3 (Gli3R) in a PKA-dependent manner (Mau *et al.*, 2007).

Fibroblast growth factor receptor 3 (FGFR3)

Another major molecular player in growth plate chondrocyte biology is fibroblast growth factor receptor 3 (FGFR3) (reviewed in Ornitz, 2005). FGF1, FGF2, and FGF9 bind FGFR3 with relatively high affinity (Ornitz and Leder, 1992); however, the ligands of FGFR3 *in vivo* and their downstream effects in individual tissues have not been precisely defined. Gain-of-function mutations in FGFR3 have been linked to several dominant skeletal dysplasias in humans, including achondroplasia (Bellus *et al.*, 1995; Rousseau *et al.*, 1994; Shiang *et al.*, 1994), thanatophoric dysplasia (TD) types I (Rousseau *et al.*, 1996; Tavormina *et al.*, 1995) and II (Tavormina *et al.*, 1995), and hypochondroplasia (Bellus *et al.*, 1995). This group of disorders is characterized by a continuum of severity, from hypochondroplasia exhibiting a lesser degree of phenotypic severity, to achondroplasia, and to TDs, two lethal neonatal forms of dwarfism distinguished by subtle differences in skeletal radiographs. Achondroplasia is the most common genetic form of dwarfism in humans and results from a mutation in the transmembrane domain (G380R) of FGFR3, whereas thanatophoric dysplasia is the most common neonatal lethal skeletal dysplasia in humans and results from any of three independent point mutations in *FGFR3*. Nearly all reported missense mutations in families with TDI were found to cluster in two locations: codon 248 involving the substitution of an arginine for a cysteine residue (R248C) and the adjacent codon 249 causing a serine to a cysteine change (S249C). In all patients with TDII, a lysine to glutamic acid substitution at position 650 (K650E) was described in the tyrosine kinase domain of the FGFR3 receptor. Heterozygous *FGFR3* mutations have been reported also in patients with hypochondroplasia. In 8 of 14 alleles examined, a single C-to-A transversion causing an asparagine-to-lysine substitution at position 540 (N540K) of the protein was demonstrated.

Clinically, all of these mutations result in a characteristic disruption of growth plate architecture and disproportionate shortening of the proximal limbs. The mechanism by which *FGFR3* mutations disrupt skeletal development has been investigated extensively. Outside the developing central nervous system, the highest level of *FGFR3* mRNA is found in the cartilage rudiments of all bones, and during endochondral ossification, *FGFR3* is restricted to the resting and proliferating zones of cartilage in the growth plates (Peters *et al.*, 1993). Inactivation of FGFR3 signaling in mice leads to an increase in the size of the hypertrophic zone, as well as a coincident increase in bone length postnatally, suggesting that FGFR3 functions as a negative regulator of bone growth (Colvin *et al.*, 1996; Deng *et al.*, 1996). *In vitro* studies indicate that FGFR3-associated mutations confer gain-of-function properties to the receptor by rendering it constitutively active (Naski *et al.*, 1996). Ligand-independent receptor tyrosine phosphorylation then leads to inhibition of cell growth and differentiation in cartilaginous growth plates (Naski *et al.*, 1998; Segev *et al.*, 2000).

The ligand that signals to FGFR3 during skeletal development is likely *Fgf18*, which is expressed in the perichondrium (Ohbayashi *et al.*, 2002). Growth plate histology of mice lacking *Fgf18* is similar to that of mice lacking *Fgfr3*. Both knockout mice show an upregulation of Ihh and Ptc expression and increased chondrocyte proliferation. These phenotypic similarities strongly suggest that FGF18 is a physiological ligand for FGFR3 in chondrocytes (Liu *et al.*, 2002; Ohbayashi *et al.*, 2002). While the molecular mechanisms that underlie the processes by which FGFR3 signaling inhibits bone growth remain sketchy at present, a model has been developed in which inhibition of chondrocyte differentiation occurs through the MAPK pathway while proliferation of chondrocytes is inhibited through a STAT1 pathway (Murakami *et al.*, 2004).

Transcription factors

Although the pivotal role of transcription factors in the development of the growth plate has been exemplified by the actions on Sox9, Runx2, and Gli3, recently, additional such factors have been implicated, rather unexpectedly at times, in this process. The fetal growth plate is a unique mesenchymal tissue, because it is virtually avascular and hence hypoxic. This prompted the question as to how chondrocytes could survive in this milieu. The transcription factor Hif-1α has emerged as the central regulator of the hypoxic response in mammals (reviewed in Provot and Schipani, 2007). Transcriptional activation by Hif-1α occurs on its binding to the hypoxia response element (HRE) within its target genes. One target of Hif-1α transcriptional activation is the angiogenic factor vascular endothelial growth factor (VEGF). Hif-1α accumulation is controlled by the von Hippel–Lindau (VHL) tumor suppressor, an E3-ubiquitin ligase that induces its degradation by the proteasome. To address the role of Hif-1α, in adaptive responses to hypoxia, a conditional knockout approach was used to remove specifically Hif-1α from chondrocytes (Schipani *et al.*, 2001). *Hif-1α-null* cells at the center of the growth plate, which is hypoxic, underwent massive cell death, a phenotype with striking similarity to the cell death observed after deletion of *Vegf* from chondrocytes (Zelzer *et al.*, 2004). This elegant work suggests that Hif-1α and VEGF are likely part of a common pathway that supports chondrocyte survival in endochondral bone development.

Histone acetylation, which is catalyzed by histone acetyltransferases (HATs) and promotes gene transcription by relaxing chromatin structure, is counterbalanced by histone deacetylation that favors chromatin condensation and transcriptional repression. The class II histone deacetylases (HDACs) HDAC 4, 5, 7, and 9 display cell type-restricted patterns with HDAC4 being highly expressed in prehypertrophic chondrocytes. Mice lacking HDAC4 display a remarkable phenotype characterized by inappropriate chondrocyte hypertrophy leading to ectopic bone formation (Vega *et al.*, 2004), abnormalities analogous to the phenotype observed in mice with constitutive expression of Runx2 in chondrocytes (Takeda *et al.*, 2001). Indeed, HDAC4 was shown to associate with and inhibit the activity of Runx2, thereby establishing HDAC4 as a key regulator of chondrocyte hypertrophy.

Another transcription factor, myocyte enhancer factor-2c (MEF2C), known to regulate muscle and cardiovascular development, was reported to be critical for normal chondrocyte hypertrophy and subsequent ossification. Chondrocytes in developing bones of Mef2c-null mice fail to express Runx2 and thereby fail to undergo hypertrophy indicating that MEF2C acts upstream of Runx2 in the induction of chondrocyte hypertrophy (Arnold *et al.*, 2007). Failure of endochondral ossification in heterozygous Mef2c-null mice can be reversed by deletion of Hdac4 alleles, and the premature ossification of Hdac4-null mice can be reversed by deletion of a Mef2c allele. These observations indicate that the balance between transcriptional activation by MEF2C and repression by HDAC4 dictates normal initiation and progression of chondrocyte hypertrophy. Since HDAC9 interacts with and suppresses the activity of MEF2 in the heart (Zhang *et al.*, 2002), it remains to be determined whether HDAC4 acts in a similar fashion in chondrocytes.

Interplay of Local Mediators

During the process of proliferation and differentiation, chondrocytes integrate a complex array of signals both from local and systemic factors. Understanding the specific role of one signaling pathway requires an appreciation of how it integrates with other signals participating in bone development. What is known about the interplay between FGFR3, PTHrP and Ihh signaling in the growth plate? The overlapping expression of FGFR3 and PTHR1 in the growth plate would suggest that these signaling pathways interact. In fact, the marked decrease in the size of the proliferative zone arising from inactivation of either PTHrP or PTHR1 in mice resembles the phenotype seen with constitutive activation of FGFR3 signaling. It is likely, therefore, that one pathway by which PTHrP can stimulate chondrocyte proliferation involves downregulation of *Fgfr3* expression. In fact, work by McEwen *et al.* (1999) supports a model whereby PKA signaling, by effectors such as the PTHR1, represses *Fgfr3* gene expression in proliferating chondrocytes of the epiphyseal growth plate and thus serves to regulate endochondral ossification (see Fig. 7). Moreover, *in vivo* studies indicate that FGFR3 signaling can repress *Ihh* and *Pthr1* expression in the growth plate (Chen *et al.*, 2001; Naski *et al.*, 1998). This would, in turn, link the Ihh/PTHrP signaling to the FGFR3 pathway in the epiphyseal growth plate and hence complete a potential feedback loop that orchestrates endochondral bone growth.

The Articular Cartilage

Little is known about the factors that control the differentiation of joint mesenchymal interzone cells to form the opposing articular cartilage surfaces. In contrast to chondrocytes in the shaft, which tend to undergo maturation, hypertrophy, mineralization, and subsequent replacement by bone, these cells resist differentiation and produce abundant extracellular matrix to maintain normal joint function throughout life. The mechanisms that drive chondrocytes to this alternative fate are only now beginning to be unveiled. Endogenous TGFβs likely maintain cartilage homeostasis by preventing inappropriate chondrocyte differentiation because expression of a dominant-negative form of the transforming growth factor (TGF-) type II receptor in skeletal tissue results in increased hypertrophic differentiation in growth plate as well as articular chondrocytes (Serra *et al.*, 1997).

Studies by Iwamoto *et al.* (2000) have identified C-1-1, a novel variant of the ets transcription factor ch-ERG, which lacks a 27-amino-acid segment upstream of the ets DNA-binding domain. C-1-1 expression has been localized in the developing articular chondrocytes, whereas ch-ERG is particularly prominent in prehypertrophic chondrocytes in the growth plate. Virally driven overexpression of C-1-1 in developing chick leg chondrocytes blocks their maturation into hypertrophic cells and prevents the replacement of cartilage by bone. It also induces the synthesis of tenascin-C, an extracellular matrix protein that is unique to developing articular chondrocytes. In contrast, expression of ch-ERG stimulates chondrocyte maturation.

When the human C-1-1 counterpart (hERG3Delta81) is expressed throughout the cartilaginous skeleton of transgenic mice using Col2a1 gene promoter/enhancer sequences, the skeletal phenotype is severe and neonatal lethal (Iwamoto *et al.*, 2007). The transgenic mice are smaller than wild-type littermates and their skeletons are largely cartilaginous. Limb long bone anlagen are entirely composed of chondrocytes actively expressing collagen IX and aggrecan as well as articular markers such as tenascin-C. Typical growth plates are absent and there is very low expression of maturation and hypertrophy markers. This work identifies hERG3Delta81, the human counterpart of C-1-1, as a transcription factor instrumental in the genesis and maintenance of epiphyseal articular chondrocytes and provides a first glimpse into the mechanisms that dictate alternative chondrocyte developmental pathways.

COUPLING CHONDROGENESIS AND OSTEOGENESIS

The Formation of Bone Collar

As illustrated in Fig. 5A, the bone collar that forms in the perichondrium is the precursor of the cortical region of long bones. Hypertrophic chondrocytes have been proposed to play a critical role in coordinating growth plate chondrogenesis and perichondrial osteogenesis, although the molecular parameters that regulate these processes remain for the most part undefined. In earlier work, it was noted that *Ihh*-null mice have no bone collar (St-Jacques *et al.*, 1999) whereas overexpression of Ihh induces bone collar formation (Vortkamp *et al.*, 1996). Follow-up observations made in growth plates from genetically altered mice have identified *Ihh* expression by prehypertrophic chondrocytes as the critical determinant in the site of bone collar formation and in the induction of mature osteoblasts in the adjacent perichondrium (Chung Ui *et al.*, 2001). The presence of mature osteoblasts in membranous bones of Ihh mutants suggests that the bone collar, which is often referred to as being similar to intramembranous ossification, is in fact developmentally distinct.

In turn, a cascade of interacting factors in the perichondrium inhibits chondrocyte maturation. This was initially suggested from studies of chicken tibia organ cultures in which removal of the perichondrium resulted in increased chondrocyte proliferation and in a larger zone of chondrocyte hypertrophy (Long and Linsenmayer, 1998; Di Nino, *et al.* 2001). It was subsequently shown that the nuclear protein Twist-1, although it is never expressed in chondrocytes, regulates Runx2 activity in perichondrial cells where *Fgf18*, a negative regulator of chondrocyte maturation, is a target gene of Runx2 (Hinoi *et al.*, 2006). As a result, through its perichondrial expression, Runx2 exerts a negative influence on chondrocyte maturation.

Vascular Invasion of the Growth Plate

Ossification begins by the invasion of calcified hypertrophic cartilage. If ossification is to occur successfully, vascular invasion of the growth plate must take place. This process presents a challenge to the system because cartilage, a tissue highly resistant to vascularization, is replaced by bone, one of the most vascular tissues in the body. As such, this process would require the coordination of expression of factors that promote neovascularization and/or removal of factors that inhibit it, along with the proteolysis of the cartilage extracellular matrix that allows for vascular invasion to take place. In support of this concept is the observation that avascular cartilage expresses potent angiogenic inhibitors such as chondromodulin I (Hiraki *et al.*, 1997; Shukunami and Hiraki, 2007), whereas a number of factors that promote neovascularization are being produced by hypertrophic chondrocytes.

Matrix metalloproteinases (MMPs), a family of extracellular matrix-degrading enzymes, have been implicated in this process (for review, see Vu and Werb, 2000). The MMPs are produced as latent proenzymes and can be inhibited by specific tissue inhibitors of metalloproteinases (TIMPs). Gene-targeting studies have implicated two particular MMPs in bone development: MMP9/gelatinase B (MMP9) and MT1-MMP (MMP14). MMP9 is highly expressed in multinucleated osteoclasts localized along the mineralized longitudinal septae and chondroclasts at the nonmineralized transverse septae of the cartilage–bone junction that lead the vascular invasion front. Endothelial cells, on the other hand, which are also abundant at the invasion front of the growth plate, do not express MMP9. Targeted disruption of *Mmp9* in mice leads to the development of abnormal growth plates in the long bones characterized by a nearly doubling in the length of the hypertrophic zone at birth with no changes noted in the reserve or proliferating zones (Vu *et al.*, 1998). By 3 weeks of age, the zone has enlarged to six to eight times the normal length. Because these cells appear normal and the matrix calcifies normally, alterations in the hypertrophic zone are attributed to a delay in the apoptosis of hypertrophic chondrocytes coupled with an impediment in vascular invasion. Because *Mmp9*-null hypertrophic cartilage exhibits a net decrease in angiogenic activity, the model for MMP9 action at the growth plate is attributed to the release of angiogenic factors sequestered in the extracellular matrix.

A variety of angiogenic factors are expressed in the growth plate, including members of the FGF family, IGF1, EGF, PDGF-A, members of the TGF- family, Cyr61 and transferrin. However, the importance of these factors in growth plate angiogenesis is still uncertain. Vascular endothelial growth factor (VEGF) is one angiogenic protein that is expressed in hypertrophic chondrocytes and binds to extracellular matrix. When made bioavailable, VEGF binds to its respective tyrosine kinase receptors Flt-1 (VEGFR1) and Flk-1/KDR (VEGFR2), both of which are expressed on endothelial cells (Ferrara and Davis-Smyth, 1997). Strong experimental evidence now links receptor activation to VEGF-induced mitogenesis, angiogenesis, and endothelial cell survival (Fong *et al.*, 1995; Gerber *et al.*, 1998; Shalaby *et al.*, 1995). Blockade of VEGF action through the systemic administration of a soluble receptor chimeric protein (Flt-(1-3)-IgG), recapitulates the phenotype of the *Mmp9*-null bones by impairing invasion of the growth plate (Gerber *et al.*, 1999). That VEGF plays a significant role in cartilage vascularization was substantiated by the observation that mice with conditional knockout of all three VEGF isoforms in chondrocytes showed delayed invasion of blood vessels into primary ossification centers and delayed removal of terminal hypertrophic chondrocytes (Zelzer *et al.*, 2004). It appears that MMP9 releases VEGF from the extracellular matrix (Bergers *et al.*, 2000), which in turn recruits endothelial cells and thus induces and maintains blood vessels. These blood vessels bring in not only nutrients, but also chondroclasts, osteoclasts, and osteoblasts as well as proapoptotic signal(s) (Engsig *et al.*, 2000). VEGF-mediated blood vessel invasion is therefore essential for coupling resorption of cartilage with bone formation. In the absence of blood vessel invasion, hypertrophic chondrocytes fail to undergo cell death, resulting in thickening of the growth plate. Therefore, the vasculature conveys the essential signals required for correct growth plate morphogenesis.

But what regulates the expression of VEGF? Recent work in mice null for *Fgf18* indicates an apparent delay in skeletal mineralization closely associated with delayed initiation of chondrocyte hypertrophy, skeletal vascularization, and osteoclast and osteoblast recruitment to the growth plate (Liu *et al.*, 2007). The concomitant observation that Ffg18 is necessary for *Vegf* expression in hypertrophic chondrocytes and the perichondrium supports a model in which Fgf18 coordinates neovascularization of the growth plate and subsequent recruitment of osteoblasts/osteoclasts, in part, through regulation of VEGF expression.

Vascular Invasion of the Epiphysis

In the epiphysis, hypertrophic cartilage is formed in the center, as chondrocyte maturation progresses inward. In order that osteogenic precursor cells come in for the ensuing ossification process, the formation of vascular canals must first take place. This involves degradation of uncalcified cartilage to clear a path for the invading blood vessels (see Fig. 3.5A). In the mouse, cartilage canals start off as invaginations of the perichondrium at day 5 after birth. At day 10, several small ossification nuclei arise around the canal-branched endings, which finally coalesce, and at day 18 a large secondary ossification center occupies the whole epiphysis. Interestingly, cartilage canal cells express collagen I and, during canal formation, several resting chondrocytes immediately around the canals are freed into the canal cavity and appear to remain viable, suggesting that cartilage canal cells belong to the bone lineage and may contribute to the formation of the bony epiphysis by differentiating into osteoblasts (Blumer *et al.*, 2007).

MT1-MMP (MMP14) is a membrane-bound matrix metalloproteinase capable of mediating pericellular proteolysis of extracellar matrix components. Its role in skeletal development was recognized following its targeted inactivation (Holmbeck *et al.*, 1999; Zhou *et al.*, 2000). In contrast to *Mmp9*-null mice, these animals display craniofacial dysmorphism and dwarfism, the former likely arising from impaired intramembranous bone formation whereas the latter reflects defects in endochondral ossification of the epiphyseal (secondary) centers of ossification. In the

Mmp14-null mice, invasion of the uncalcified epiphyseal hyaline cartilage by vascular canals, which represents a critical early step in the development of the secondary centers of ossification, fails to occur, leading to a delay in ossification. For reasons that are not exactly clear, this delay has profound consequences on the growth of the epiphyseal plate including thinning, disorganization, and lack of chondrocyte proliferation. It is speculated that the delay of epiphyseal vascularization results in a shortage of chondrocyte precursors and subsequent growth plate atrophy.

A role for CCN2/connective tissue growth factor (CCN2/CTGF) during secondary ossification center formation has also been suggested, although the evidence here remains circumstantial. CCN2/CTGF expression is reported in the central region of the epiphysis, where the chondrocytes become hypertrophic and the cartilage canals enter into the hypertrophic mass (Oka *et al.*, 2007). Nevertheless, the proposed role of CCN2/CTGF in supporting angiogenesis during the development of the secondary ossification center remains to be validated.

SUMMARY

In each phase of skeletal development, it is the appropriate interplay of a number of gene products that will determine the final phenotypic outcome. In this chapter, we have reviewed the developmental biology of the skeleton, the complex array of signals that influence each developmental stage, and finally, a number of inherited disorders of the skeleton arising from mutant gene products that influence primarily, although not exclusively, one of these specific phases. Knowledge of how specific gene defects contribute to bone pathophysiology will guide future efforts in the treatment of inherited and metabolic skeletal disorders.

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Chapter 4

Mesenchymal Stem Cells and Osteoblast Differentiation

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INTRODUCTION

Bone has the potential to repair itself throughout the life of an organism through the coordinated activities of osteoclasts, derived from hemopoietic stem cells (HSCs), and osteoblasts, derived from mulitpotential stem cells that have been variously termed stromal stem cells, mesenchymal stromal cells, skeletal stem cells, stromal fibroblastic stem cells, and most recently, mesenchymal stem cells (MSCs) among other monikers. Notwithstanding the limitations of the name in terms of developmental origin and ontogeny and the relative lack of evidence for the stemness of stromal-derived multipotent cells [sometimes also designated mesenchymal progenitor cells (MPCs)], as will be discussed further later, the term MSC has gained popularity and will be used in this chapter for convenience. I will review currently available paradigms governing development of osteoblasts and other mesenchymal cell types such as chondrocytes, adipocytes, and myoblasts among other mesenchymal cell types. I will also discuss evidence for MSC and osteoprogenitor deficiencies in disease and disease models, and the rapidly expanding literature on potential use of MSC in regenerative medicine applications, not only for bone, but also for other tissues and organs.

ONTOGENY OF OSTEOBLASTS AND CONTROL OF OSTEOBLAST DEVELOPMENT

CFU-F Assays and Osteogenic Cell Lineage Hierarchies

Friedenstein first showed that bone marrow stroma contains cells that have both significant proliferative capacity and the capacity to form bone when transplanted *in vivo* in diffusion chambers. Subsequently, he and others demonstrated that, in addition to bone, cartilage, marrow adipocytes, and fibrous tissue also formed *in vivo* and that all the tissues could arise from single colonies or colony-forming units-fibroblastic (CFU-F) [Friedenstein (1990); summarized in Bianco *et al.* (2001)]. *In vivo* analyses of stromal cells have been augmented by functional assays *in vitro* that show formation of a range of differentiated cell phenotypes and have led many to identify stromal populations as MSCs. However, the kinds of experiments needed to address whether marrow stroma contains a definitive stem cell—by the definition of self-renewal capacity and the ability to repopulate all the appropriate differentiated lineages or even by less stringent definitions—are only beginning to be done. For example, although expanded populations of stromal cells are routinely reported to express capacity to undergo differentiation along multiple mesenchymal lineages, CFU-F are heterogeneous in size, morphology, and potential for differentiation (Friedenstein, 1990; Kuznetsov *et al.*, 1997), consistent with the view that they are developmentally heterogeneous and belong to a lineage hierarchy in which only some of the cells are multipotential stem or primitive progenitors whereas others are more restricted. A growing body of data supports the view that only some single-cell-derived colonies of stromal cells express multilineage capacity whereas others express more restricted pontentiality, both by marker expression profiling (Zhang *et al.*, 2006a) and by functional/differentiation endpoints *in vitro* and *in vivo* (Gronthos *et al.*, 2003; Pittenger *et al.*, 1999; Rider *et al.*, 2007). There is also evidence that stromal cells are biochemically heterogeneous, i.e., that different stromal cell subpopulations express different regulatory proteins that function in angiogenesis, hematopoiesis, neural activities, and immunity and defense (Phinney, 2007). These data are consistent with limiting dilution and very-low-density plating studies that show that only a proportion of CFU-F are CFU-alkaline phosphatase (CFU-ALP) and, further, that only a proportion of these are CFU-osteogenic (CFU-O,

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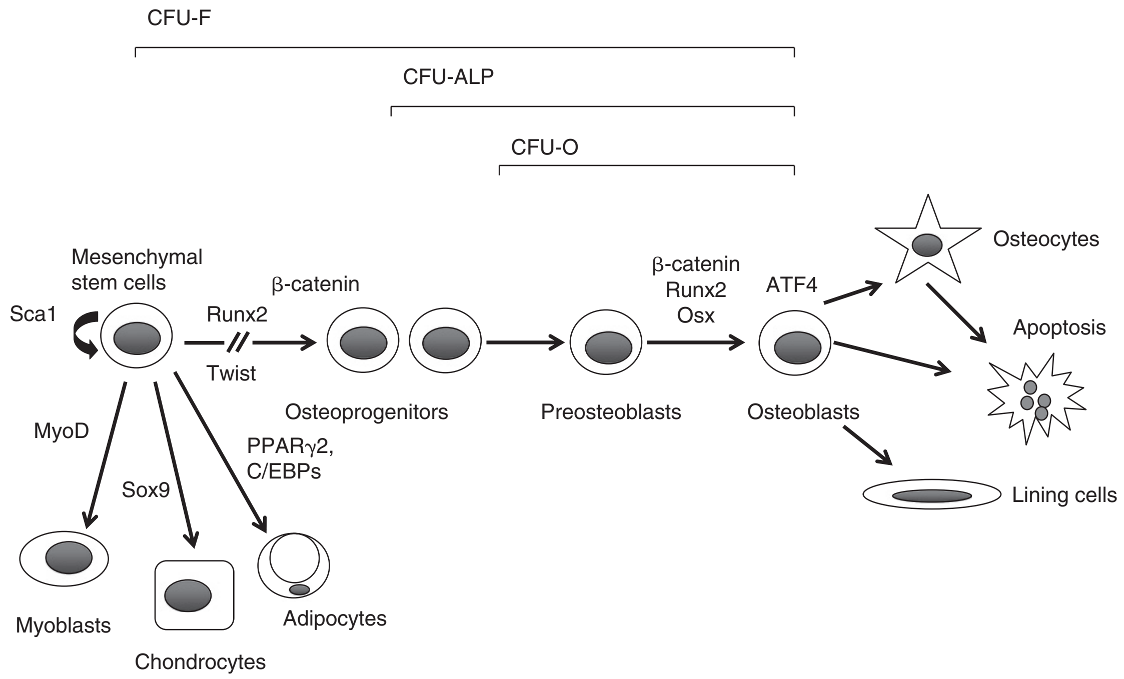


FIGURE 1 Schematic illustration of stem cell commitment to various end-stage mesenchymal cell types, including osteoblast lineage cells, with some of the known regulatory transcription factors indicated.

clonogenic bone colonies or bone nodules) with some variation reported between different species; CFU-adipocytic (CFU-A) also constitute a subset of CFU-Fs (Aubin, 1999; Wu *et al.*, 2000) (Fig. 1). What would help advance the field are assays comparable to those achievable for hemopoietic stem cells (HSCs), long-term culture-initiating cells (LTC-IC), and HSC/LTC-IC capable of long-term repopulating ability detected by their ability to serially repopulate lethally irradiated mice at limiting dilution (reviewed in Bryder *et al.*, 2006; Sauvageau *et al.*, 2004). Although such assays may be difficult to achieve for MSCs, especially *in vivo*, clear quantification and understanding of the clonality of mesenchymal cell progeny, the ratios of stem to other more restricted progenitors in various stromal populations, the identifiable commitment and restriction points in the stromal cell hierarchy, the self-renewal capacity, and the repopulation capacity of individual precursor cells should be goals. Attempts to combine retrospective assays for specific progenitor cell types with quantitative approaches *in vitro* and *in vivo* [e.g., gene marking, reviewed in Prockop (1997); limiting dilution, reviewed in Aubin (1999), and single cell transplantation experiments] are beginning to aid in the determination of the frequency and biological properties of various mesenchymal precursor cell populations and address where biology and concepts of HSCs versus MSCs may converge or diverge (Benveniste *et al.*, 2003; Ema *et al.*, 2006; Waller *et al.*, 1995b). A recent analysis of human stromal cells is particularly notable in showing that some single-cell-derived colonies with high expression of CD146 (melanoma-associated cell adhesion molecule, also known as Mel-CAM or MCAM) express at least some capacity for self-renewal and multipotentiality, including capacity to generate osteoblasts and the HSC niche, whereas CD146-negative/CD146$^{\text{low}}$ can make osteoblasts but not the HSC niche [Sacchetti *et al.* (2007); see commentary (Aubin, 2008)]. Such observations will become increasingly important as work on stromal populations increases based on their proposed utility for tissue regeneration and as vehicles for gene therapy (see later).

Differentiation analyses of clonally derived immortalized (e.g., spontaneously or via large T antigen expression) cell lines derived from stroma, bone-derived cells, or other mesenchymal/mesodermal tissues, such as the mouse embryonic fibroblast line C3H10T1/2, the fetal rat calvaria-derived cell lines RCJ3.1 and ROB-C26, and the teratocarcinoma mesodermally derived C1 line, have also provided evidence for the existence of multipotential mesenchymal progenitor or stem cells capable of giving rise to multiple differentiated cell phenotypes, including osteoblasts, chondroblasts, myoblasts, and adipocytes (Aubin and Liu, 1996). Studies on these cell lines have led to suggestions of two different kinds of events underlying MSC commitment: a stochastic process with an expanding hierarchy of increasingly restricted progeny [e.g., RCJ3.1 (Aubin, 1998); see also that stromal cell clonal analysis as described earlier would fit this model] and a nonrandom, single-step process in which multipotential progenitors become exclusively restricted to a single lineage by particular culture conditions [e.g., the C1 line in an environment of soluble inducers, substrate, and/or cell density (Poliard *et al.*, 1995)] have been proposed to underlie mesenchymal stem cell restriction (see discussion in Aubin, 1998). These models may be different endpoints on a single continuum, as particular culture restraints or environmental or local conditions *in vivo* may shift the frequency or probability of what might otherwise be random or stochastic commitment/restriction events to favor particular outcomes. This is consistent with the observation that at least certain hormones [e.g., 1,25-dihydroxyvitamin D_3 (Zhang *et al.*, 2006a); dexamethasone followed sequentially by triiodothyronine (Locker *et al.*, 2004)] and cytokines [e.g., leukemia inhibitory factor (Falconi *et al.*, 2007)] can regulate fate choices of multipotential cells *in vitro*.

Committed osteoprogenitors, i.e., progenitor cells restricted to osteoblast development and bone formation, at least under standard conditions, can also be identified by functional assays of their differentiation capacity *in vitro* (as so-called earlier, the CFU-O assay) in not only stromal cell populations, but also populations derived from calvariae, vertebrae, and other bones. However, as already indicated earlier, under some conditions and from some tissues, mixed colony types can also be seen. A number of studies on human bone-derived cells, both populations derived from human trabecular bone and clonally derived lines of human bone marrow stromal cells, have supported the observations on rodent marrow stromal populations that a bipotential adipocyte-osteoblast precursor cell exists [reviewed in Duque (2007); Gimble *et al.* (2006)]. It has also been suggested that the inverse relationship sometimes seen between expression of the osteoblast and adipocytic phenotypes in marrow stroma (e.g., in osteoporosis or in some culture manipulations) may reflect the ability of single or combinations of agents to alter the commitment or at least the differentiation pathway these bipotential cells will transit. In many cases, individual colonies are seen in which both osteoblast and adipocyte markers are present simultaneously. However, whether a clearly distinguishable bipotential adipo-osteoprogenitor can be identified or other developmental paradigms, such as transdifferentiation or plasticity, underlie expression in these two lineages needs to be analyzed further and is of significant clinical interest in osteoporosis and the aging or immobilized skeleton [summarized in Gimble *et al.* (2006)].

A variety of observations have suggested that a bipotential osteochondroprogenitor may also exist. However, as raised earlier for adipocyte-osteoblast phenotypes, the ability to transdifferentiate or change expression profiles may also characterize osteoblast-chondroblast lineages. The possible presence of undifferentiated/uncommitted stem cells and multi- and bipotential progenitors in cultures that may also contain monopotential but plastic progenitors at higher frequencies often complicates the ability to unambiguously discriminate the nature of the cells being affected. Difficulties in establishing unambiguous evidence for multipotentiality underscore the need for more markers and experiments to distinguish the molecular mechanisms underlying the ability of cells to express multipotentiality, the number and nature of commitment steps to a restricted phenotype(s), and both physiological and pathological mechanisms that may govern phenotypic conversions.

MSCs were originally isolated from the bone marrow but MSCs—or populations with similar developmental potential—have now been identified in many other tissues including adipose tissue (Gimble and Guilak, 2003; Tholpady *et al.*, 2003), umbilical cord blood (Karahuseyinoglu *et al.*, 2007; Kern *et al.*, 2006; Sarugaser *et al.*, 2005), muscle (Jiang *et al.*, 2002; Shefer and Yablonka-Reuveni, 2007), dental pulp (Shi and Gronthos, 2003), amniotic fluid (Roubelakis *et al.*, 2007), and skin (Toma *et al.*, 2005). Compact bone has also been suggested to be a source, perhaps even a richer source than bone marrow, of multipotential mesenchymal progenitors (Guo *et al.*, 2006; Short *et al.*, 2003). Interestingly, it has been suggested that the earliest embryonic appearance of MSCs is from neuroepithelium, not mesoderm, and that the neural source is replaced by nonneural sources as development continues (Takashima *et al.*, 2007). The isolation of MSCs from these various tissues usually involves adherence of the cells to tissue culture plastic, with or without subfractionation or enrichment strategies, and in most cases the adherent population is termed MSCs (or the CFU-F population). However, it is worth considering how adherent MSCs relate to nonadherent stem and progenitor cells for osteoblasts and other mesenchymal lineages described in the bone marrow and peripheral circulation.

Support for the existence of circulating mesenchymal precursor cells comes from several groups and models. For example, Zvaifler *et al.* (2000) reported human peripheral blood cells with a multipotentiality for mesenchymal lineages, including osteogenic cells, *in vitro*. This is consistent with data from Kuznetsov *et al.* (2001) who found that a small population of cells within the peripheral blood of humans, mice, rabbits, and guinea pigs adhered to tissue culture plates *in vitro*, a property of stromal elements. These cells also formed colonies with osteogenic capacity in ceramic-based implants placed subcutis in immunocompromised mice. Notably, the frequency of circulating osteogenic precursors measured by Kuznetsov and colleagues was extremely low, raising questions about their physiological significance. On the other hand, as many as 5% of circulating cells have been designated as potential osteoprogenitors in studies in which nonadherent cells were assayed directly from the peripheral blood, rather than the subpopulation of cells that are tissue culture plastic-adherent (Eghbali-Fatourechi *et al.*, 2005). These data support those of Long and colleagues (1995, 1999) who had reported cells with the capacity to form colonies in semisolid medium, express osteoblastic markers and deposit mineralized matrix within a population of low-density, nonadherent marrow cells. Scutt and Bertram (1995) also found that some bone marrow cells that are initially nonadherent on plastic culture dishes with time adhere and form fibroblast colony-forming units *in vitro*. More recently, Horwitz and colleagues showed that plastic-nonadherent bone marrow cells have more than 10 times the bone-repopulating activity of plastic-adherent bone marrow cells in lethally irradiated mice (Dominici *et al.*, 2004). Even more striking in these latter studies was the finding that at least some of the cells in the plastic-nonadherent population, which were retrovirally marked to distinguish intrinsic development potential from fusion-related events, were able to generate both functional osteoblasts/osteocytes and hematopoietic cells supporting the idea that bone marrow may contain a primitive cell able to generate both the hematopoietic and osteoblastic lineages. Circulating stem and/or osteoprogenitor cells are unexpected based on earlier parabiosis experiments suggesting distinct precursors for hematopoietic versus stromal mesenchymal cells including osteoblasts (Walker, 1975). It is therefore worth considering whether the pool size of putative circulating osteoblastic cells changes under particular conditions that would make them easier or harder to detect. Eghbali-Fatourechi and colleagues (2005) hypothesized that the frequency of circulating osteoblasts changes with age and in states of high bone remodeling where they are regulated by particular bone-responsive hormones and growth factors, e.g., IGF-I and IGFBP3, as required for their active participation in bone formation. Much more work will be required to address whether and how the birth, life (differentiation/maturation), and/or death of putative circulating osteoblasts are regulated by a variety of factors. However, such studies suggest that marrow mesenchymal stem cells or their progeny, using as yet molecularly and biochemically uncharacterized transmigration and homing strategies, may have access to the blood stream directly from marrow, especially if mobilized by specific recruitment signal(s), much as hematopoietic stem cells do. This is consistent with the fact that the blood stream is a viable route of delivering at least some marrow-derived cells to the bone (Horwitz *et al.*, 1999, 2001). Their relationship to a putative vascular pericyte pool of osteogenic (Doherty *et al.*, 1998) or multipotential progenitor (Farrington-Rock *et al.*, 2004) cells also needs to be addressed [reviewed in Modder and Khosla (2008)].

Control of Osteoblast Development

Significant strides have been made in identifying the regulatory mechanisms underlying lineage restriction, commitment, and/or differentiation within some of the mesenchymal lineages (see Fig. 1). Master genes, exemplified by the MyoD, myogenin, and Myf-5 helix-loop-helix transcription factors in muscle lineages, are one paradigm in which one transcription factor is induced and starts a cascade that leads to the sequential expression of other transcription factors and of phenotype-specific genes (Berkes and Tapscott, 2005). A factor of a different transcription factor family, PPARγ2 mentioned earlier, together with other transcription factors, including the CCAAT/enhancer-binding (C/EBP) protein family, plays a key role in adipocyte differentiation (Rosen, 2005). Sox9, a member of yet another transcription factor family, is essential for chondrocyte differentiation, expression of various chondrocyte genes, and cartilage formation (Lefebvre and Smits, 2005).

With respect to osteoblasts, it is well-established that Runx2 (formerly called Cbfa1), a member of the runt homology domain transcription factor family, plays a crucial role in osteoblast development. Deletion of Runx2 in mice leads to animals in which the skeleton comprises only chondrocytes and cartilage without any evidence of bone (Komori, 2006; Komori *et al.*, 1997). Haploinsufficiency in mice (Komori *et al.*, 1997) and humans leads to the cleidocranial dysplasia phenotype [Mundlos *et al.*, 1997; Otto *et al.*, 1997; reviewed in Otto *et al.* (2002)]. Runx2 is the earliest of osteoblast differentiation markers currently known, its expression during development and after birth is high in osteoblasts, and it is upregulated in cultures treated with bone morphogenetic proteins (BMPs) and other factors that stimulate bone formation [reviewed in Komori (2006)]. Interestingly, studies have shown that Runx2 is also required alone or together with another Runx family member Runx3, depending on the skeletal element, for maturation of hypertrophic chondrocytes [Enomoto *et al.*, 2000; Yoshida, 2004; reviewed in Yoshida and Komori (2005)].

Many molecules that interact with and modify Runx2 activity have now been identified and it is beyond the scope of this chapter to review all of them; interested readers are referred to several other recent reviews (Franceschi *et al.*, 2007; Karsenty, 2007; Komori, 2006; Lian *et al.*, 2006) (see Fig. 1). However, some specific examples point toward a number of different mechanisms and pathways of importance. The basic helix-loop-helix (bHLH)-containing transcription factors Twist1 and Twist 2 are inhibitors of Runx2 in the craniofacial skeleton and in the appendicular skeleton, respectively (Bialek *et al.*, 2004). On the other hand, Schnurri 3, a zinc finger protein that controls Runx2 protein levels by promoting its degradation through the recruitment of the E3 ubiquitin ligase WWP1, also regulates osteoblast development (Jones *et al.*, 2006). Runx2 activity is also regulated via its interaction with signal transducers of transforming growth factor beta superfamily receptors, Smads. For example, Runx2 functions synergistically with Smad1 and Smad5 to regulate expression of bone-specific genes (Lee *et al.*, 2000; Zhang *et al.*, 2000) and to drive osteoblastogenesis *in vitro* (Phimphilai *et al.*, 2006) and *in vivo* (Ito and Zhang, 2001). The importance of the tightly regulated expression of not only Runx2 but also the factors that regulate it and that it regulates is underscored by the fact that

Runx2 can also inhibit chondrocyte and osteoblast differentiation. In cells of the bone collar and the perichondrium, Runx2 regulates expression of a secreted molecule, FGF18, which in turn inhibits osteoblast and chondrocyte differentiation (Hinoi *et al.*, 2006; Liu *et al.*, 2002).

Osterix (Osx), another zinc finger protein that belongs to the SP family of transcription factors, acts downstream of Runx2 and in its absence osteoblasts also do not form (Nakashima *et al.*, 2002). The regulation of Osx is still relatively poorly understood, but its transcription is positively regulated by Runx2 (Celil *et al.*, 2005) and negatively regulated by p53 (Wang *et al.*, 2006) with consequent reciprocal effects on osteoblast development and bone formation. In the absence of another zinc finger protein, Schnurri 2, Osx levels are also reduced leading to decreased bone formation (Saita *et al.*, 2007). Although it is clear that Osx acts to direct the commitment of preosteoblasts toward an immature osteoblast fate and away from a chondrogenic fate (Akiyama *et al.*, 2005; Nakashima *et al.*, 2002), there is as yet little information on exactly how Osx functions. It is known to form a complex with nuclear factor of activated T cells (NFAT) leading to cooperative regulation of bone formation, at least in part, via the transcriptional regulation of the Osx and of the COLIA1 promoters but not the Runx2-dependent activation of the osteocalcin promoter by the NFAT-Osx complex (Koga *et al.*, 2005).

Recently, the importance of Wnt signaling in osteoblast development and bone formation has emerged. Wnts are a family of 19 secreted proteins that bind to a membrane receptor complex composed of Frizzled (FZD) G protein-coupled receptors (GPCRs) and low-density lipoprotein (LDL) receptor-related proteins (LRPs) and activate intracellular signaling pathways. The best characterized is the canonical or Wnt/β-catenin pathway that signals through LRP-5 or LRP-6 and leads to inhibition of glycogen synthase kinase (GSK)-3β and subsequent stabilization of β-catenin, which translocates to the nucleus and activates lymphoid-enhancer-binding factor (LEF)/T-cell-specific transcription factors (TCFs); Wnts also activate noncanonical pathways [reviewed in Baron and Rawadi (2007); Krishnan (2006); Chan (2007)]. Wnts activate the canonical pathway by interacting with receptors of the Frizzled family and coreceptors of the LRP5/6 family. Loss-of-function mutations in Lrp5 result in low bone mass, whereas gain-of-function mutations in Lrp5 result in high bone mass [reviewed in Balemans and Van Hul (2007); Baron and Rawadi (2007); Krishnan *et al.* (2006)]. Conditional deletion of the β-catenin gene in neural crest cell precursors results in loss of cranial bones derived from neural crest cells (Brault *et al.*, 2001) and conditional deletion of β-catenin gene in mesenchymal progenitors revealed an essential role of β-catenin in osteoblast versus chondrocyte differentiation (Day *et al.*, 2005; Hill *et al.*, 2005, 2006) indicating that β-catenin plays a role in commitment of mesenchymal precursors to the osteoblast lineage. An additional mechanism by which Wnt may control osteoblast commitment is by blocking adipogenesis via the inhibition of the adipogenic transcription factors CCAAT/enhancer-binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) as demonstrated *in vivo* in Wnt10b transgenic mice (Bennett *et al.*, 2005) or *in vitro* (Rawadi *et al.*, 2003). Other recent data support the view that commitment of mesenchymal precursors to an osteoblast rather than an adipocytic fate is via the ability of the noncanonical Wnt signaling pathway to suppress PPARγ function through chromatin inactivation triggered by recruitment of a repressing histone methyltransferase (Takada *et al.*, 2007).

The link between Wnts and other transcription factors has also been noted. Indian hedgehog (Ihh) null mice completely lack endochondral ossification owing to the lack of osteoblasts at those skeletal sites (St-Jacques *et al.*, 1999). In Ihh$^{-/-}$ mice, Runx2 is expressed in chondrocytes but not in perichondrial cells that differentiate into osteoblasts in wild-type mice, indicating that Ihh is required for Runx2 expression in perichondrial cells. Runx2 and Runx3 are essential for chondrocyte maturation and induce Ihh expression in prehypertrophic chondrocytes, which in turn induces Runx2 expression in perichondrial cells. Nuclear β-catenin is not detected in the perichondrial cells of Ihh$^{-/-}$ mice, indicating that Ihh is required for canonical Wnt signaling in perichondrial cells (Hu *et al.*, 2005). Very recently, it was shown that the noncanonical Wnt signaling pathway also regulates osteoblast development; Wnt-5a through the noncanonical Wnt signaling pathway suppressed PPARγ function through chromatin inactivation triggered by recruitment of a repressing histone methyltransferase promoting osteoblastogenesis versus adipogenesis in mesenchymal precursors *in vitro* and in mice (Takada *et al.*, 2007).

As already mentioned, other soluble factors beyond the Wnts appear also to alter commitment of MSCs and their more restricted progeny. Although review of all of them is beyond the scope of this chapter, it is worth noting that certain hormones [e.g., $1,25(OH)_2$ vitamin D_3 (Zhang *et al.*, 2006a)] and cytokines [e.g., leukemia inhibitory factor (LIF) (Falconi *et al.*, 2007)] (see also later) alter the frequency of specific mesenchymal colony types in bone-derived populations, an issue worthy of further investigation. Later I will address further a variety of other transcription factors and receptor signaling pathways that clearly also influence osteoblast activity, the rate of bone formation, and the amount of bone deposited.

IMMUNOPHENOTYPING AND ENRICHING MSC AND OSTEOPROGENITOR CELLS

In hematopoiesis, immune cell biology, and oncology, the availability of large panels of stem and progenitor cell antibodies has been invaluable for defining subpopulation makeup, purifying highly enriched stem cell populations and analyzing cell commitment and developmental potential (Herzenberg and De Rosa, 2000). Fewer markers have been available for MSCs and their progeny, although the situation is beginning to change, especially for human and mouse cells, and combinations of antibodies and flow cytometry procedures are being used to subfractionate both marrow stromal cells and other osteogenic populations.

There is no one single antigen, cell surface or otherwise, that is able to unambiguously identify MSCs, and species specificity of some available reagents further complicates the analysis. However, many studies now suggest that both human and mouse multipotent populations, albeit heterogeneous by various functional criteria, express CD105 (transforming growth factor-B coreceptor), CD73 (membrane-bound ecto-5′-nucleotidase), and CD44 (hyaluronan receptor), but do not express CD45 (common leukocyte antigen), CD11b (Mac-1a; integrin α_M chain; highly expressed in a variety of hemopoietic cells including monocytes/macrophages, microglia, and others) or CD31 [platelet-endothelial cell adhesion molecule (PECAM)-1; highly expressed on endothelial cells; reviewed in Deans and Moseley (2000); Wagner (2005); Phinney (2007)]. There are conflicting data on whether MSCs do or do not express CD34 (a heavily glycosylated type I transmembrane protein/sialomucin), a marker routinely used to enrich for HSCs, and also expressed by hematopoietic progenitors of various sorts [see, e.g., Delorme and Charbord (2007); Simmons and Torok-Storb (1991a); Waller *et al.* (1995a)], but this may reflect, at least in part, changes with culture expansion and/or differences inherent in different strains of mice (Peister *et al.*, 2004). Sca-1 (stem cell antigen 1, Ly-6A/E; an 18-kDa phosphatidylinositol-anchored protein), another marker used in HSC enrichment especially in mice, has been used to enrich progenitor populations in other tissue types (Welm *et al.*, 2002). Some data suggest that Sca-1 is coexpressed with side population (SP) activity (ability to efficiently efflux Hoechst dye via the ATP-binding cassette transporter protein ABCG2), another phenotypic marker used to enrich for HSCs (Goodell *et al.*, 1996), and bone-derived multipotential mesenchymal cell populations (Zhang *et al.*, 2006b). Consistent with the view that Sca-1 may be a marker of MSCs, *in vivo* and *in vitro* analyses of Sca-1-deficient mice demonstrated that Sca-1 is required for the self-renewal of mesenchymal progenitors (Bonyadi *et al.*, 2003).

A few other monoclonal antibodies that react with surface antigens on MSCs *in vitro* have been generated and used frequently to fractionate marrow stromal cells and enrich for MSCs and/or osteoblastic cells, including the STRO-1 antibodies that react with human (but not other species) MSCs (Simmons and Torok-Storb, 1991b). The STRO-1+ fraction of adult human bone is still markedly heterogeneous, but when combined with an antibody directed against vascular cell adhesion molecule 1 (VCAM-1/CD106), yields a highly enriched population of human marrow stromal precursor cells (Gronthos *et al.*, 2003). Several analyses demonstrated that these cells have at least some stem cell characteristics, as defined by extensive proliferative capacity and retention of differentiation capacity for osteogenesis, chondrogenesis, and adipogenesis. Expression of STRO-1 has been used in a number of other combinations, including with antibodies recognizing the bone/liver/kidney isoform of alkaline phosphatase (ALP) in human marrow stromal cell populations in which an inverse association was found between the expression of STRO-1 and ALP (Gronthos *et al.*, 1999; Stewart *et al.*, 1999). Osteogenic cells were sorted from mouse bone marrow based on light scatter characteristics, Sca-1 expression, and their binding to wheat germ agglutinin (WGA) (Van Vlasselaer *et al.*, 1994). Osteopontin (OPN) expression combined with cell size and granularity was used to sort rat calvaria and bone marrow stromal cells to enrich for cells responsive to BMP-7; these were said to have stem-like properties (Zohar *et al.*, 1997, 1998). Fractionation based on ALP alone or in combination with parathyroid hormone/parathyroid hormone-related protein receptor (PTHR1) expression has also been used to significantly enrich osteoprogenitors from rat calvaria populations (Purpura *et al.*, 2003; Turksen and Aubin, 1991) and rat bone marrow stromal cell populations (Herbertson and Aubin, 1997).

Better understanding of the surface profiles and their temporal changes with commitment of MSCs and the development of osteogenic cells would advance the field. Further, although all the studies summarized have fractionated populations into subpopulations with different characteristics and differentiation potentials, few attempts have yet been made to quantify the most primitive progenitors or stem cells in these populations and their functional characteristics: self-renewal, proliferation, and differentiation potentialities at single-cell or colony levels so that clear lineage relationships and hierarchies can be established analogous to those that have been achieved in hematopoietic populations. Many studies have defined MSCs based mainly on their ability to differentiate into multiple mesenchymal lineage cells (adipocytic, osteogenic, chondrogenic, etc.) *in vitro*, but a growing number suggest at least some capacity of MSC populations to contribute to bone and cartilage formation *in vivo* (Meirelles Lda and Nardi, 2003; Peister *et al.*, 2004; Sun *et al.*, 2003). Particularly powerful are recent studies that have attempted to characterize *in situ* localization of marker expression within a tissue combined with functional assays to distinguish between tissue-specific resident stem/progenitor cells and multipotent hematopoietic, endothelial, or mesenchymal progenitors *in vitro* and *in vivo*. In this regard, in a series of *in vitro* analyses and *in vivo* transplantation experiments, Sacchetti *et al.* (2007) recently demonstrated that the CD146-positive subendothelial reticular cell subpopulation of human bone marrow stromal progenitors (BMSCs) form all assayable CFU-F and their clonal progeny *in vitro*, have at least some capacity for self-renewal, and regenerate bone, stroma, and the hemopoietic microenvironment *in vivo*. Further analyses of human bone marrow-derived CD146$^+$ cells and analogous cells in mouse and other species will be crucial not only for understanding MSCs and osteoblasts, but also for understanding the bone niche for hematopoiesis (see also later).

OSTEOPROGENITOR CELLS AND REGULATION OF OSTEOBLAST DIFFERENTIATION AND ACTIVITY

Osteoprogenitor Cells

The morphological and histological criteria by which osteoblastic cells, including osteoprogenitors, preosteoblasts, osteoblasts, and lining cells or osteocytes, are identified have been reviewed extensively and will not be reiterated in detail here [see, e.g., Aubin and Heersche (2002)]. Morphological definitions are now routinely supplemented by the analysis of the expression of cell- and tissue-specific macromolecules, including the ecto-enzyme ALP, bone matrix proteins [type I collagen (COLL-I), osteocalcin (OCN), OPN, and bone sialoprotein (BSP), among others] and transcription factors that regulate them and commitment/differentiation events (e.g., Runx2, AP-1 family members, Msx-2, Dlx-5, etc.). Committed osteoprogenitors, i.e., progenitor cells restricted to osteoblast development and bone formation under default differentiation conditions, can be identified in bone marrow stromal cell populations and populations derived from calvaria and other bones by functional assays of their proliferation and differentiation capacity *in vitro* or, as often designated, the CFU-O assay. As mentioned earlier, CFU-Os appear to constitute a subset of CFU-F and CFU-ALP (Purpura *et al.*, 2003). Cells morphologically essentially identical to cells described *in vivo* and subject to many of the same regulatory activities can be identified, and the deposited matrix contains the major bone matrix proteins [see Bhargava *et al.* (1988); Malaval *et al.* (1994)].

Much has been learned from the *in vitro* bone nodule assay in which both the nature of the osteoprogenitors and their more differentiated progeny have been investigated by functional (the nature of the colonies they form, e.g., mineralized bone nodules), immunological (e.g., immunocytochemistry, Western blot analysis), and molecular (e.g., Northerns, PCR of various sorts, *in situ* hybridization, arrays and microarrays) assays. Bone nodules represent the *in vitro* end product of the proliferation and differentiation of CFU-O or osteoprogenitor cells present in the starting cell population. Estimates by limiting dilution and functional endpoints *in vitro* have indicated that osteoprogenitor cells are relatively rare in primary cell populations digested from fetal rat calvaria [i.e., <1% (Bellows and Aubin, 1989)] and rat (Aubin, 1999) and mouse (Falla *et al.*, 1993) bone marrow stroma (i.e., $0.5–1 \times 10^{-5}$ of the nucleated cells of unfractionated marrow) under standard isolation and differentiation culture conditions (ascorbic acid and a phosphate source, usually β-glycerophosphate). Estimates of osteoprogenitor frequency in adherent stromal cell layers, including those from humans, is significantly higher and ranges from ~1% up to ~10% of adherent cells by functional assays *in vitro* and *in vivo* (cells transplanted with ceramic particles) (Bianco *et al.*, 2006). Many authors have counted the number of nodules or colonies forming bone with or without limiting dilution for an estimate of osteoprogenitor numbers in other bone cell populations, e.g., from vertebrae (Bellows *et al.*, 2003; Ishida *et al.*; 1997; Lomri *et al.*, 1988), femoral trabecular bone fragments (Robey, 1995), primary spongiosa of femoral metaphysis (Onyia *et al.*, 1997), and compact bone (Short *et al.*, 2003), and in other tissues and organs, including peripheral blood where frequencies also range markedly [1 in 106 peripheral blood mononuclear cells in mice and guinea pigs, 1 in 107 in rabbits, and 1 in 108 (or less) in humans] depending on whether enrichment strategies were employed [for discussion, see, Modder and Khosla (2008)].

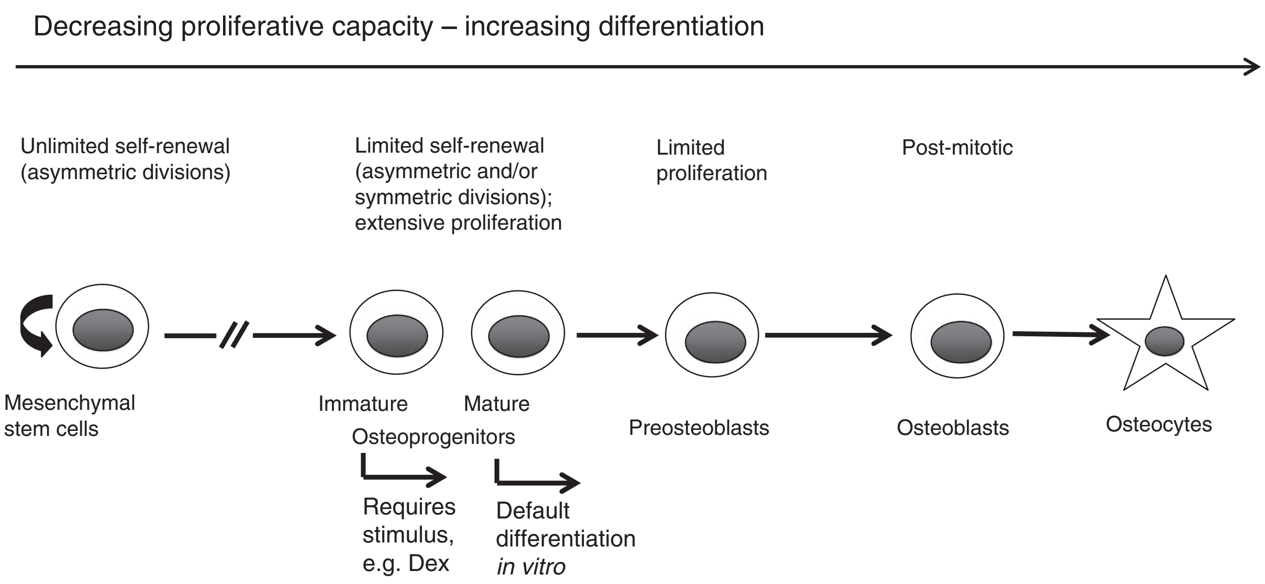
It is worth noting that many of the frequency estimates of progenitor numbers must be viewed with caution in the absence of unambiguous markers. Culture conditions significantly influence frequency estimates, and many base their estimates not on a Poisson analyses of limiting dilution (which quantifies absence or loss of progenitors over serial dilutions) or clonal analysis of single-cell plating in microtiter wells, but based on counting nodules in mass cultures at one or more plating densities, an approach subject to influences from "accessory" cells present in the population, some of which may be physiologically relevant to regulation of osteogenesis and others not (Aubin, 1999; Bord *et al.*, 2005; Eipers *et al.*, 2000; Kacena *et al.*, 2006). Second, based on data first reported in rat calvaria (Bellows and Aubin, 1989; Bellows *et al.*, 1987) and rat bone marrow stromal cell (Maniatopoulos *et al.*, 1988) cultures, additives expected to be stimulatory to osteogenesis such as dexamethasone are routinely added to osteogenic cultures *in vitro*, even though they may be without effect or even inhibitory in some species, at certain concentrations and in certain batches of serum [see, e.g., the inhibitory effect of dexamethasone in mouse stromal cultures (Falla *et al.*, 1993; Ishida and Heersche, 1998)]. On the other hand, the fact that many additives have been found to enhance osteogenesis supports the presence of "inducible" osteoprogenitor cells in bone marrow- and bone-derived populations (see later). Elucidation of species differences, the nature (i.e., multipotential or various committed progenitors) of progenitors present, at what proliferation and/or differentiation stages they are induced, and whether comparable activities can be discerned *in vivo* as well as *in vitro* warrants further attention, an issue that is advancing rapidly as various genetically modified mouse and other models become increasingly available.

Whether all progenitors from different sources that differentiate to osteoblasts and make bone belong to the same unidirectional lineage pathway (i.e., immature progenitors undergoing differentiation to mature osteoblasts), whether osteoprogenitor cells must transit all recognizable differentiation stages (or may skip steps under appropriate conditions) under all developmental situations, or whether recruitment from other parallel lineages and pathways can result in functional osteoblasts remains to be established. However, as discussed in more detail later, they do appear to reach similar endpoints with respect to the ability to make and mineralize a bone matrix, but they may not be identical. At least some precursor cell pools appear to undergo osteogenic differentiation as a default pathway *in vitro* whereas others require a stimulus beyond the presence of serum. Clearly, the presence of factors from accessory cells in bone-derived (Purpura *et al.*, 2003) and bone marrow-derived (Aubin, 1999; Eipers *et al.*, 2000; Miao *et al.*, 2004) cell populations points toward the intrinsic differentiation capacity of precursor cells being modulated by cell nonautonomous factors *in vitro* and *in vivo* (Kacena *et al.*, 2006). As already discussed, the relationship of inducible osteoprogenitors that apparently reside in the nonadherent fraction of bone marrow and are assayable under particular culture conditions, e.g., in the presence of PGE_2 [rat (Scutt and Bertram, 1995)] or as colonies in soft agar or methylcellulose [human (Long *et al.*, 1995)] to adherent osteoprogenitors and MSCs, also remains to be explicitly determined. Direct and unambiguous comparisons will require more markers for the most primitive progenitors, including stem cells.

Morphologically recognizable osteoblasts associated with three-dimensional nodules appear in long-term bone cell cultures at predictable and reproducible periods after plating. Time-lapse cinematography of individual progenitors forming colonies in low-density rat calvaria cultures indicated that primitive (glucocorticoid-requiring) osteoprogenitors divide ~8 times prior to overt differentiation, i.e., to achieving cuboidal morphology and matrix deposition (Malaval *et al.*, 1999). Interestingly, however, the measurement of large numbers of individual bone colonies in low-density cultures shows that the size distribution of fully formed bone colonies covers a large range but is unimodal, suggesting that the coupling between proliferation and differentiation of osteoprogenitor cells may be governed by a stochastic element, but distributed around an optimum, corresponding to the peak colony size/division potential (Malaval *et al.*, 1999). Osteoprogenitors measurable in functional bone nodule assays also appear to have a limited capacity for self-renewal in both calvaria (Bellows *et al.*, 1990a; Purpura *et al.*, 2004) and stromal (McCulloch *et al.*, 1991) populations *in vitro*, consistent with their being true committed progenitors with a finite life span [reviewed in Aubin and Heersche (2002)]. However, in comparison with certain other lineages, most notably hematopoietic cells, relatively little has been done to assess the regulation of self-renewal in different osteogenic populations beyond the effects of glucocorticoids. According to signaling threshold models, the differentiation of hematopoietic stem cells and embryonic stem cells is regulated by threshold levels of ligands and receptors (Davey *et al.*, 2007; Viswanathan *et al.*, 2005; Zandstra *et al.*, 2000). Very little has been done regarding osteoblast lineage to assess comparable pathways, yet the differential expression of a variety of receptors for cytokines, hormones, and growth factors during osteoblast development and in different cohorts of osteoblasts predicts that similar mechanisms may play a role in bone formation (see also later).

Differentiation of Osteoprogenitor Cells to Osteoblasts

A fundamental question in osteoblast development remains how progenitors progress from a stem or primitive state to a fully functional matrix-synthesizing osteoblast. Inherently based on observations *in vivo* and in bone nodule formation *in vitro*, the process has been conceived of as deterministic and subdivided into three stages: (i) proliferation, (ii) extracellular matrix development and maturation, and (iii) mineralization, with characteristic changes in gene expression at each stage; some apoptosis can also be seen in mature nodules. In many studies, it has been found that genes associated with proliferative stages, e.g., histones and proto-oncogenes such as c-fos and c-myc, characterize the first phase, whereas certain cyclins, e.g., cyclins B and E, are upregulated postproliferatively [Kalajzic *et al.*, 2005, Malaval *et al.*, 1994, Owen *et al.*, 1990; reviewed in Aubin (2001)]. Expression of the most frequently assayed osteoblast-associated genes COLLI, ALP, OPN, OCN, BSP, and PTH1R is upregulated asynchronously, acquired, and/or lost as the progenitor cells differentiate and the matrix matures and mineralizes. In general, ALP increases and then decreases when mineralization is well progressed; OPN peaks twice during proliferation and then again later but prior to certain other matrix proteins, including BSP and OCN; BSP is expressed transiently very early and is then upregulated again in differentiated osteoblasts forming bone; and OCN appears approximately concomitantly with mineralization [summarized in Aubin and Heersche (2002)] (Fig. 2). Notably, however, use of global amplification poly(A) PCR, combined with replica plating and immunolabeling, showed that all these osteoblast-associated markers are upregulated prior to the cessation of proliferation in osteoblast precursors except OCN, which is upregulated only in postproliferative osteoblasts; in other words, differentiation is well progressed before osteoblast precursors leave the proliferative cycle. Based on the simultaneous expression patterns of up to 12 markers, osteoblast differentiation can be categorized into a minimum of seven


Decreasing proliferative capacity – increasing differentiation
Unlimited self-renewal (asymmetric divisions)
Limited self-renewal (asymmetric and/or symmetric divisions); extensive proliferation
Limited proliferation
Post-mitotic
Mesenchymal stem cells
Immature
Mature
Osteoprogenitors
Preosteoblasts
Osteoblasts
Osteocytes
Requires stimulus, e.g. Dex
Default differentiation *in vitro*

Transcription factors							
Runx2	+?	+		+	+	+	+
Osx	−	−		−	+	+	+
Enzymes							
ALP	−	−	−	+	++	+++	−
Phex	−	−		−	−	++	+++
Extracellular matrix							
ColI	−?	−	+	++	+	++	−
OPN	+?	−/+	−/+	−/+	− → +	− → +++	− → +++
BSP	−	−	++	−	− → +++	− → +++	− → +++
OCN	−	−	−	−	−	− → +++	−
Factors and receptors							
PTH1R	?	−	−	−/+	++	++	++
PTHrP	?	−/+	−/+	+	++	++	−
PDGFRα	?						
Sclerostin	−	−		−	−	−	+++
DMP1	−	−		−	−	−	+++

FIGURE 2 Sequential proliferation–differentiation steps in the osteoblast lineage as detectable from *in vitro* and *in vivo* experiments. Superimposed on this scheme are several well-established markers of osteoblastic cells with an indication of when during the differentiation sequence they are expressed, but also denoting heterogeneous expression of many of the markers. The list is not exhaustive, but does show some important categories of molecules in the lineage and their utility to help define transitions in osteoblast differentiation-maturation. −, no detectable expression; −/+ to + + +, expression ranging from detectable to very high; − ? + + +, intercellular heterogeneous expression.

transitional stages (Liu *et al.*, 2003), not just the three stages mentioned earlier. An interesting issue is whether osteoprogenitor cells in all normal circumstances must transit all stages or can "skip over" some steps under the action of particular environmental stimuli or regulatory agents.

Most or all of these molecules are downregulated as cells transition to ostecytic osteoblasts and to osteocytes. However, over the past several years, several markers have been found to be upregulated at these terminal differentiation stages. One of these is sclerostin, a Wnt antagonist that is mutated in human sclerosteosis and Van Buchem disease and that blocks BMP-induced bone formation; sclerostin is the subject of much current interest as a potential new therapeutic for bone disease (Chan *et al.*, 2007; van Bezooijen *et al.*, 2005). Among others are molecules regulating phosphate homeostasis and mineralization. For example, the mRNA for Phex, a phosphate-regulating gene with homology to endopeptidases on the X chromosome, which is mutated in X-linked hypophosphatemia (XLH), is expressed differentially as osteoblasts differentiate (Ecarot and Desbarats, 1999; Guo and Quarles, 1997; Ruchon *et al.*, 1998) and, depending on the species studied and the antibody used, the protein is reported to be expressed by osteoblasts and osteocytes (Ruchon *et al.*, 2000) or osteocytes alone (Westbroek *et al.*, 2002). Matrix extracellular phosphoglycoprotein (MEPE), a recently isolated RGD-containing matrix protein that acts as the tumor-derived phosphaturic factor in oncogenic hypophosphatemic osteomalacia, is much more highly expressed in osteocytes than osteoblasts (Nampei *et al.*, 2004). Dentin matrix protein 1 (encoded by DMP1) is highly expressed in osteocytes (Toyosawa *et al.*, 2001) and, when deleted in mice and in humans with a newly identified disorder, autosomal recessive hypophosphatemic rickets, rickets, and osteomalacia result with renal phosphate-wasting associated with elevated fibroblast growth factor 23 (FGF23) levels (Feng *et al.*, 2006).

Although many osteoblast models have been reported to follow the general proliferation–differentiation outlined earlier, some differences have been reported. At least some of the variations may reflect inherent differences in the populations being analyzed, e.g., species differences or different mixtures of more-or-less primitive progenitors and more mature cells. However, as underscored by a couple of the preceding examples, there is also growing evidence from both *in vitro* and *in vivo* observations that different gene expression profiles may underlie developmental events in different osteoblasts. In another example, a study of ROS cells differentiating and producing mineralizing bone matrix in diffusion chambers *in vivo*, neither proliferation nor most differentiation markers followed the pattern described previously (Onyia *et al.*, 1999). One possible explanation is that different subpopulations of cells within the chambers were undergoing different parts of the proliferation—matrix synthesis—death cycle at different times, such that activities of some subpopulations may have been obscured among larger subpopulations engaged in other activities in the chamber at the same time. At least some data supported this view. Another possibility is that there are differences in proliferation–differentiation coupling and/or the nature of the matrix and process of mineralization in osteosarcoma cells versus normal diploid osteoblasts. In addition, however, it is growing clear that high levels of genes typical of some normal osteoblasts may not be required in others, i.e., that some pathways by which mineralized matrix can be formed *in vivo* or *in vitro* are different from others and that there is heterogeneity among osteoblast developmental pathways and/or the resulting osteoblasts.

The possibility that marked intercellular heterogeneity in expressed gene repertoires may characterize osteoblast development and differentiation is an important concept. It has been evident for some time that not all osteoblasts associated with bone nodules *in vitro* are identical (Liu *et al.*, 1994; Malaval *et al.*, 1994; Pockwinse *et al.*, 1995). Single-cell analysis of the most mature cells in mineralizing bone colonies *in vitro* showed that the heterogeneity of expression of markers by cells classed as mature osteoblasts is extensive and appears not to be related to cell cycle differences (Liu *et al.*, 1997). That this extensive diversity is not a consequence or an artifact of the *in vitro* environment was confirmed by the analysis of osteoblastic cells *in vivo*. When individual osteoblasts in 21-day fetal rat calvaria were analyzed, only two markers of nine sampled, ALP and PTH1R, appeared to be globally or ubiquitously expressed by all osteoblasts. All other markers analyzed (including OPN, BSP, OCN, PTHrP, c-fos, Msx-2, and E11) were expressed differentially at both mRNA and protein levels in only subsets of osteoblasts, depending on the maturational state of the bone, the age of the osteoblast, and the environment (endocranium, ectocranium) and the microenvironment (adjacent cells in particular zones) in which the osteoblasts reside (Candeliere *et al.*, 2001). The biological or physiological consequences of the observed differences are not known, but they support the notion raised earlier that not all mature osteoblasts develop via the same regulatory mechanisms nor are they identical molecularly or functionally. They predict that the makeup of different parts of bones may be significantly different, as suggested previously by the observations that the presence of and amounts of extractable noncollagenous bone proteins are different in trabecular versus cortical bone and in different parts of the human skeleton [for discussion, see Candeliere *et al.* (2001)]. They also suggest that the globally or ubiquitously expressed molecules serve common and nonredundant functions in all osteoblasts and that only small variations in the expression of these molecules may be tolerable; e.g., all bones display mineralization defects in ALP knockout mice (Fedde *et al.*, 1999; Wennberg *et al.*, 2000). Differentially expressed lineage markers, however, e.g., BSP, OCN, and OPN, vary much more, both between osteoblasts in different zones and between adjacent cells in the same zone and may have specific functions associated with only some positionally or maturationally defined osteoblasts. In this regard, it is striking that all of the noncollagenous bone matrix molecules are extremely heterogeneously expressed and that ablation of many of those studied to date, e.g., OCN (Ducy *et al.*, 1996) and OPN (Yoshitake *et al.*, 1999), does not result in a complete failure of osteoblast differentiation and maturation, although the amount, quality, and remodeling of the bone formed differs from normal.

The nature of the signals leading to the diversity of osteoblast gene expression profiles is not known, but the fact that the heterogeneity is apparently controlled both transcriptionally and post-transcriptionally implies that regulation is complex. The observations also suggest that it will be important to analyze the expression of regulatory molecules, including transcription factors, not only globally but at the individual osteoblast level. Another unanswered question remains as to whether the striking diversity of marker expression in different osteoblasts is nonreversible or reversible in a stochastic manner, governed by changes in a microenvironmental signal or receipt of hormonal or growth factor cues, or both. Because the heterogeneity observed extends to the expression of regulatory molecules, such as cytokines and their receptors, it also suggests that autocrine and paracrine effects may be elicited on or by only a subset of cells at any one time and that the responses to such stimuli could themselves be varied.

The observed differences in mRNA and protein expression repertoires in different osteoblasts may also contribute to the heterogeneity in trabecular microarchitecture seen at different skeletal sites (Amling *et al.*, 1996), to site-specific differences in disease manifestation such as seen in osteoporosis (Riggs *et al.*, 1998), and to regional variations in the response to therapeutic agents (Compston, 2007). Further analysis of differential expression profiles for a variety of receptors in different skeletal sites, and at different maturational age of the cells and skeleton, should provide further insight into site-specific effects of various agents.

Many other molecules are now known to be made by osteoblast lineage cells, often with differentiation stage-specific changes in expression levels, but not all have been tested for a bone-related function. It is beyond the scope of this chapter to review all of them, but those markers that may comprise regulatory molecules within the HSC niche are increasingly of interest. The presence of HSCs close to endosteal bone surfaces and a role for osteoblasts in supporting hematopoietic progenitors *in vitro* are now well-accepted concepts [reviewed in Taichman (2005)]. However, the lack of markers for unambiguous identification of specific stromal cell subtypes and various osteoblastic cell subpopulations has led to some confusion about the exact nature of the HSC niche, and both a bone niche and a vascular niche have been proposed. For example, osteoblasts in the bone niche have been variously characterized as PTH-activatable Jag1+ (Notch ligand jagged 1-positive) osteoblastic cells (Calvi *et al.*, 2003), a spindle-shaped N-cadherin + CD45− subset of osteoblastic (SNO) cells at the bone surface (Lymperi *et al.*, 2007; Zhang *et al.*, 2003), and angiopoietin 1 (Ang1)+ osteoblasts regulating HSC number through the activation of the Tie-2/Ang1 signaling pathway (Arai *et al.*, 2004). As already mentioned, more recently, Sacchetti *et al.* (2007) demonstrated that human CD146+ osteoblast precursors, which also express Jag1, N-cadherin, Ang1, and CXCL12 [also known as stromal-cell-derived factor (SDF)-1], are cells that may comprise both the bone and the vascular niche [for discussion, see Aubin (2008)]. These and other studies underscore the interest in and necessity for additional markers by which to discriminate osteoblast maturational stage and osteoblast subpopulations.

An increasing number of studies using genomewide expression profiling of MSC and osteoblast transcriptomes, and a few protein profiles, are beginning to address the current paucity of markers and to add new insights into diverse aspects of osteoblast development and activity. These include analyses with cells under standard differentiation conditions (Jeong *et al.*, 2005; Qi *et al.*, 2003; Roman-Roman *et al.*, 2003), with specific gene mutations [those underlying craniosynostosis (Coussens *et al.*, 2007)] or treated with such agents as siRNA or DNA modifiers/regulators of DNA expression [histone deacetylase inhibitors (Schroeder *et al.*, 2007)], hormones and growth factor treatments [BMP, Nakashima *et al.* (2002); Wnt3a (Jackson *et al.*, 2005), mechanical loading and combinations of treatments (mechanical loading and estrogen effects in wild-type and $ER^{-/-}$ cells (Armstrong *et al.*, 2007)). Large databases of genes previously unrecognized as osteoblast-associated and falling into many functional clusters (e.g., transcription factors, transport molecules, cell cycle- and apoptosis-related molecules, cytokines-chemokines, extracellular matrix proteins, proteases, receptors, members of signal transduction pathways, molecules involved in lipid metabolism, cytoskeletal proteins, growth and differentiation factors, and others) are becoming available. Although the availability of such databases is increasing daily, in many, but not all, cases the data remain descriptive and issues remain not the least of which is the need for more highly purified populations of cells for analysis, more sophisticated bioinformatics approaches and more functional analysis of differentially expressed genes and gene families. Nevertheless, such interesting and important osteoblast-regulatory molecules as Osx (Nakashima *et al.*, 2002) were identified by such a strategy.

TRANSCRIPTION FACTOR, HORMONE, CYTOKINE, AND GROWTH FACTOR REGULATION OF CFU-F AND OSTEOPROGENITOR CELL PROLIFERATION AND DIFFERENTIATION

Transcription factors, hormones, cytokines, growth factors, and their receptors can serve both as markers and as stage-specific regulators of osteoblast development and differentiation (Fig. 1). It is beyond the scope of this chapter to review every factor known to influence osteoblast differentiation and bone formation at some level, because many will be covered in other chapters. However, a few examples are given that emphasize other issues discussed in this chapter, including heterogeneity of osteoblast response, proliferation–differentiation coupling, and differentiation stage-specific regulatory mechanisms.

Regulation by Transcription Factors

Transcription factors known to regulate osteoblast recruitment, osteoblast number, and the rate and duration of osteoblast activity are growing in number. For example, it is already evident from the preceding summary that Runx2 is necessary for osteoblast development, but Ducy *et al.* (1999) used a dominant-negative strategy in transgenic mice to show that Runx2 plays a role beyond osteoblast development, i.e., it also regulates the amount of matrix deposited by osteoblasts in postnatal animals. Several transcription factors that belong to homeobox protein families [msh homeobox homologue 1 and 2 (Msx1 and Msx2) and Distal-less homeobox 5 and 6 (Dlx5 and Dlx6)] also play a role in osteoblast differentiation. For example, an activating mutation in Msx2 underlies Boston-type craniosynostosis (Ma *et al.*, 1996) whereas Msx2 inactivation in mice delays skull ossification, an outcome associated with decreased Runx2 expression (Satokata *et al.*, 2000). However, although it is clear that Msx2 is a positive regulator of bone formation *in vivo* (Ichida *et al.*, 2004; Ishii *et al.*, 2003), there are discrepant data on the underlying cellular mechanisms, i.e., effects of Msx2 on proliferation–differentiation. Differentiation stage-specific effects may provide at least a partial answer because endogenous Msx2 is expressed at higher levels in osteoprogenitors and is downregulated during differentiation (Hassan *et al.*, 2004). Thus, Msx2 stimulates proliferation and osteoblast differentiation in some mesenchymal cell lines (Cheng *et al.*, 2003) but inhibits Runx2 activity and osteoblast gene expression in more mature osteoblasts *in vitro* (Newberry *et al.*, 1998; Shirakabe, 2001; Lee, 2005). Other studies *in vitro* (Dodig *et al.*, 1999) and in mice (Liu *et al.*, 1999) suggest that enhanced expression of Msx2 keeps osteoblast precursors transiently in a proliferative state, delaying osteoblast differentiation, resulting in an increase in the osteoblast pool and ultimately in an increase in bone growth. It is worth noting that Msx2 promotes vascular calcification by activating paracrine Wnt signaling (Shao *et al.*, 2005).

Another example of skeletal site-specific effects is with Dlx5, which is expressed in osteoblasts in the entire skeleton, but whose deletion in mice results in abnormalities restricted to craniofacial bones. With a phenotype reminiscent of that seen in people with split-hand/split-foot malformation (SHFM), Dlx5$^{-/-}$Dlx6$^{-/-}$ mice have severe abnormalities in craniofacial bone formation, with complete absence of the calvaria, maxilla, and mandible, and anomalies in the axial and appendicular skeletons (Robledo *et al.*, 2002). It is not yet clear whether the defects in osteoblastogenesis are secondary to the retarded chondrocyte maturation that is also seen in Dlx5$^{-/-}$Dlx6$^{-/-}$ mice. However, Dlx5 is expressed in osteoprogenitors and expression levels increase in more mature osteoblasts (Hassan *et al.*, 2004; Newberry *et al.*, 1998; Ryoo *et al.*, 1997) where it activates expression of Runx2, BSP, and OCN (Holleville *et al.*, 2007).

Many members of the AP1 subfamily of leucine zipper-containing transcription factors regulate osteoblast differentiation and activity and two striking examples are summarized here [for review see Wagner and Eferl (2005)]. Osteosclerosis results when either Fra-1 (Jochum *et al.*, 2000), a Fos-related protein encoded by the c-Fos target gene *Fosl1* (referred to as *fra-1*), or ΔFosB, a naturally occurring splice variant of FosB (Sabatakos *et al.*, 2000), are overexpressed in transgenic mice. In both cases, the mice appear normal at birth, but with time, much increased bone formation is evident throughout the skeleton (endochondral and intramembranous bones). The osteosclerotic phenotype derives from a cell autonomous modulation of osteoblast lineage cells that is characterized by accelerated and more osteoblast differentiation and bone nodule formation *in vitro*. In a reciprocal experiment, when Fra-1 was deleted conditionally in embryonic tissues of mice, postnatal mice became progressively osteopenic; although osteoblasts were present in normal numbers, they appeared less mature and showed severely reduced bone-forming activity that included reduced expression of Col1a2, OCN, and matrix Gla protein (MGP) (Eferl *et al.*, 2004). It is still not clear mechanistically how ΔFosB expression leads to osteosclerosis. Initial suggestions that ΔFosB favored commitment of mesenchymal precursors cells toward an osteoblast fate rather than an adipogenic fate were consistent with the low fat mass of the osteosclerotic mice; it was subsequently shown that the changes in osteoblast and adipocyte differentiation in the ΔFosB mice resulted from independent cell autonomous mechanisms in adipocyte and osteoblast lineages (Kveiborg *et al.*, 2004). It is, therefore, worth noting that it is the further truncated Δ2ΔFosB isoform, rather than ΔFosB itself, that is responsible for the increased bone formation seen in ΔFosB transgenic mice. This suggests that further study of differentiation stage-specific aspects of the mechanism may shed light on the underlying osteoblast lineage perturbation, as differentiation stage-specific alternative splicing of fosB mRNA and selective initiation site use of ΔFosB appear to be involved (Sabatakos *et al.*, 2000).

Mice homozygous for mutations in the gene (AIM) encoding the nonreceptor tyrosine kinase c-Abl also have a bone phenotype, including osteoporotic (both thinner cortical and reduced trabecular) bones and reduced mineral apposition rates (Li *et al.*, 2000), apparently reflecting no change in osteoclast number or activity but an osteoblast defect manifested by delayed maturation *in vitro*. Whether a cell autonomous defect in osteoblasts is responsible for the osteopenia seen in the spontaneous mouse mutant staggerer (sg/sg) (Meyer *et al.*, 2000), which carries a deletion within the retinoic acid receptor-related orphan receptor a (RORα) gene, remains to be determined, but because RORα appears to regulate BSP and OCN transcriptionally, this is clearly one possibility, given the increased bone formation seen earlier in OCN null mice (Ducy *et al.*, 1996). The leucine-zipper containing protein ATF4, which is highly enriched in osteoblasts, regulates bone formation by regulating amino acid import (Yang *et al.*, 2004). This function requires the phosphorylation of ATF4 by the kinase Rsk2, which is inactivated in Coffin–Lowry syndrome and increased in osteoblasts in another disease, neurofibromatosis type 1 (Elefteriou *et al.*, 2006). Given the preceding summary of some of the AP1 factors, it is worth noting that Jun proteins can also interact with ATF family members, raising the possibility that heterodimerization with ATF4 may be one mechanism by which these proteins can regulate osteoblast-specific gene expression (Chinenov and Kerppola, 2001).

Regulation by Hormones, Growth Factors, and Cytokines

Bone marrow injury associated with local bleeding, clotting, and neovascularization recapitulates a process similar to the normal developmental process and callus formation during fracture repair, with the induction of an environment rich in growth factors (e.g., PDGF, FGF, TGFβ, VEGF) followed by a process of very active bone formation [reviewed in Carano and Filvaroff (2003); Gerstenfeld *et al.* (2003); Harada and Rodan (2003)]. Similarly, in inflammatory bone disease (e.g., the subchondral bone loss seen in rheumatoid arthritis or the alveolar bone loss seen in periodontal disease), high levels of numerous cytokines (e.g., interferons, TNFα, IL1, IL6, and other gp130 family cytokines, etc.) are seen that often associated with stimulation of osteoclastogenesis but which may play direct roles in osteoblasts as well, either directly or as a consequence of release from the bone matrix [(reviewed in Findlay and Haynes (2005); Franchimont *et al.* (2005)]. Similar cytokines and growth factors are likely to contribute to the "vicious cycle" of osteolytic and osteoblastic bone disease seen in tumor metastases (reviewed in Chirgwin (2007)]. These and other cytokines may play roles in normal bone remodeling and osteoporosis (Eriksen *et al.*, 2007; Martin and Sims, 2005; Raisz, 2005). Thus, a growing list of systemic or local growth factors, cytokines, and hormones are being tested in animal models and cellular models *in vitro* and combinations of the two. Differing requirements and opposite results have sometimes been reported depending on the species studied and the model cell system under study (e.g., bone marrow stroma versus calvariae-derived populations), whether total CFU-F or specific subpopulations (e.g., CFU-ALP, CFU-O) are quantified, and the presence or absence of other factors. Nevertheless, because of increasingly detailed evaluation of bone parameters *in vivo* and careful documentation of proliferation and differentiation stages underlying the formation of CFU-F and bone nodules/CFU-O *in vitro*, the models are helping to clarify the nature of perturbations in a carefully orchestrated osteoprogenitor proliferation–osteoblast differentiation and activity sequence. As previously, it is not possible to summarize the effects of all hormones, cytokines, and growth factors that are being investigated in osteoblast lineage cells, but a few representative examples will highlight important concepts by which the target cells responding (stem cells, mesenchymal precursors, committed progenitors) and the precise nature of the responses in bone and nonbone cells in these complex environments are being elucidated.

There is substantial evidence that at least some of the actions of growth and differentiation factors depend on the relative stage of differentiation (either more or less mature) of the target cells, with the same factor sometimes having opposite effects on proliferative/progenitor stages and differentiation stages; the situation is even more complex when effects are differentiation stage-specific and mature osteoblast activity is also affected (Fig. 3). For example, the inflammatory cytokine IL-1 is stimulatory to CFU-O formation when calvaria-derived cultures are exposed transiently during proliferative culture stages, and inhibitory when cells are exposed to the same doses transiently during differentiation stages; the inhibitory effects dominate when cells are exposed chronically through proliferation and differentiation stages in culture (Ellies and Aubin, 1990). Many other factors of current interest similarly have

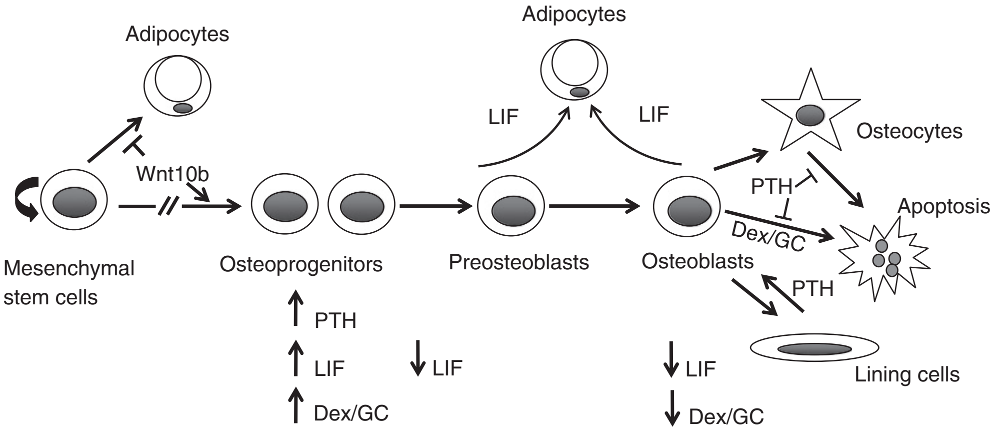


FIGURE 3 Regulation of osteoblast differentiation by hormones and cytokines. The list is not exhaustive, but examples are given to highlight some of the concepts discussed in the text.

biphasic or multiphasic effects *in vitro*, including EGF, TGFβ, and PDGF [for review see Aubin (2001)].

Members of the leukemia inhibitory factor (LIF)/IL6 family, including IL6, IL11, oncostatin M (OSM), and cardiotrophin-1 (CT-1) also fall into the category of factors with biphasic or multiphasic effects. These cytokines have long been studied as positive regulators of osteoclastogenesis and bone resorption. However, a wide variety of data indicate that at least some of the cytokines that signal through the gp130 receptor are also regulators of osteoblast lineage cells and bone formation. Metcalf and Gearing showed almost 20 years ago that mice engrafted with cells overexpressing LIF developed a fatal syndrome characterized by, among other features including cachexia, excessive new bone formation and ectopic calcifications in skeletal muscles and heart (Metcalf and Gearing, 1989). That LIF/IL-6 cytokine signaling is normally required in bone is exemplified by Stuve–Wiedemann syndrome, a severe autosomal recessive genetic disease whose prominent features include bowing of long bones, cortical thickening, and altered trabecular pattern, recently shown to result from null mutations of LIFR (Dagoneau *et al.*, 2004). Further, a series of mouse knockout and knockin studies showed that LIFR and gp130 are necessary for normal mouse bone development, because their absence leads to profound osteopenia accompanied by increased osteoclast number (Ware *et al.*, 1995; Yoshida *et al.*, 1996). With respect to bone formation, lack of gp130 signaling alters osteoblast phenotype and activity (Shin *et al.*, 2004), and loss of SHP2/ras/MAPK activation by gp130 induces high bone turnover with increased formation and resorption (Sims *et al.*, 2004). However, the nature of the target cells in bone and their response to changes in gp130 signaling are generally not yet clear. Further, a plethora of often contradictory data on effects of LIF/IL-6 family members on stromal and osteoblast lineage cells *in vitro* has been reported, with stimulatory, inhibitory, and biphasic effects on osteoprogenitor proliferation and differentiation. For example, LIF inhibits osteoprogenitor differentiation (i.e., bone nodule formation) in the rat calvaria (RC) cell culture model in a very differentiation stage-specific manner, i.e., at a late osteoprogenitor–early preosteoblast stage (Malaval *et al.*, 1995, 1998). In contrast, in rat bone marrow stromal cell cultures, LIF over the same concentration ranges stimulates nodule formation in early proliferation stages, but inhibits it at later times similarly to what is seen in RC cultures (Malaval and Aubin, 2001). Similarly, although IL-11 has also been described to inhibit osteogenesis in RC cultures (Hughes and Howells, 1993), it stimulates it in bone marrow stromal cell cultures (Suga *et al.*, 2001) and promotes osteoblast differentiation and bone apposition in transgenic mice and IL-11 overexpressing stromal cell cultures through STAT potentiation of the action of Smads (Takeuchi *et al.*, 2002). mOSM is the only member of the LIF/IL-6 family to increase osteoprogenitor recruitment (bone nodule formation) in RC cell cultures, and it does so during proliferation stages or at relatively early stages of differentiation (days 1–3/4 of culture) (Malaval *et al.*, 2005). Thus, LIFR and OSMR appear to display differential effects on differentiation and phenotypic expression of osteoblasts, apparently via different signals. The data suggest that IL-6, IL-11, which shares the gp130/gp130 receptor, and other family members act differently on the periosteum/suture and bone-tissue-derived RC cell population compared with stromal/endosteal cells. The observation that the inhibitory effect of LIF in RC cell cultures may occur at least in part through the ability of LIF to alter the fate of precursors away from the osteoblast lineage and toward the adipocytic lineage (Falconi *et al.*, 2007), together with the increased expression of LIF/IL-6 cytokines after menopause (Manolagas and Jilka, 1995) and PTH treatment (Greenfield *et al.*, 1996), and that the bone matrix synthesized by LIF-treated osteoblasts is abnormal (Cornish *et al.*, 1993; Falconi and Aubin, 2007) suggests that further detailed analyses of the gp130 family of cytokines are warranted.

An example of clinical significance is the effect of PTH on osteoblast lineage cells and its catabolic (chronic PTH treatment) versus anabolic (intermittent) action on bone. PTH receptor (PTH1R) is expressed throughout osteoblast differentiation, although the levels of expression and activity appear to increase as osteoblasts mature (Liu *et al.*, 2003). It is therefore not surprising that PTH elicits widespread but diverse effects on both early osteoprogenitor cells and mature osteoblastic cells (osteoblasts, osteocytes, lining cells) *in vivo* and *in vitro*, but analyses continue to reveal the basis of the bone effects seen. Analyses of bones from people and animals treated intermittently with PTH show that the increase in bone formation appears to be caused by an increase in the number of matrix-synthesizing osteoblasts; increased osteoblast development, attenuation of osteoblast apoptosis (Jilka *et al.*, 1999), and activation of quiescent lining cells (Dobnig and Turner, 1995) have all been proposed as explanations for this effect of PTH as either direct or indirect (from changes in expression of other growth factors) consequences [for reviews see Aubin and Heersche (2001); Jilka (2007); Rosen (2003)]. Chronic exposure to PTH inhibited osteoblast differentiation and bone nodule formation in an apparently reversible manner at a relatively late preosteoblast stage in rat calvarial cells *in vitro* (Bellows *et al.*, 1990b). However, when the cells were treated for 1-hour versus 6-hour pulses in 48-hour cycles during a 2- to 3-week culture period, either inhibition (1-hour pulse; apparently related to cAMP/PKA pathways) or stimulation (6-hour pulse; apparently related to cAMP/PKA, Ca^{2+}/PKC, and IGF-I) in osteoblast differentiation and bone nodule formation was seen (Ishizuya *et al.*, 1997). On the other hand, analysis of numbers of colony numbers in marrow stromal cell cultures from mice treated with intermittent PTH showed no increase in CFU-O (Jilka *et al.*, 1999). In mice deficient in PTH1R, not only is a well-studied defect in chondrocyte differentiation seen (as also seen in PTHrP knockout mice), but also increased osteoblast number and increased bone mass (a phenotype not seen in PTHrP-deficient mice) (Lanske *et al.*, 1999), supporting the view that PTH plays an important role in the regulation of osteoblast number and bone volume. When Calvi *et al.* (2001) expressed constitutively active PTH1R in bone, osteoblastic function was increased in the trabecular and endosteal compartments, but decreased in the periosteum of both long bones and calvaria. Interestingly, a decrease in osteoblast apoptosis and an apparent increase in both osteoblast precursor proliferation and mature osteoblasts were seen in trabecular bone. The latter is also important in relation to the observation that bone comprises the HSC niche and that constitutively active PTH1R signaling with the expanded osteoblastic cell pool (Calvi *et al.*, 2003) and PTH treatment (Adams *et al.*, 2007) expands the HSC pool.

Review of all regulatory factors is beyond the scope of this chapter, and indeed, I have not touched on a growing number of reports on matrix and matrix–integrin effects on osteoblast development and/or activity *in vitro* and *in vivo* [see, e.g., Franceschi *et al.* (2003); Rubin *et al.* (2006)]. However, given their inclusion in the majority of CFU-F and CFU-O assays *in vitro* reported in this chapter, it is worth considering glucocorticoids (most often dexamethasone in *in vitro* assays) in more detail. Glucocorticoid effects *in vivo* and *in vitro* are complex and often opposite, i.e., stimulating osteoprogenitor self-renewal and differentiation *in vitro* in calvaria (and other bones)-derived and stromal cell models [Aubin, 1999; Purpura *et al.*, 2004; reviewed in Aubin (2001)] while stimulating osteoblast apoptosis (Gohel *et al.*, 1999; Weinstein *et al.*, 1998) and resulting in glucocorticoid-induced osteoporosis *in vivo* (Weinstein, 2001; Weinstein *et al.*, 1998). In an interesting recent report, Teitelbaum and colleagues found that whereas glucocorticoids delay osteoclast apoptosis, they retard the capacity of osteoclasts to resorb bone, *in vitro* and *in vivo*, by disrupting the cytoskeleton (Kim *et al.*, 2006). Notably, the arrested resorption induced by glucocorticoids translated to dampened osteoblast activity, in other words suppression of the resorptive phase of remodeling, contributes to the retarded bone formation central to glucocorticoid-induced osteoporosis (Kim *et al.*, 2007). The mechanisms by which osteoclasts signal to control osteoblast formation and activity deserve more attention (Kim *et al.*, 2007; Martin and Sims, 2005).

A mechanism by which glucocorticoids may act is through autocrine or paracrine regulatory feedback loops in which the production of other factors is modulated, including growth factors and cytokines that themselves regulate the osteoblast differentiation pathway. For example, in rat calvaria cultures, glucocorticoids downregulate the endogenous production of LIF, which, as summarized earlier, is inhibitory to bone nodule formation when cells are treated at a late progenitor/preosteoblast stage (Malaval *et al.*, 1998), and upregulate BMP-6, which is stimulatory possibly through LMP-1, a LIM domain protein (Boden *et al.*, 1998). These are but two of a growing list of examples of glucocorticoid regulation of endogenously produced factors with apparently autocrine or paracrine activities on osteoblast lineage cells (Liberman *et al.*, 2007).

Many factors of interest have effects on gene expression in mature osteoblasts that may correlate with effects on the differentiation process and may be opposite for different osteoblast genes, with glucocorticoids being a case in point. The molecular mechanisms mediating these complex effects are generally poorly understood; however, the ability to form particular transcription factor complexes, localization and levels of endogenous expression of cytokine/hormone/growth factor receptors, and expression of cognate or other regulatory ligands within specific subgroups of osteogenic cells as they progress from a less to a more differentiated state may all play roles. As mentioned earlier, growing evidence shows that the probability for self-renewal versus differentiation of hemopoietic stem cells is regulated, at least in part, by the maintenance of required/critical signaling ligands (soluble or matrix or cell-bound) above a threshold level. Although there are few explicit data or experiments examining these issues in MSCs or osteoprogenitor populations, it seems likely that similar threshold controls may apply.

STEM CELL/OSTEOPROGENITOR CELL CHANGES IN DISEASE AND AGING

The formation of colonies reflecting specific progenitor cell types (i.e., CFU-F, CFU-O, etc.) provides an estimate of the stem/progenitor cell status in normal or diseased states, as reflected in the numerous examples summarized earlier with genetically modified mice or animals treated with hormones and factors of interest. However, many discrepancies exist when such colony-counting methods have been used to assess changes in stem/progenitor frequency in aging people or animals and in certain disease models, probably reflecting such variables as age groups tested and culture parameters including whether cell enrichment/fractionation strategies were used. A decline in CFU-F, CFU-O, and CFU-ALP size and number with age has been reported in some studies done on stromal cell populations isolated from mice, rats, and humans, but in others no significant changes have been found [Bellows *et al.*, 2003; Bonyadi *et al.*, 2003; D'Ippolito *et al.*, 1999; Jilka *et al.*, 1996; Kajkenova *et al.*, 1997; Nishida *et al.*, 1999; Oreffo *et al.*, 1998; Stenderup *et al.*, 2001; for review, see Stolzing and Scutt (2006)]. Beyond a potential change in number, some data suggest an age-related loss of differentiation potential, loss of proliferation potential, loss of self-renewal capacity, impaired production and/or response to hormones and factors stimulating osteogenesis, and increases in senescence

[Gazit, 1998; Erdmann, 1999; Fujieda, 1999; Stenderup, 2003; Abdallah, 2006; for recent reviews, see Carrington (2005); Manolagas and Almeida (2007)].

Taken together, the known loss of bone with aging or menopause may be caused not only by a decrease in MSC or osteoprogenitor number, but also by a reduced responsiveness of osteoprogenitor cells to biological factors resulting in an alteration in their subsequent differentiation potentials or to local bone environment changes in these factors. This has fundamental and strategic implications regarding therapeutic intervention to prevent bone loss and to increase bone mass in postmenopausal women and in aging populations. It also opens up possibilities for experimental studies to test whether the necessary growth factors can be supplied to the deficient site by the transfer of marrow stroma from one bone tissue site to another or by genetically engineered autologous cell therapy. There has been an explosion of interest in the potential use of adult MSCs—among other cell sources including embryonic stem cells—for therapy in many degenerative disorders of metabolic, environmental, and genetic origins. The interest is documented by the rapid rise in research publications in this area, and MSCs have been noted to have potentials far beyond skeletal reconstruction and augmentation of skeletal mass [for recent reviews, see Delorme *et al.* (2006); Kassem (2004); Phinney and Prockop (2007); Shefer and Yablonka-Reuveni (2007)]. However, much remains to be done to define both MSC and osteoprogenitor number and differentiation capacity in normal aging and in various disease states including osteoporosis.

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Chapter 5

Transcriptional Control of Osteoblast Differentiation and Function

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Transcriptional regulation of gene expression is a key mechanism controlling cellular differentiation and function in all cell types. Osteoblasts differentiate from mesenchymal precursor cells and then produce the bone-specific extracellular matrix. The identification of the key transcriptional events required for osteoblast differentiation and function was not as easy using *in vitro* or *ex vivo* approaches, which is best explained by the fact that osteoblast differentiation in the culture dish is not associated with obvious changes in cellular morphology. However, in the past decade these limitations have been overcome owing to a combination of molecular efforts and genetic studies in mice and humans. This chapter summarizes our current knowledge about the transcriptional control of osteoblast differentiation and function.

Runx2, A MASTER CONTROL GENE OF OSTEOBLAST DIFFERENTIATION AND FUNCTION

The power of a combined effort between molecular biologists and human geneticists in identifying key genes regulating cell differentiation, which has been so beneficial for our understanding of skeletal biology, is best illustrated by the discovery of Runx2 as the master gene of osteoblast differentiation. Runx2, previously termed Pebp2a1, Aml3, or Cbfa1, was originally cloned in 1993 as one of three mammalian homologues of the *Drosophila* transcription factor Runt (Kagoshima *et al.*, 1993; Ogawa *et al.*, 1993). Based on the finding that it is expressed in thymus and T-cell lines, but not in B-cell lines, Runx2 was thought to be involved in T-cell differentiation (Ogawa *et al.*, 1993; Satake *et al.*, 1995). However, 4 years after the cloning of Runx2, its crucial role as a transcriptional activator of osteoblast differentiation was demonstrated by several investigators at the same time using different experimental approaches (Ducy *et al.*, 1997; Komori *et al.*, 1997; Lee *et al.*, 1997; Mundlos *et al.*, 1997; Otto *et al.*, 1997).

One approach, purely molecular, aimed at the identification of osteoblast-specific transcription factors by using the *Osteocalcin* gene as a tool. The analysis of a proximal promoter fragment of one of the two mouse *Osteocalcin* genes, active only in osteoblasts, led to the identification of the only two known osteoblast-specific *cis*-acting elements, termed OSE1 and OSE2 (Ducy and Karsenty, 1997). Sequence inspection of OSE2 revealed homology to the DNA-binding site of Runt family transcription factors, and subsequent analysis demonstrated that the factor binding to OSE2 is related immunologically to transcription factors of the Runt family (Geoffroy *et al.*, 1995; Merriman *et al.*, 1995). Eventually, screening of a mouse primary osteoblast cDNA library revealed that only one of the three mammalian *Runx* genes, namely *Runx2*, is expressed predominantly in cells of the osteoblast lineage (Ducy *et al.*, 1997).

In situ hybridization further revealed that during mouse development, *Runx2* is first expressed in the lateral plate mesoderm at 10.5 days post-coitus (dpc), and later in cells of the mesenchymal condensations. Until 12.5 dpc these cells, which prefigure the future skeleton, represent common precursors of osteoblasts and chondrocytes. At 14.5 dpc osteoblasts first appear and maintain the expression of *Runx2*, whereas in chondrocytes *Runx2* expression is decreased significantly and restricted to prehypertrophic and hypertrophic chondrocytes. After birth, *Runx2* expression is strictly restricted to osteoblasts and cells of the perichondrium. Taken together, this spatial and temporal

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expression pattern suggested a critical role for Runx2 as a regulator of osteoblast differentiation (Ducy *et al.*, 1997).

The demonstration that this was the main function of Runx2 came from several lines of molecular and genetic evidence. First, in addition to the *Osteocalcin* promoter, functional OSE2-like elements were identified in the promoter regions of most other genes that are expressed at relatively high levels in osteoblasts, such as *α1(I) collagen*, *Osteopontin*, and *Bone sialoprotein* (Ducy *et al.*, 1997). Second, and more importantly, the forced expression of *Runx2* in nonosteoblastic cell lines or primary skin fibroblasts induced osteoblast-specific gene expression in these cells, demonstrating that Runx2 acts as a transcriptional activator of osteoblast differentiation *in vitro* (Ducy *et al.*, 1997). Third, the ultimate demonstration that Runx2 is an indispensable transcriptional activator of osteoblast differentiation came from genetic studies in mice and humans.

At the same time, two groups deleted the *Runx2* gene from the mouse genome, both expecting an immunological phenotype based on the assumption that *Runx2* was involved in T-cell differentiation (Komori *et al.*, 1997; Otto *et al.*, 1997). However, unexpectedly, all *Runx2*-deficient mice died immediately after birth owing to respiratory failure caused by the complete absence of endochondral and intramembranous bone formation. Most importantly, there are no osteoblasts in *Runx2*-deficient mice, which was confirmed by the lack of expression of osteoblast marker genes, as demonstrated by *in situ* hybridization.

The critical importance of Runx2 for osteoblast differentiation was further underscored by the finding that mice lacking only one allele of *Runx2* display hypoplastic clavicles and delayed closure of the fontanelles, i.e., defects of intramembranous ossification (Otto *et al.*, 1997). This phenotype is identical to what is seen in the human disease cleidocranial dysplasia (CCD), and subsequent genetic analysis of CCD patients revealed disease-causing heterozygous mutations of the *RUNX2* gene, thereby demonstrating the relevance of Runx2 for osteoblast differentiation also in humans (Lee *et al.*, 1997; Mundlos *et al.*, 1997). Taken together, this overwhelming molecular and genetic evidence has led to the generally accepted view that Runx2 is a master control gene of osteoblast differentiation, providing a molecular switch inducing osteoblast-specific gene expression (Lian and Stein, 2003).

In addition to its prominent role in osteoblast differentiation, Runx2 is also involved in the regulation of bone formation beyond development. This has been demonstrated in several ways. First, transgenic mice expressing a dominant-negative variant of Runx2 specifically in fully differentiated osteoblasts are viable, but develop severe osteopenia caused by a decreased rate of bone formation, in the light of normal osteoblast numbers (Ducy *et al.*, 1999). This phenotype is readily explained by the finding that several Runx2 target genes encoding bone extracellular matrix proteins are expressed at much lower levels. Second, mice lacking *Stat1*, a transcription factor attenuating the nuclear translocation of Runx2, as discussed later, display a high-bone-mass phenotype that is not only explained by increased osteoblast differentiation, but also by increased bone matrix deposition (Kim *et al.*, 2003). Third, a similar, but even more severe phenotype is observed in mice lacking the nuclear adapter protein Shn3. Because Shn3, as discussed later, is involved in the ubiquitination and proteasomal degradation of Runx2, the increased bone formation of the *Shn3*-deficient mice is readily explained by increased Runx2 levels in osteoblasts that in turn lead to enhanced bone matrix deposition (Jones *et al.*, 2006). Given these results, it came as a surprise that another transgenic mouse model, overexpressing intact *Runx2* under the control of an osteoblast-specific *α1(I)-Collagen* promoter fragment, did not display the expected high-bone-mass phenotype, but a severe osteopenia accompanied by an increased fracture risk (Liu *et al.*, 2001). Although these mice had increased numbers of osteoblasts, their bone formation rate was strikingly reduced, which likely illustrates the fact that the dosage of Runx2 needs to be tightly regulated in order to orchestrate proper bone formation *in vivo*.

Runx2 FUNCTIONS DURING SKELETOGENESIS BEYOND OSTEOBLAST DIFFERENTIATION

Although most of these results demonstrating a key role of Runx2 in osteoblasts were already discussed in the last edition of this book, there is accumulating novel evidence that the role of Runx2 in skeletogenesis is much more complex than previously anticipated. The starting point for these findings was the observation that *Runx2*-deficient mice also display defects of chondrocyte hypertrophy in some skeletal elements (Inada *et al.*, 1999; *Kim et al.*, 1999). Moreover, because *Runx2* is transiently expressed in prehypertrophic chondrocytes of mouse embryos, there was a possibility for a function of Runx2 in chondrocyte differentiation. One way to address this possibility was the generation of a transgenic mouse model expressing *Runx2* in nonhypertrophic chondrocytes, using a *α1(II)-Collagen* promoter/enhancer construct (Takeda *et al.*, 2001).

In line with the suspected role of Runx2 as a positive regulator of chondrocyte hypertrophy, these transgenic mice displayed accelerated chondrocyte maturation in the growth plates, but also evidence of ectopic cartilage formation in the rib cage or in the trachea, among other locations. Moreover, the presence of this transgene in a *Runx2*-deficient genetic background prevented the absence of skeletal mineralization that is normally associated with the *Runx2* deficiency. However, the skeleton of these mice only contained hypertrophic cartilage, but no bone matrix, thereby demonstrating that Runx2 induces chondrocyte hypertrophy, but not a transdifferentiation into osteoblasts (Takeda *et al.*, 2001).

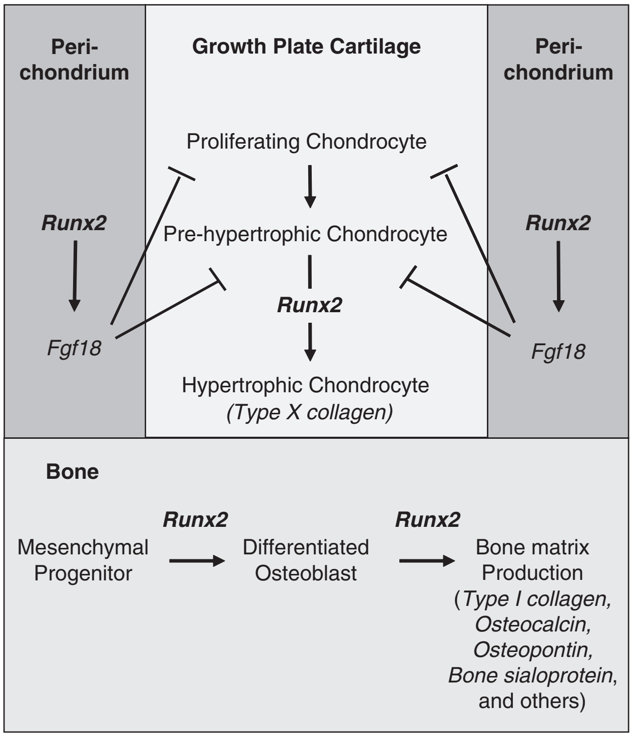

FIGURE 1 Runx2, a major regulator of skeletal development. Trough is transient embryonic expression in prehypertrophic chondrocytes. Runx2 positively regulates chondrocyte hypertrophy by activating the expression of target genes, such as type X collagen (Zheng *et al*., 2003). Additionally, Runx2 has a negative effect on chondrocyte maturation by increasing the expression of Fgf18 in cells of the perichondrium (Hinoi *et al*., 2007). Besides this regulation of skeletal growth, Runx2 plays a key role in osteoblast differentiation and function, where it activates the expression of several target genes (Ducy *et al*., 1997; Vaes *et al*., 2006; Hecht *et al*., 2007).

Because *Runx2* expression in prehypertrophic chondrocytes is transient, the main function of Runx2 here is probably to establish the growth plate. However, through another site of expression, namely in perichondrial cells, Runx2 has an additional function in the regulation of chondrogenesis. In these cells Runx2 positively regulates the expression of Fgf18, a diffusible molecule that inhibits chondrocyte maturation and osteoblast differentiation (Hinoi *et al*., 2006; Liu *et al*., 2002; Ohbayashi *et al*., 2002). Taken together, these results establish that Runx2 is more than the master gene of osteoblast differentiation, it is, in fact, along with Sox9, the major transcriptional regulator of cell differentiation during skeletogenesis, acting positively and negatively on osteoblast and chondrocyte differentiation (Fig. 1).

REGULATORS OF Runx2 AND THEIR IMPORTANCE FOR SKELETAL BIOLOGY

Given the central role played by Runx2 in skeletal cell differentiation, it is not surprising that other transcription factors playing a role in the regulation of osteoblast differentiation and bone formation exert their functions in a Runx2-dependent manner. Some of these factors regulate, directly or indirectly, transcription of the *Runx2* gene, whereas others, as already mentioned earlier for Stat1 and Shn3, interact with the Runx2 protein and modulate its activity. Although the interplay of these factors is still far from being completely understood, it is increasingly clear, that there are positive and negative influences on Runx2 expression and function, playing a role at different stages of development, but also in different skeletal elements. Therefore, this part of the chapter will focus on some of the best-characterized regulators of Runx2, whose function in skeletal biology was demonstrated by the genetic analysis of mouse models or human patients, as well as by molecular biology experiments.

For instance, there are several lines of evidence showing that certain homeodomain-containing transcription factors are involved in the regulation of *Runx2* expression. One of these proteins is Msx2, whose role in skeletal development was demonstrated through the identification of gain- and loss-of-function mutations in human patients suffering from Boston-type craniosynostosis or enlarged parietal foramina, respectively (Jabs *et al.*, 1993; Wilke *et al.*, 2000). Likewise, *Msx2*-deficient mice display defective ossification of the skull and of bones developing by endochondral ossification (Satokata *et al.*, 2000). Moreover, because the expression of *Osteocalcin* and *Runx2* is strongly reduced in *Msx2*-deficient mice, it appears that Msx2 acts upstream of Runx2 in a transcriptional cascade regulating osteoblast differentiation. A similar observation has been described for mice lacking the homeodomain-containing transcription factor Bpx (Tribioli and Lufkin, 1999). These mice die at birth owing to a severe dysplasia of the axial skeleton, whereas the appendicular skeleton is virtually unaffected. *Runx2* expression in *Bpx*-deficient mice is strongly reduced in osteochondrogenic precursor cells of the prospective vertebral column, thereby indicating that Bpx is required for *Runx2* expression specifically in these skeletal elements.

There are also negative regulators of *Runx2* expression. One of them is the homeodomain-containing transcription factor Hoxa2. *Hoxa2*-deficient mice display ectopic bone formation in the second branchial arch, which is readily explained by an induction of *Runx2* expression exactly in this region (Kanzler *et al.*, 1998). Consistently, transgenic mice expressing *Hoxa2* in craniofacial bones under the control of an *Msx2* promoter fragment lack several bones in the craniofacial area. Beyond development there is at least one factor required to limit *Runx2* expression in osteoblast precursor cells, namely, the high-mobility group containing transcription factor Sox8. *Sox8*-deficient mice display an osteopenia that is caused by accelerated osteoblast differentiation accompanied by enhanced expression of *Runx2* (Schmidt *et al.*, 2005). Likewise, transgenic mice expressing *Sox8* under the control of an osteoblast-specific *α1(I)-Collagen* promoter fragment virtually lack differentiated osteoblasts because of decreased expression of *Runx2*.

In addition to the existence of transcriptional regulators of *Runx2* expression, there are also factors interacting with the Runx2 protein, thereby activating or repressing its activity. One recently identified positive regulator of Runx2 action is the nuclear matrix protein Satb2. The importance of this protein in skeletogenesis was first discovered in human patients with cleft palate that carry a heterozygous chromosomal translocation inactivating the *SATB2* gene (Fitzpatrick *et al.*, 2003). The generation of a *Satb2*-deficient mouse model confirmed the importance of this gene in craniofacial development, skeletal patterning, and osteoblast differentiation (Dobreva *et al.*, 2006). The latter function was in part attributed to an increased expression of *Hoxa2*, a negative regulator of bone formation discussed earlier, whose expression is repressed by the binding of Satb2 to an enhancer element of the *Hoxa2* gene.

In addition to this type of action, there is also a Hoxa2-independent influence of Satb2 on the transcription of *Bone Sialoprotein* and *Osteocalcin*. Whereas, in the case of *Bone Sialoprotein,* Satb2 directly binds to an osteoblast-specific element in the promoter of this gene, the activation of *Osteocalcin* expression by Satb2 requires a physical interaction with Runx2. This was demonstrated by cotransfection assays using a *Luciferase* reporter gene under the control of an osteoblast-specific *Osteocalcin* promoter fragment, but also by coimmunoprecipitation experiments. Moreover, the synergistic action of Satb2 and Runx2 in osteoblasts was genetically confirmed by the generation of compound heterozygous mice lacking one allele of each gene (Dobreva *et al.*, 2006). Taken together, these results identified Satb2 as an important regulator of osteoblast differentiation in mice and humans. Moreover, the finding, that Satb2 also interacts with ATF4, another transcription factor involved in osteoblast differentiation and function that will be discussed later, illustrates that the transcriptional network regulating bone formation is much more complex than previously anticipated.

This is underscored by the discovery of other proteins that physically interact with Runx2, thereby attenuating its activity. One of these proteins is Stat1, a transcription factor regulated by extracellular signaling molecules, such as interferons. *Stat1*-deficient mice, as already mentioned, are viable, but develop a high-bone-mass phenotype explained by enhanced bone formation (Kim *et al.*, 2003). The increase of osteoblast differentiation and function in these mice is molecularly explained by the lack of a Stat1-mediated inhibition of the transcriptional activity of Runx2. Interestingly, the physical interaction of both proteins is independent of Stat1 activation by phosphorylation, and it inhibits the translocation of Runx2 into the nucleus. Thus, overexpression of Stat1 in osteoblasts leads to a cytosolic retention of Runx2, and the nuclear translocation of Runx2 is much more prominent in Stat1-deficient osteoblasts (Kim *et al.*, 2003).

Another protein interacting with Runx2, thereby decreasing its availability in the nucleus, is Shn3. Shn3 is a zinc finger adapter protein originally thought to be involved in VDJ recombination of immunoglobulin genes (Wu *et al.*, 1993). Unexpectedly, however, the generation of a *Shn3*-deficient mouse model revealed a major function of this protein in bone formation. In fact, the *Shn3*-deficient mice display a severe adult-onset osteosclerotic phenotype owing to a cell-autonomous increase of bone matrix deposition (Jones *et al.*, 2006). Interestingly, although several Runx2 target genes are expressed at higher rates in *Shn3*-deficient osteoblasts, *Runx2* expression itself is not affected by the absence of Shn3. Importantly, however, the Runx2 protein level is strikingly increased in *Shn3*-deficient osteoblasts.

This latter finding is molecularly explained by the function of Shn3 as an adapter molecule linking Runx2 to the E3 ubiquitin ligase WWP1. The Shn3-mediated recruitment of WWP1 in turn leads to an enhanced proteasomal degradation of Runx2, which is best underscored by the finding that RNAi-mediated downregulation of WWP1 in osteoblasts leads to increased Runx2 protein levels and enhanced extracellular matrix mineralization, thus virtually mimicking the defects observed in the absence of Shn3 (Jones *et al.*, 2006). Taken together, these data identify Shn3 as a key regulator of Runx2 actions *in vivo*. Moreover, given the postnatal onset of the bone phenotype of the *Shn3*-deficient mice, it is reasonable to speculate that compounds blocking the interaction of Runx2, Shn3, and WWP1 may serve as specific therapeutic agents for the treatment of bone loss diseases, such as osteoporosis.

While Stat1 and Shn3 provide examples for the importance of a negative regulation of Runx2 in postnatal bone remodeling, there is also a need for a negative regulation of Runx2 activity during bone development. This is underscored by the finding that *Runx2* expression in the lateral plate mesoderm is already detectable as early as E10 of mouse development, whereas the expression of molecular markers of differentiated osteoblasts cannot be detected before E13 (Ducy, 2000). A molecular explanation for this delay between *Runx2* expression and osteoblast differentiation came from the functional analysis of Twist proteins that are transiently coexpressed with *Runx2* early during development and inhibit osteoblast differentiation by interacting with Runx2. In essence, the initiation of osteoblast differentiation occurs only when *Twist* gene expression fades away.

That Runx2 expression precedes osteoblast differentiation more than 4 days led to the assumption that Runx2 function must be regulated negatively in *Runx2*-expressing cells. The identification of such a factor relied on human genetic evidence. Haploinsufficiency at the *RUNX2* locus causes a lack of bone in the skull, whereas haploinsufficiency at the *TWIST1* locus causes essentially too much bone in the skull, a condition called craniosynostosis (El Ghouzzi *et al.*, 1997; Howard *et al.* 1997). Because the same phenotypes are observed in the corresponding mouse models lacking one allele of either gene, it was possible to demonstrate a genetic interaction of *Runx2* and *Twist1* by the generation of compound heterozygote mice. In fact, these mice did not display any detectable defects of skull development and suture fusion (Bialek *et al.*, 2004). In contrast, the defects of clavicle development caused by haploinsufficiency of *Runx2* were not rescued by heterozygosity of *Twist1*, but by heterozygosity of *Twist2*. Thus, both Twist proteins have similar functions in different skeletal elements, which is consistent with their expression pattern in mouse embryos (Bialek *et al.*, 2004).

Both Twist proteins are basic helix-loop-helix transcription factors, yet this function of Twist is not determined by the bHLH domain, but rather by the C-terminal 20 amino acids, the so-called Twist-box, whose sequence is fully conserved in both Twist proteins, in mice and humans. Through the Twist-box, Twist proteins interact with the Runx2 DNA-binding domain and prevent its DNA binding. The importance of this sequence motif for Twist function *in vivo* was confirmed by the existence of a Twist-box mutation within the human *TWIST1* gene that causes a severe form of craniosynostosis (Gripp *et al.*, 2000). Moreover, an ethyl-nitroso-urea-mutagenesis approach in mice led to the identification of an amino acid substitution within the Twist-box of Twist1 that causes premature osteoblast differentiation *in vivo* (Bialek *et al.*, 2004).

Unexpectedly, this mouse model, termed *Charlie Chaplin* (*CC/CC*), also displayed decreased chondrocyte maturation, thereby suggesting an additional physiological role of Twist1, independent of its antiosteogenic function (Hinoi *et al.*, 2006). Because *Twist1* is not expressed in chondrocytes, but in mesenchymal cells of the perichondrium, it appears that it is required to inhibit the induction of *Fgf18* expression in these cells by the action of Runx2, which was described earlier. Indeed, whereas transgenic mice overexpressing *Twist1* under the control of an osteoblast-specific *α1(I)-Collagen* promoter fragment displayed enhanced chondrocyte maturation, the decreased chondrocyte maturation in the *CC/CC* mice was normalized by haploinsufficiency of *Runx2* (Hinoi *et al.*, 2006). Taken together, these data demonstrate that Twist1, through inhibition of Runx2 DNA binding, not only limits osteoblast differentiation and bone formation, but also enhances chondrocyte maturation during skeletal development.

OSTERIX, A Runx2-DEPENDENT OSTEOBLAST-SPECIFIC TRANSCRIPTION FACTOR REQUIRED FOR BONE FORMATION

Besides Runx2, there is at least one more transcription factor, termed Osterix (Osx), whose activity is absolutely required, in mice, for osteoblast differentiation. Osx is a zinc finger-containing transcription factor that is specifically expressed in osteoblasts of all skeletal elements. Inactivation of Osx in mice results in perinatal lethality owing to a complete absence of bone formation (Nakashima *et al.*, 2002). Unlike the *Runx2*-deficient mice, however, whose skeleton is completely nonmineralized, the *Osx*-deficient mice did only lack a mineralized matrix in bones formed by intramembranous ossification. In contrast, the bones formed by endochondral ossification contained mineralized matrix, but this resembled calcified cartilage, and not mineralized bone matrix. This finding shows, that Osx, unlike Runx2, is not required for chondrocyte hypertrophy, thereby demonstrating that it is specifically inducing osteoblast differentiation and bone formation *in vivo*. The comparative expression analysis by *in situ* hybridization further revealed that *Osx* is not expressed in *Runx2*-deficient embryos, and *Runx2* is normally expressed in *Osx*-deficient embryos (Nakashima *et al.*, 2002). These results demonstrated that Osx is acting downstream of Runx2 in a transcriptional cascade of osteoblast differentiation, and its expression is apparently directly regulated by the binding of Runx2 to a responsive element in the promoter of the *Osx* gene (Nishio *et al.*, 2006).

In contrast to the steadily increasing knowledge about the function of Runx2 and its regulation by other molecules, the molecular mechanisms underlying the action of Osx in osteoblasts are less well understood. Moreover, unlike for *RUNX2*, no human mutations of the *OSX* gene have yet been identified that would be associated with decreased bone formation. Nevertheless, there is one recent publication providing evidence for a contribution of Osx to the negative effects of nuclear factor of activated T-cells (NFAT) inhibitors on bone mass (Koga *et al.*, 2005). NFAT inhibitors, such as FK506 or cyclosporin A, are commonly used as immunosuppressants, e.g., after organ transplantation. However, this treatment is often accompanied by the development of osteopenia in the respective patients (Rodino and Shane, 1998). Likewise, treatment of mice with FK506 leads to decreased bone mass owing to impaired bone formation, and the same phenotype was observed in mice lacking the transcription factor Nfatc1. The deduced role of Nfatc1 as a physiological activator of osteoblast differentiation and function can be molecularly explained by an interaction with Osx. In fact, both proteins synergistically activate transcription of a *Luciferase* reporter gene driven by an osteoblast-specific *α1(I)-Collagen* promoter fragment, which is based on the formation of a DNA-binding complex of Nfatc1 and Osx (Koga *et al.*, 2005). Although this is just a first example, it is likely that the complexities of the transcriptional control of osteoblast differentiation will increase further when more Osx-interacting molecules are identified in the future.

ATF4, A TRANSCRIPTIONAL REGULATOR OF OSTEOBLAST FUNCTIONS AND A MEDIATOR OF THE NEURAL REGULATION OF BONE MASS

The role of ATF4 in skeletal biology also arose from a combination of molecular biology and human and mouse genetic data. *RSK2,* which encodes a kinase, is the gene mutated in Coffin–Lowry syndrome, an X-linked mental retardation condition associated with skeletal abnormalities (Trivier *et al.*, 1996). Likewise *Rsk2*-deficient mice display decreased bone mass owing to impaired bone formation (Yang *et al.*, 2004). *In vitro* kinase assays demonstrated that ATF4 is strongly phosphorylated by Rsk2, and that this phosphorylation is undetectable in osteoblasts derived from *Rsk2*-deficient mice. The subsequent analysis of an *ATF4*-deficient mouse model revealed that this transcription factor plays a crucial role in bone formation. In fact, *ATF4*-deficient mice display a delayed skeletal development, and thereafter develop a severe low-bone-mass phenotype caused by decreased bone formation (Yang *et al.*, 2004).

Molecularly, ATF4 was identified as the factor binding to the osteoblast-specific element OSE-1 in the *Osteocalcin* promoter, thereby directly activating the transcription of this gene. Moreover, ATF4 is required for proper synthesis of type I collagen, although this function is not mediated by a transcriptional regulation of *type I collagen* expression. In fact, because type I collagen synthesis in the absence of nonessential amino acids is specifically reduced in primary osteoblast cultures lacking ATF4, it appears that ATF4 is required for efficient amino acid import into osteoblasts, as it has been described for other cell types (Harding *et al.*, 2003). Because a reduced type I collagen synthesis was subsequently also observed in mice lacking Rsk2, these data provided evidence that the diminished ATF4 phosphorylation in the absence of Rsk2 may contribute to the skeletal defects associated with Coffin–Lowry syndrome (Yang *et al.*, 2004).

In addition to its role in bone formation, ATF4, through its expression in osteoblasts, regulates bone resorption (Elefteriou *et al.*, 2005). This function is molecularly explained by the binding of ATF4 to the promoter of the *Rankl* gene, which is expressed by osteoblasts in order to promote osteoclast differentiation (Teitelbaum and Ross, 2003). Likewise, *ATF4*-deficient mice have decreased osteoclast numbers because of reduced *Rankl* expression. Most importantly, however, this function of ATF4 is involved in the control of bone resorption by the sympathetic nervous system. In fact, treatment of normal osteoblasts with isoprotenerol, a surrogate of sympathetic signaling, enhanced osteoclastogenesis of cocultured bone marrow macrophages through an induction of osteoblastic *Rankl* expression (Elefteriou *et al.*, 2005). As expected, this effect was blunted, when the osteoblasts were derived from mice lacking the β2-adrenergic receptor Adrb2. However, the effect of isoprotenerol was also blunted by an inhibitor of protein kinase A, or by using osteoblasts derived from *ATF4*-deficient mice (Elefteriou *et al.*, 2005). Taken together, these results demonstrated that ATF4 is an important mediator of extracellular signals, such as β-adrenergic stimulation, in osteoblasts.

Thus, it is not surprising that the function of ATF4 is mostly regulated post-translationally. For example, as already mentioned, ATF4 is also interacting with other proteins, such as the nuclear matrix protein Satb2 (Dobreva *et al.*, 2006). As described earlier, the proximal *Osteocalcin* promoter contains two osteoblast-specific elements, termed OSE-1 and OSE-2, that serve as binding sites for ATF4 and Runx2, respectively (Ducy and Karsenty, 1995; Ducy *et al.*, 1997; Schinke and Karsenty, 1999; Yang *et al.*, 2004). Because of the proximity of both elements, there is indeed a physical interaction of the two proteins, which is stabilized by Satb2, that acts a scaffold enhancing the synergistic activity of Runx2 and ATF4, which is required for sufficient *Osteocalcin* expression (Xiao *et al.*, 2005; Dobreva *et al.*, 2006).

Other aspects of ATF4 biology are also regulated post-translationally. In fact, even the osteoblast specificity of ATF4 function is not determined by osteoblast-specific *ATF4* expression, but by a selective accumulation of the ATF4 protein in osteoblasts, which is explained by the lack of proteosomal degradation (Yang and Karsenty, 2004). This is best demonstrated by the finding that the treatment of nonosteoblastic cell types with the proteasome inhibitor MG115 leads to an accumulation of the ATF4 protein, thereby resulting in ectopic *Osteocalcin* expression. Taken together, these data provided the first evidence for the achievement of a cell-specific function of a transcriptional activator by a post-translational mechanism. They are therefore of general importance for our understanding of the transcriptional networks controlling cellular differentiation and function.

Remarkably, ATF4 biology further illustrates how the molecular understanding of a disease-causing gene can translate into therapeutic interventions. Indeed, an increased Rsk2-dependent phosphorylation of ATF4 may also be involved in the development of the skeletal abnormalities in human patients suffering from neurofibromatosis (Ruggieri *et al.*, 1999; Stevenson *et al.*, 1999). This disease, which is primarily known for tumor development within the nervous system, is caused by inactivating mutations of the *NF1* gene that encodes a Ras-GTPase-activating protein (Klose *et al.*, 1998). The generation of a mouse model lacking *Nf1* specifically in osteoblasts ($Nf1_{ob-/-}$) led to the demonstration that this gene plays a major physiological role in bone remodeling. In fact, the $Nf1_{ob-/-}$ mice displayed a high-bone-mass phenotype that is caused by increased bone turnover and is accompanied by an enrichment of nonmineralized osteoid (Elefteriou *et al.*, 2006). The further analysis of this phenotype revealed an increased production of type I collagen in the absence of *Nf1*, which is molecularly explained by an Rsk2-dependent activation of ATF4. Likewise, transgenic mice overexpressing *ATF4* in osteoblasts display a phenotype similar to the $Nf1_{ob-/-}$ mice, and the increased type I collagen production and osteoid thickness in the latter ones is significantly reduced by haploinsufficiency of ATF4 (Elefteriou *et al.*, 2006).

These molecular findings may also have therapeutic implications. Given the previously discussed function of ATF4 in amino acid import, it appeared reasonable to analyze whether the skeletal defects of the $Nf1_{ob-/-}$ mice can be affected by dietary manipulation. Indeed, the increased bone formation and osteoid thickness of $Nf1_{ob-/-}$ mice can be normalized by a low-protein diet, and the same was the case in the transgenic mice overexpressing *ATF4* in osteoblasts (Elefteriou *et al.*, 2006). Likewise, the defects of osteoblast differentiation and bone formation observed in both the *ATF4*- and the *Rsk2*-deficient mice were corrected by feeding a high-protein diet. Taken together, these data not only underscore the importance of ATF4 in osteoblast biology, but they also demonstrate how the knowledge about its specific functions in osteoblasts can be useful for the treatment of skeletal diseases.

AP1 REGULATION OF OSTEOBLAST DIFFERENTIATION AND FUNCTION

Activator protein 1 (AP1) is a heterodimeric transcription factor composed of members of the Jun and Fos family of basic leucine zipper proteins (Karin *et al.*, 1997). These include the Jun proteins c-Jun, JunB, and JunD, as well as the Fos proteins c-Fos, Fra1, Fra2, and Fosb, respectively. Although AP1-transcription factors have been demonstrated to fulfill various functions in different cell types, it is striking that some of the family members play specific roles in bone remodeling, as demonstrated by several loss- or gain-of-function studies in mice (Wagner and Eferl, 2005). For instance, the deletion of *c-Fos* from the mouse genome results in severe osteopetrosis owing to an arrest of osteoclast differentiation, whereas the transgenic overexpression of *c-Fos* results in osteosarcoma development (Grigoriadis *et al.*, 1993, 1994). Moreover, transgenic mice overexpressing either *Fra1*, or *ΔfosB*, a splice variant of *FosB*, display a severe osteosclerotic phenotype caused by increased osteoblast differentiation and function (Jochum *et al.*, 2000; Sabatakos *et al.*, 2000). Likewise, mice lacking Fra1 in extraplacental tissues display an osteopenia associated with reduced bone formation, indicating a physiological role of Fra1 in osteoblasts (Eferl *et al.*, 2004). When the same approach was used to inactivate JunB in extraplacental tissues, thereby circumventing the embryonic lethality caused by a complete genomic deletion of *JunB*, the resulting mice developed a state of low bone turnover, owing to cell-autonomous defects of osteoblast, but also of osteoclast differentiation (Kenner *et al.*, 2004).

Taken together, these data provide evidence for a crucial role of AP1-transcription factors in the regulation of bone formation, although their connection to the other transcriptional regulators described earlier still needs to be further investigated. For instance, it is known from other cell types that Jun proteins can also interact with ATF family members, thus raising the possibility that heterodimerization with ATF4 may be one mechanism by which these proteins can regulate osteoblast-specific gene expression (Chinenov and Kerppola, 2001). Interestingly, it has recently been demonstrated that the osteosarcoma development of *c-Fos* transgenic mice is dramatically decreased in an *Rsk2*-deficient genetic background (David *et al.*, 2005). This observation is molecularly explained by the lack of c-Fos phosphorylation by Rsk2, thereby leading to increased proteosomal degradation. Thus, Rsk2 is apparently not only involved in the physiological regulation of bone formation via phosphorylation of ATF4, but may also have an influence on the development of osteosarcomas via phosphorylation of c-Fos.

Another potential mechanism by which AP1-family members might be involved in the regulation of bone formation came from the analysis of mouse models with impaired circadian regulation. These mice, which lack components of the molecular clock, namely the *Per* or *Cry* genes, display a high-bone-mass phenotype caused by increased bone formation (Fu *et al.*, 2005). Moreover, they respond to intracerebroventricular infusion of leptin by a further increase of bone mass, suggesting that the components of the molecular clock are involved in the regulation of bone formation via the sympathetic nervous system. Interestingly, virtually all genes encoding members of the AP1-transcription factor family were expressed at higher levels in osteoblasts derived from mice lacking either the *Per* genes, or the *β*2-adrenergic receptor Adrb2 (Fu *et al.*, 2005). This increase was especially pronounced in the case of the *c-Fos* gene, whose expression can also be induced by the addition of isoprotenerol in wild-type osteoblasts. In turn, c-Fos leads to a direct activation of *c-Myc* transcription, thereby indirectly increasing the intracellular levels of cyclin D1 and promoting osteoblast proliferation. Taken together, these data demonstrated that the expression of AP1-components is activated via sympathetic signaling, and that this induction is counteracted by the activity of clock gene products.

TRANSCRIPTION FACTORS ACTING DOWNSTREAM OF WNT SIGNALING

The recent discovery of the *LRP5* gene as a major determinant of bone mass in humans has paved the way to the identification of another class of transcription factors acting downstream of the Wnt signaling pathway (Gong *et al.*, 2001; Boyden *et al.*, 2002; Little *et al.*, 2002). Lrp5 is a transmembrane protein that acts as a coreceptor for ligands of the Wnt family, thereby triggering a cascade of intracellular signaling events that leads to a nuclear translocation of β-catenin, that in turn activates transcription factors of the Tcf/Lef family (Mao *et al.*, 2001; Huelsken and Birchmeier, 2001). Mice lacking *Lrp5* recapitulate the phenotype of patients with inactivating *LRP5* mutations, namely low bone mass owing to decreased bone formation, and also persistent embryonic eye vascularization (Kato *et al.*, 2002). Because bone resorption is virtually unaffected in the absence of Lrp5, it came as a surprise that mouse models with altered levels of the downstream effector β-catenin in osteoblasts primarily display defects of bone resorption (Glass *et al.*, 2005).

Although an osteoblast-specific mutational activation of β-catenin led to an osteopetrotic phenotype, the osteoblast-specific inactivation of β-catenin resulted in a low-bone-mass phenotype, caused by increased bone resorption. Because of the strategy used to unravel these functions of β-catenin, it was expected that both phenotypes would be explained by a cell-autonomous defect of osteoblasts, which was subsequently confirmed in coculture experiments (Glass *et al.*, 2005). The molecular explanation for the regulation of osteoclast differentiation by β-catenin expression in osteoblasts came from the expression analysis of *Osteoprotegerin (Opg)*, a well known inhibitor of bone resorption, blocking the activity of Rankl (Simonet *et al.*, 1997; Yasuda *et al.*, 1998). Surprisingly, although *Opg* expression was markedly increased in osteoblasts from mice expressing the stabilized form of β-catenin, its expression was decreased in mice harboring an osteoblast-specific deletion of β-catenin (Glass *et al.*, 2005).

One of the transcription factors activated by β-catenin in osteoblasts has already been identified as Tcf1, whose importance for the regulation of gene expression in osteoblasts is underscored by several lines of evidence. First, *in situ* hybridization revealed that *Tcf1* is expressed in osteoblasts during bone development and after birth. Second, *Tcf1*-deficient mice display a low-bone-mass phenotype, caused by increased bone resorption. Third, the compound heterozygosity of *Tcf1* and *β-catenin* in osteoblasts also results in low bone mass, which is not observed, when one allele of each gene is inactivated alone. Fourth, *Opg* expression is decreased in osteoblasts of *Tcf1*-deficient mice, and the molecular analysis of the *Opg* promoter revealed the existence of a Tcf-binding site, whose functional activity was subsequently proven by chromatin immunoprecipitation and cotransfection assays (Glass *et al.*, 2005). Thus, although these results raise the question of why the inactivation of Lrp5 does not result in similar abnormalities, they do show that Tcf1 is one transcription factor acting downstream of the Wnt signaling pathway that regulates gene expression in osteoblasts.

PERSPECTIVES

Since the last edition of this book, it is obvious that many new insights into this field have accumulated. These include the recent discoveries of novel transcriptional regulators, such as Osx, ATF4, and others, playing a key role in bone formation. Moreover, the molecular networks between these factors are much better understood than a few years ago, although still more research is necessary to answer the many remaining questions (Fig. 2). These

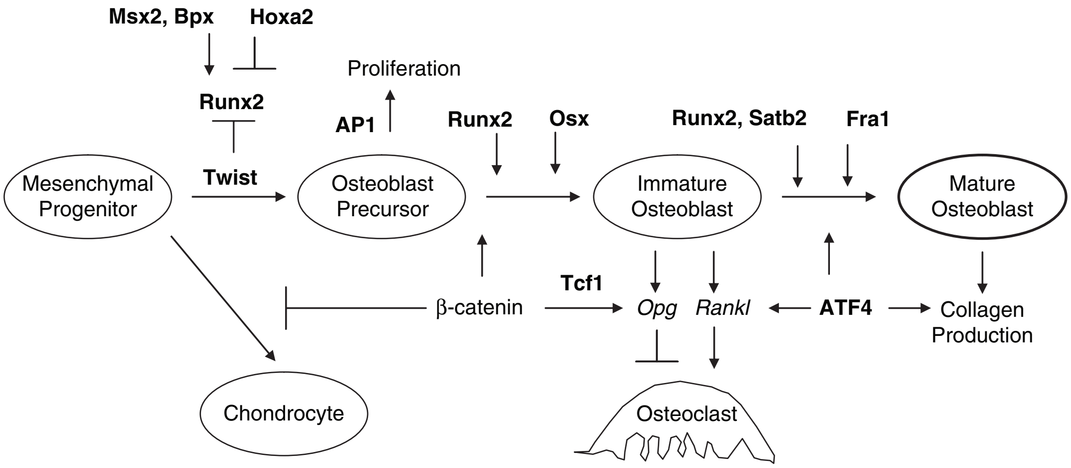

FIGURE 2 Transcriptional control of osteoblast differentiation and function. Runx2 expression in mesenchymal progenitor cells is controlled by homeodomain transcription factors such as Msx2, Bpx, and Hoxa2. The activity of Runx2 is first limited by the action of Twist proteins, whose expression ceases to allow Runx2-mediated differentiation along the osteoblast lineage. Besides Runx2 there are other important transcriptional activators, such as Osx, Satb2, Fra1, and ATF4, that are required for proper osteoblast differentiation and bone matrix deposition. Through the regulation of Opg and Rankl expression by Tcf1 and ATF4, respectively, osteoblasts also control bone resorption by osteoclasts. Moreover, components of the AP1-transcription factor are involved in osteoblast proliferation, and an intact Wnt signaling pathway in mesenchymal progenitor cells is also required to suppress their differentiation into chondrocytes (Day *et al.*, 2005; Hill *et al.*, 2005).

include the definition of specific target genes, the regulation by outside signals, and still a deeper understanding about the interactions of all factors playing a role at various stages of development in the different skeletal elements. Moreover, the most important goal for the future, which should be the major aim of basic research in general, is the use of this accumulating knowledge to define novel therapeutic strategies for the treatment of skeletal diseases.

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Chapter 6

Wnt Signaling and Bone

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INTRODUCTION

The past decade has witnessed a literal explosion in our understanding of fundamental biological processes, catalyzed in large part by the human genome project and the vast repertoire of technologies that are now available to identify genes and study gene function. The field of bone biology has certainly benefited from these advances as illustrated by many of the chapters in this book. Not surprisingly perhaps, the genetic dissection of single-gene human disorders that present with skeletal abnormalities has led to advances in our understanding of bone biology, often in directions that could not have been anticipated by the current state of knowledge. The discovery of mutations in the low-density lipoprotein receptor-related protein 5 (LRP5) gene that give rise to conditions of decreased (Gong *et al.*, 2001) or increased bone mass (Little *et al.*, 2002; Boyden *et al.*, 2002) is an example of how fundamental principles are often revealed through the identification of causal mutations underlying inherited human conditions. Prior to these studies the role of Wnt/β-catenin signaling in bone was limited mainly to skeletal development, but because of the explosion of research that occurred as a result of those seminal publications we now appreciate the role of this pathway in bone cell differentiation, proliferation, and apoptosis, bone mass regulation, and the ability of bone to respond to changes in mechanical load. Although Wnts can signal through multiple pathways, in this chapter, the focus will be on what is known about the Wnt/β-catenin signaling pathway and its regulation as it pertains to bone.

Wnt GENES AND PROTEINS

There are currently 19 known *Wnt* genes in humans. Wnt proteins are secreted, highly post-translationally modified proteins that play key roles in development and homeostasis through their involvement in cell differentiation, proliferation, and apoptosis. Nusse and Varmus (1982) first described Wnt as the mouse proto-oncogene integration site/locus (*int-1*) of the murine mammary tumor virus that resulted in breast tumors. Later the *Drosophila* segmentation gene *Wingless* (*Wg*) was shown to be homologous to the *int-1* gene (Cabrera *et al.*, 1987; Rijsewijk *et al.*, 1987) and the name *Wnt* was coined.

The Wnt proteins have proven difficult to study because of their post-translational modifications (glycosylation and palmitoylation) that make them extremely insoluble and, until recently, refractory to purification to any degree. Therefore, much of what we know about Wnt proteins at a structural level is based on their DNA sequence analysis and comparisons. The various Wnt genes share general homology in the range of 35%, although within subgroups the homology can be as high as 83%. The molecular weight of the various Wnt proteins ranges from 39,000 to 46,000. All Wnts contain 23 or 24 conserved cysteine residues that are spaced similarly between proteins, suggesting an important conservation of function, possibly proper folding of the protein, which is required for Wnt activity (Miller, 2001).

Nusse and colleagues successfully purified Wnt3a from mouse L cells (Willert *et al.*, 2003). Subsequently Schulte *et al.* (2005) used a similar strategy to purify Wnt5a. Both groups demonstrated that the addition of a palmitate to the first, most amino-terminally conserved of the cysteine residues is absolutely required for Wnt3a and Wnt5a activity. In *Drosophila* the gene *porcupine* and in *Caenorhabditis elegans* the gene *mom-1* encode acyltransferase enzymes that are strong candidates for the proteins responsible for the palmitoylation of Wnts (Kadowaki *et al.*, 1996; Nusse, 2003). Interestingly, these proteins are membrane bound and found in the endoplasmic reticulum indicating that the palmitoylation occurs intracellularly. Although palmitoylation of secreted proteins is somewhat unusual (Dunphy and Linder, 1998), the hedgehog family of proteins is also palmitoylated and secreted in this manner (Nusse, 2003). Wnts are known to act developmentally as morphogens (Aulehla and Herrmann, 2004; Aulehla *et al.*, 2003; Zecca *et al.*, 1996), but the role of the palmitate in Wnt function

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is not clear. Possible roles for the palmitoylation include anchoring the Wnt proteins to the cell surface membrane, binding to a transport protein (unknown) and binding of Wnts to Lrp5/6 and/or frizzled (Miura and Treisman, 2006). Little is known about the role of the glycosylation of Wnts, although this modification is not essential for activity (Mason *et al.*, 1992). Recently, two groups have shown that the multipass transmembrane protein Wntless/Evi, which is located in the Golgi and/or the plasma membrane, is involved in the transport and secretion of Wnt proteins (Banziger *et al.*, 2006; Bartscherer *et al.*, 2006).

COMPONENTS OF THE Wnt/β-CATENIN SIGNALING PATHWAY

A detailed description of the various components of the Wnt/β-catenin signaling pathway can be found at the Wnt homepage website maintained by Roel Nusse (http://www.stanford.edu/~rnusse/) and in several reviews in the literature (Clevers, 2006; Gordon and Nusse, 2006; He, 2003; He *et al.*, 2004a; Johnson and Summerfield, 2005; Kikuchi, 2003; Logan and Nusse, 2004; Mikels and Nusse, 2006; Nusse, 2003; Prunier *et al.*, 2004; Reya and Clevers, 2005). The major players in the Wnt/β-catenin signaling pathway in bone are: (1) the cell surface coreceptors Lrp5/6 and frizzled; (2) the intracellular proteins Dishevelled (Dsh), glycogen synthase kinase-3β (GSK-3β), Axin, and β-catenin; and (3) the TCF/LEF nuclear transcription factors that bind β-catenin and regulate gene expression. In addition, there are a number of other extracellular and intracellular proteins involved in regulation of this pathway that serve to modify the functionality of one of these key proteins. Particularly important are several proteins that bind Lrp5/6 to modulate its binding of Wnt ligand.

The details of the Wnt/β-catenin pathway are illustrated in Figure 1. Ultimately the Wnt/β-catenin signaling pathway controls the intracellular levels of free β-catenin. In the absence of Wnt ligand, the intracellular levels of β-catenin are extremely low because of the activity of a degradation complex composed of Axin, GSK-3β, the adenomatous polyposis coli (APC) protein, and several other proteins. This complex, specifically GSK-3β, is responsible for the phosphorylation of β-catenin, which leads to its ubiquitination and degradation by the 26S proteosome complex (Aberle *et al.*, 1997) (Fig. 1A). Wnt binding to the Lrp5/6-frizzled coreceptors results in Dsh activation and Axin binding to the cytoplasmic tail of Lrp5/6. Dsh activation leads to GSK-3β phosphorylation and inhibition of its activity. β-Catenin is no longer phosphorylated and this, coupled with the binding of Axin to the cytoplasmic tail of Lrp5/6, releases β-catenin. β-Catenin accumulates in the cytoplasm and then translocates into the nucleus where it binds to the TCF/LEF family of transcription proteins and regulates the expression of specific target genes (Fig. 1B).

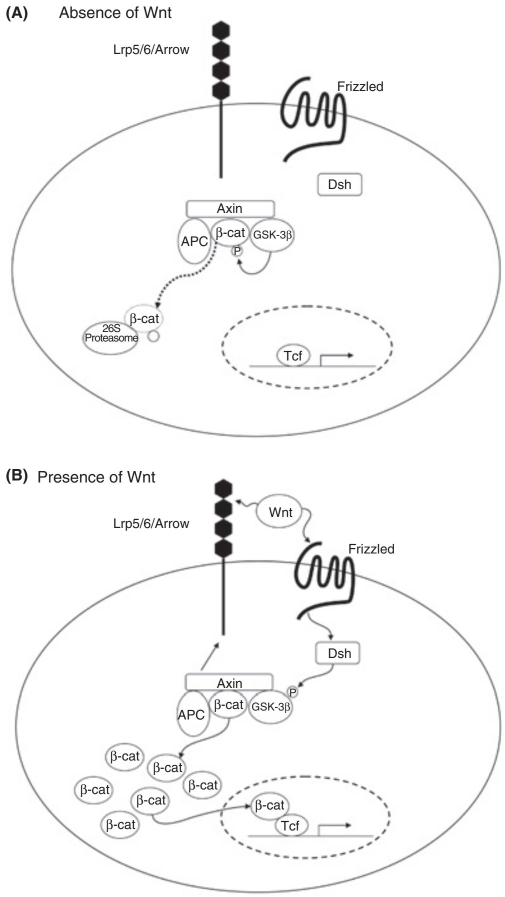

FIGURE 1 The Wnt/β-catenin signaling pathway. The Wnt/β-catenin signaling pathway is controlled by two cell surface coreceptors, Frizzled and one of the low-density lipoprotein receptor-related proteins (Lrp) 5 or 6 in vertebrates, or arrow in *Drosophila* that cooperatively bind the Wnt protein ligand. (**A**) In the absence of Wnt, the degradation complex consisting of the scaffolding protein Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase-3β (GSK-3β), along with several other proteins (not shown), coordinate the phosphorylation of β-catenin, which is then ubiquitinated and degraded by the 26S protesome complex. This serves to keep the intracellular levels of free β-catenin at a low level. (**B**) When Wnt ligand is bound by the Lrp5/6/arrow and frizzled coreceptors the protein Dishevelled (Dsh) is activated, which results in the phosphorylation of GSK-3β, thereby inhibiting its activity. The degradation complex is also induced to bind to the cytoplasmic tail of Lrp5/6/arrow and the combination of events leads to the intracellular accumulation of high levels of free β-catenin. β-Catenin can then translocate into the nucleus and, on binding to the Tcf/LEF family of transcription factors, regulate the expression of a number of target genes.

Lrp5, Lrp6, and Frizzled

Lrp5 and Lrp6 (Arrow in *Drosophila*) are highly homologous proteins that, until their role in bone biology was described, were considered orphan members of the low-density lipoprotein receptor (LDLR) family, which includes at least 13 members (Strickland *et al.*, 2002). One of the hallmark structural characteristics of this family is the presence of tyrosine-tryptophan-threonine-aspartic acid

(YWTD) repeat motifs framed by EGF repeats in the extracellular domain of the proteins. These repeat clusters form β-propellor structures that are important for ligand binding. Based on the amino acid sequence of Lrp5 and Lrp6 derived from cDNA cloning (Brown *et al.*, 1998; Dong *et al.*, 1998; Hey *et al.*, 1998; Kim *et al.*, 1998), Lrp5/6 each contain four of these repeat clusters. However the YWTD motif is degenerate and one of the six repeat sequences comprising each motif in Lrp5 is substituted by leucine-phenylalanine-alanine-asparagine (LFAN) or phenylalanine-phenylalanine-threonine-asparagine (FFTN) sequences (see review by Johnson and Summerfield, 2005). Many of these LDLR family members have a consensus sequence of asparagine-proline-x-tyrosine (NPxY) in their cytoplasmic tail that is required for endocytosis of the receptor (Chen *et al.*, 1990). Lrp5 and Lrp6 lack an NPxY sequence. Also the orientation of the their extracellular domain is reversed relative to other members of the LDLR family in that LDL-type A repeats (complement-like repeats) are adjacent to the transmembrane-spanning region rather than at the amino-terminal end (or clustered throughout the extracellular domain). There are also only three of these LDL-A type repeats in Lrp5 and Lrp6.

Originally human *LRP5* was proposed to be a candidate gene for susceptibility to type I diabetes in the IDDM 4 locus that was mapped to chromosome 11q13 (Figueroa *et al.*, 2000; Twells *et al.*, 2001). However, subsequent genetic association studies failed to confirm this functional role (Twells *et al.*, 2003). At the same time, Arrow (Wehrli *et al.*, 2000) and Lrp6 (Tamai *et al.*, 2000) were shown to mediate Wnt/β-catenin signaling and *Lrp6*$^{-/-}$ mutant mouse embryos displayed skeletal abnormalities similar to those found in mice carrying mutations in different Wnt genes (Pinson *et al.*, 2000). Subsequently, mutations in human *LRP5* were shown to cause osteoporosis pseudoglioma syndrome (OPPG) (Gong *et al.*, 2001), which is characterized by low bone mass and progressive blindness, and the high-bone-mass (HBM) mutation G171V was reported in two separate kindreds (Little *et al.*, 2002; Boyden *et al.*, 2002). At present, a large number of additional mutations that give rise to conditions of increased or decreased bone mass have been reported in the literature (Ai *et al.*, 2005; Jiao *et al.*, 2004; Jin *et al.*, 2004; Johnson *et al.*, 2004; Rickels *et al.*, 2004, 2005; Streeten *et al.*, 2003, 2004; Toomes *et al.*, 2004; Van Wesenbeeck *et al.*, 2003; Whyte *et al.*, 2004). Also, allelic variants of *LRP5* have been shown to contribute to variation in bone mass in several studies, although the relative degree varies and sex differences are noted in many of the studies (Bollerslev *et al.*, 2005; Choudhury *et al.*, 2003; Crabbe *et al.*, 2005; Deng *et al.*, 2002; Ezura *et al.*, 2007; Ferrari *et al.*, 2004; Giroux *et al.*, 2007; Hartikka *et al.*, 2005; Kiel *et al.*, 2007; Koay *et al.*, 2004; Koh *et al.*, 2004; Koller *et al.*, 2005; Mizuguchi *et al.*, 2004; Okubo *et al.*, 2002; Saarinen *et al.*, 2007; van Meurs *et al.*, 2006; Xiong *et al.*, 2007).

The role of LRP6 in human bone density variation has only been studied to a limited degree. One report indicates that the *LRP6* Ile1062Val allele contributes to a higher risk for fragility and vertebral fracture in men and that this allelic variant, in combination with the *LRP5* A1330V allele, accounted for 10% of the fractures in males (van Meurs *et al.*, 2006). It has also been shown that *Lrp6*$^{+/-}$:*Lrp5*$^{-/-}$ mice have lower adult bone mineral density than *Lrp6*$^{+/+}$:*Lrp5*$^{-/-}$ mice, suggesting a role for Lrp6 in bone mass accrual (Holmen *et al.*, 2004). A recent report identified a mutation in LRP6 (LRP6^{R611C}) that is linked to early-onset coronary artery disease in a family and the five mutation carriers that were studied also had low bone densities, confirming a role for LRP6 in bone mass accrual as suggested by the studies in mice (Mani *et al.*, 2007). Also a hypomorphic *Lrp6* mutation in the mouse (called *ringleschwanz*) has been identified and studies in that model have confirmed a role for Lrp6 in somitogenesis and osteogenesis (Kokubu *et al.*, 2004).

Understanding the distinct and/or overlapping functional roles of Lrp5 and Lrp6 remains an important unanswered question in the bone field at this point in time, especially because both appear to be coexpressed in most cells/tissues that have been examined. If Lrp5 and Lrp6 served totally redundant roles then one would expect to observe the dramatic phenotypes observed by either the lose- or gain-of-functions mutations that are known. Clearly, Lrp6 plays an important role in embryonic skeletal patterning (Kokubu *et al.*, 2004; Pinson *et al.*, 2000), whereas Lrp5 seems to play little, if any, role in this aspect of development. Lrp5 seems to be mainly involved in the regulation of adult bone mass, whereas Lrp6 plays only a lesser role. Recently, the role of Lrp5 and the Wnt/β-catenin signaling pathway in the response of bone to mechanical loading has been firmly established. Based on our analysis of the phenotype of affected members of the HBM trait kindred we had speculated that the *LRP5* G171V mutation gene might somehow have altered the sensitivity of the skeleton to mechanical loading (Johnson *et al.*, 2002). Several lines of evidence now support a central role for Lrp5 and the Wnt/β-catenin signaling pathway in the response of bone/bone cells to mechanical loading. Sawakami *et al.* (2006) have shown that the bone-formation response after loading is reduced by 88% in male and 99% in female Lrp5$^{-/-}$ mice compared with wild-type loaded mice. Mice carrying the LRP5 cDNA containing the G171V mutation have increased sensitivity to mechanical loading (Cullen *et al.*, 2004) and these mice lose less bone in response to disuse than wild-type mice (Bex *et al.*, 2003). Changes in the expression of Wnt/β-catenin signaling pathway target genes has been shown to occur both *in vivo* in bone (Robinson *et al.*, 2006) and *in vitro* in bone cells (Lau *et al.*, 2006; Robinson *et al.*, 2006) in response to mechanical loading. Given that Lrp5 is absolutely required for the response of bone to mechanical loading, the question that next needs to be answered is where does Lrp5/Wnt/β-catenin signaling fit into the cellular cascade of events that occur in response to mechanical loading? A partial answer to this question will be discussed further later in this chapter. Another unanswered question is does Lrp6 play a role in the response to loading? At present, we have no definitive answer, but given the results of the ulna-loading studies performed with the Lrp5$^{-/-}$ mice and that these mice have normal alleles of Lrp6, it would seem that Lrp6 plays little or no role in mechanoresponsiveness in bone.

There are currently 10 known members of the frizzled family of proteins in humans and a homologous protein called smoothened that functions in hedgehog signaling. The frizzled proteins contain a cysteine-rich domain (CRD) at the amino terminus and seven transmembrane-spanning domains. The cysteine-rich domain is responsible for binding of Wnt ligands (Bhanot *et al.*, 1996). The frizzled proteins are generally thought to be coupled to trimeric G-proteins (Katanaev *et al.*, 2005; Sheldahl *et al.*, 1999). It also seems evident that different frizzleds regulate different intracellular signaling cascades. However, the specific combinations are not completely understood. The functioning of specific frizzleds in combination with Lrp5 or Lrp6 in the binding of specific Wnt proteins leads to activation of β-catenin signaling, but some frizzleds acting alone appear to be involved in the activation of three other Wnt signaling pathways. These other Wnt pathways are the planar cell polarity (PCP) (Mlodzik, 2002), the Wnt/Ca^{2+} involving protein kinase C (Kuhl *et al.*, 2000) and a protein kinase A pathway involved in muscle myogenesis (Chen *et al.*, 2005). Dishevelled also appears to be involved in the first two of these frizzled regulated pathways. It is still not clear which of the 10 different frizzleds are involved in the activation of each of the four Wnt pathways. However, it is known that some of the Wnt proteins only activate the β-catenin signaling pathway (e.g., Wnt1 and Wnt3A), whereas other Wnts only activate one of the other pathways (e.g., Wnt5A activates the Wnt/Ca^{2+} signaling pathway) (Miller, 2001).

The discovery that Lrp5/6/Arrow was also required for the activation of the Wnt/β-catenin signaling pathway suggested a model in which Lrp5/6/Arrow and frizzled function as coreceptors (Tamai *et al.*, 2000; Wehrli *et al.*, 2000). The role of Wnt was proposed to perhaps facilitate the association/interaction of the coreceptors and this was the key event leading to pathway activation. Several lines of evidence obtained from the construction of various fusion proteins suggest that this is most likely the case (Holmen *et al.*, 2002, 2005; Tolwinski *et al.*, 2003). Wnt binding and the formation of this ternary complex leads to a frizzled-dependent activation of Dishevelled (Dsh). However, the exact molecular mechanism mediating Dsh activation is not clear. Dsh phosphorylation appears to be one common feature in all of the signaling pathways through which it is known to act. The kinases casein kinase 1 (Peters *et al.*, 1999) and 2 (Willert *et al.*, 1997) and PAR-1 (Sun *et al.*, 2001) are the leading candidates for carrying out the phosphorylation of Dsh, which can occur through both Lrp5/6-dependent (leading to the β-catenin signaling pathway) and -independent mechanisms (Wnt binding to frizzled in the absence of Lrp5/6) (Gonzalez-Sancho *et al.*, 2004). Three different models of how frizzled and Dsh may interact have been proposed (He *et al.*, 2004a), but it is not clear which one functions in conjunction with Wnt and Lrp5/6/Arrow.

One might predict that loss-of-function mutations in the frizzled gene important in bone would lead to a reduction in bone mass similar to loss-of-function mutations in *LRP5*. Mutations in the frizzled family member, *FZD4*, as well as *LRP5* have been identified that result in Familial Exudative Vitreoretinopathy (FEVR), a hereditary disorder that results in blindness (Jiao *et al.*, 2004; Qin *et al.*, 2005; Robitaille *et al.*, 2002; Toomes *et al.*, 2004). In a recent study of patients with FEVR (Qin *et al.*, 2005) it was found that bone density, on average, was much lower in FEVR patients with *LRP5* mutations than in those with *FZD4* mutations. This study of FEVR patients suggests that possibly a different member of the frizzled family of proteins is important in bone mass versus FZD4, which seems to function more specifically perhaps in vision. Nine different frizzleds (Hens *et al.*, 2005) have been detected by RT-PCR in primary murine calvarial cells. However, calvarial cell populations are a heterogeneous mixture and so which frizzled is important in bone cell function and bone mass regulation is an unsolved question at this time.

Dishevelled, Glycogen Synthase Kinase-3β, Axin, and β-Catenin

Dishevelled (Dsh) serves an important intermediary function between frizzled and glycogen synthase kinase-3β in the Wnt/β-catenin signaling pathway. Dsh also serves as an intermediary in the Wnt/Ca^{2+} signaling pathway (Kuhl *et al.*, 2000) and the planar cell polarity (PCP) pathway (Mlodzik, 2002). The PCP pathway is responsible for the proper orientation of wing hairs and thoracic bristles in *Drosophila*. Mutants with hairs/bristles that were improperly oriented led to the identification of the *Frizzled* and *Dishevelled* genes [see review (Adler, 2002)]. Recent evidence suggests that Frizzled signals through the Gαo subunit in both the Wnt/β-catenin pathway and the PCP pathway (Katanaev *et al.*, 2005). Although it is not fully understood how Dsh becomes activated on Wnt binding and how it can discriminate between which pathway(s) to activate, a model has been proposed that suggests that differential intracellular localization of Dsh may be involved in determining some aspects of its function. Dsh associated with cytoplasmic vesicles has been proposed to be linked to signaling through the Wnt/β-catenin pathway, whereas Dsh associated with actin and the plasma membrane is proposed to signal via the PCP pathway (Capelluto *et al.*, 2002; Povelones and Nusse, 2002). It may also be that Frizzled localization, the specific Frizzled present in the cell, along with the distribution of downstream components and the specific Wnt ligand participating in the initial binding events, are critical as well (Bejsovec, 2005; Katoh, 2005).

The mechanism responsible for the activation of Dsh is not fully understood nor is the mechanism whereby Dsh acts known. There are four domains in Dsh that appear to be critical for its function. There is a DIX domain, which is also found in Axin, a PDZ domain, a conserved stretch of basic amino acids and a DEP domain that is found in other vertebrate proteins that interact with trimeric G-proteins (Cadigan and Nusse, 1997; He *et al.*, 2004a). Activation of Dsh occurs through phosphorylation and this appears to occur in the other Wnt signaling pathways that involve Dsh. The kinases casein kinase 1 (Peters *et al.*, 1999) and 2 (Willert *et al.*, 1997) and PAR-1 (Sun *et al.*, 2001) are the leading candidates to carry out the phosphorylation of Dsh. Gonzalez-Sanchez *et al.* (2004) have recently suggested that Frizzled-Dsh-Axin and Lrp5/6/Arrow-Axin associations occur in parallel and both are required for stabilization of β-catenin. However, much remains to be understood about the role of Dsh in Wnt/β-catenin signaling and in the other Wnt signaling pathways.

Glycogen synthase kinase-3 (GSK-3) has two main isoforms, α and β. Although most of the current evidence implicates GSK-3β in the control of intracellular levels of β-catenin, the GSK-3α isoforms may also play a role (Asuni *et al.*, 2006; McManus *et al.*, 2005). GSK-3β was first identified as playing a key role in the regulation of glycogen synthesis by phosphorylating (and thereby inhibiting) glycogen synthase [see review (Hardt and Sadoshima, 2002)]. GSK-3α and -β are themselves both inhibited by phosphorylation at an amino-terminal serine residue; serine-21 (α) (Sutherland and Cohen, 1994) or serine-9 (β) (Stambolic and Woodgett, 1994; Sutherland *et al.*, 1993). This phosphorylation is known to be catalyzed by a number of different kinases including MAP kinase (Sutherland and Cohen, 1994), protein kinase B (PKB)/Akt (Haq *et al.*, 2000), protein kinase C (Cook *et al.*, 1996), and protein kinase A (Fang *et al.*, 2000). Integrin-linked kinase also regulates GSK-3β, but apparently not through phosphorylation at serine-9 (Delcommenne *et al.*, 1998). Although there are many other regulators of GSK-3β (Hardt and Sadoshima, 2002), the kinases noted earlier all have described roles in bone and, as will be discussed later concerning the Wnt/β-catenin signaling pathway, suggests possible crosstalk between pathways.

The kinase activity of GSK-3β appears to be enhanced by a priming phosphorylation at nearby sites. In proteins such as Lrp5/6, where multiple GSK-3β phosphorylation sites are present in the cytoplasmic tail, then GSK-3β can self-prime. The initial priming phosphorylation of β-catenin needed for the subsequent GSK-3β phosphorylation is carried out by casein kinase I (CKI) (Liu *et al.*, 2002). GSK-3β has also been shown to phosphorylate two sites within Axin (T609 and S614), which is prerequisite for the binding of β-catenin (Jho *et al.*, 1999). It has been proposed that Wnt signaling leads to a dephosphorylation of these sites and this is part of the mechanism leading to the release of β-catenin from the degradation complex. In addition, protein phosphatase 2A (PP2A), which binds to Axin, APC, and Dsh (Hsu *et al.*, 1999; Seeling *et al.*, 1999; Yamamoto *et al.*, 2001) plays an important counterbalancing role opposing GSK-3β. When CK1 phosphorylates its substrates within the degradation complex PP2A dissociates, which favors further net phosphorylation and the release of β-catenin (Gao *et al.*, 2002). Phosphorylated β-catenin is then ubiquitinated and degraded by the 26S proteosome complex (Aberle *et al.*, 1997).

Axin acts as a scaffolding protein directly interacts/binds Dsh, GSK-3β, APC, PP2A, and β-catenin (Behrens *et al.*, 1998; Fagotto *et al.*, 1999; Farr *et al.*, 2000; Hart *et al.*, 1998; Ikeda *et al.*, 1998; Itoh *et al.*, 1998; Nakamura *et al.*, 1998; Sakanaka *et al.*, 1998; Yamamoto *et al.*, 1998). Axin was first identified as the product of the mouse fused locus (Zeng *et al.*, 1997). In vertebrates there are two forms of Axin: Axin 1, which is constitutively expressed and is the main form of the degradation complex, and Axin 2 (also known as Conductin). Axin 2 expression is induced by Wnt signaling and functions as a negative feedback inhibitor of β-catenin signaling (Jho *et al.*, 2002). Axin has several functional domains required for its interactions with the various other proteins of the degradation complex. It appears that amino acids 581 to 616 are responsible for binding β-catenin, whereas the RGS domain interacts with APC, and GSK-3β appears to bind to amino acids 444 to 543 (Nakamura *et al.*, 1998), although GSK-3β was observed only when β-catenin was present. The Armadillo repeats in β-catenin mediate the interaction with Axin, whereas a Dix domain similar to the one found in Dsh likely mediates the interactions between Axin and Dsh. Mutations in the Human AXIN2 gene have been linked to familial tooth agenesis and are predisposing for colon cancer (Lammi *et al.*, 2004).

The collapse of the degradation complex and the release of β-catenin is a complex cascade of poorly understood events. Wnt binding to the coreceptors induces Axin to bind to the cytoplasmic tail of Lrp5/6/Arrow (after the coreceptor is phosphorylated and FRAT-1 binds). When Wnt is not present, β-catenin is bound to APC and APC to Axin as a consequence of specific phosphorylations mediated by GSK-3β (and other kinases). In the presence of Wnt and the activation of Dsh, these phosphorylation events are inhibited and binding of Axin to the cytoplasmic tail of Lrp5/6/Arrow leads to collapse of the complex and release of β-catenin into the cytoplasm.

β-Catenin is highly homologous to the *Drosophila* segment polarity gene *armadillo* (McCrea *et al.*, 1991). The human β-catenin gene (*CTNNB*) is located on Cs 3p21 and encodes a protein of 781 amino acids (~85 kDa). Two pools of β-catenin exist within the cell; one associated with E-cadherin and the other in the cytoplasm (Nelson and Nusse, 2004). β-Catenin contains a cassette of 12 Armadillo repeats in the middle of the protein that are the interaction sites with E-cadherin, APC, and the nuclear transcription factors (Cadigan and Nusse, 1997; Willert and Nusse, 1998). A number of mutations in β-catenin have been identified that give rise to human cancers. The amino-terminal end of the protein (mainly between amino acids 29 and 49) contains key residues whose phosphorylation by GSK-3β plays a critical role in the subsequent degradation of the protein, and when one or more of these residues are mutated, the protein is more stable and the increased signaling results in tumor formation/growth (Polakis, 2000; Yost *et al.*, 1996). Exactly how β-catenin is translocated from the cytoplasm into the nucleus is not fully understood.

Transcriptional Regulation by β-Catenin

Once inside the nucleus, β-catenin binds to the TCF/LEF family of transcription factors and regulates the expression of a large (and ever increasing) list of target genes. Recently, it has been shown that β-catenin can also interact with the FOXO family of transcriptional regulators and that this association is particularly important during oxidative stress (Essers *et al.*, 2005), which may play a role in age-associated bone loss (Almeida *et al.*, 2006). A consensus TCF/LEF core sequence has been identified that is required for binding of the TCF/LEF proteins [CCTTTGATC] (Korinek *et al.*, 1997). The TCF/LEF proteins have DNA-binding ability but require the transactivating domain of β-catenin (at the C-terminal end) to regulate transcription.

A large number of proteins (see http://www.stanford.edu/~rnusse/ for a detailed listing) participate in the transcriptional regulation mediated by β-catenin. The current model for how β-catenin regulates target gene transcription involves the formation of a larger complex of proteins that induces a change in chromatin structure (Barker *et al.*, 2001). In *Drosophila* when Wnt is not present, TCF acts as a repressor of Wnt/Wg target genes by forming a complex with Groucho (Cavallo *et al.*, 1998), whose repressing ability is regulated by the histone deacetylase enzyme, Rpd3 (Chen *et al.*, 1999). Studies in *Xenopus* have shown that β-catenin interacts with the acetyltransferases p300 and CBP to activate the *siamois* gene promoter (Hecht *et al.*, 2000). Other proteins that control β-catenin signaling include the antagonist Chibby that binds to the C-terminal end (Takemura *et al.*, 2003) and the protein ICAT (Tago *et al.*, 2000) that negatively regulates the interaction between β-catenin and TCF-4. TCF/LEF can also be phosphorylated by the MAP kinase-related protein NLK/Nemo (Ishitani *et al.*, 2003), which reduces the affinity for β-catenin. Considerably less is known about the interaction of β-catenin with the FOXO family of transcription factors, but presumably it also involves the cooperative and repressive interactions with a number of proteins. Also, it is not understood how choices are made as to which interactions are favored under any given set of cellular circumstances.

There are a large number of known Wnt/β-catenin target genes. Interestingly, many of these targets are components of the Wnt signaling pathway (Logan and Nusse, 2004) (see http://www.stanford.edu/~rnusse/ for a more complete listing), which creates a complicated set of feedback loops that can potentially serve to further amplify or inhibit signaling through the pathway. Some of these target genes will be discussed further in the following sections as they play important roles in bone.

Wnt SIGNALING AND BONE CELL FUNCTION

Understanding how the Wnt/β-catenin signaling pathway is regulated and what the role of the pathway in skeletal biology is (are) still unsolved questions, despite considerable progress during the past 5 years. We now know that Wnt/β-catenin signaling regulates osteogenesis through multiple mechanisms. At the level of the mesenchymal precursor cell, Wnts suppress the differentiation pathways that lead to chondrocytes or adipocytes (Day *et al.*, 2005; Hill *et al.*, 2005; Ross *et al.*, 2000). In the case of adipogenesis versus osteoblastogenesis, Wnts work in favor of the osteoblast lineage through blocking the induction of transcription factors such as C/EBPα and PPARγ (Ross *et al.*, 2000). Secreted Frizzled Related Protein-1 (sFRP1) (see discussion following) has been identified as an important negative regulator of Wnt/β-catenin signaling and the progression of chondrocyte differentiation induced by BMP2 (Gaur *et al.*, 2006). Wnt/β-catenin signaling has been shown to promote osteogenesis by direct stimulation of *Runx2* gene expression (Gaur *et al.*, 2005). Activation of Wnt/β-catenin signaling also promotes further osteoblast cell proliferation and mineralization activity, reduces osteoblast apoptosis, and can suppress osteoclast differentiation induced by osteoblasts (Glass *et al.*, 2005) through regulating the expression of OPG (Simonet *et al.*, 1997).

Much of our understanding of the role of Wnt/β-catenin signaling in the control of skeletal development and bone cell differentiation, proliferation, apoptosis, and function is derived from studies of mutations that gave rise to abnormal skeletal phenotypes in both humans and animal models. For example, the Lrp6 knockout mouse (Pinson *et al.*, 2000) displays many of the skeletal patterning defects that were observed in the mice carrying Wnt gene mutations (Greco *et al.*, 1996; Takada *et al.*, 1994; Yoshikawa *et al.*, 1997) and these observations provided the first clue about the role of the Wnt/β-catenin signaling pathway in skeletal development. Subsequently, the identification of mutations in *LRP5* that gave rise to OPPG (Gong *et al.*, 2001) or increased bone mass (Little *et al.*, 2002; Boyden *et al.*, 2002; Van Wesenbeeck *et al.*, 2003) in human kindreds implicated Wnt/β-catenin signaling in the control of bone mass. The development of Lrp5 and Lrp6 knockout (Kato *et al.*, 2002; Kokubu *et al.*, 2004) and HBM (*LRP5*^{G171V}) transgenic mice (Babij *et al.*, 2003) has also helped to further define the mechanisms by which the pathway regulates bone cell functions. Gong *et al.* (2001) provided initial evidence that LRP5 regulates osteoblast differentiation and proliferation and this was confirmed by studies in the Lrp5$^{-/-}$ mouse (Kato *et al.*, 2002). Adding to the complexity of the affects of Wnt/β-catenin signaling pathway on bone cell activities is the finding that osteoclasts express high levels of LRP6, instead of LRP5 (Spencer *et al.*, 2006), which these investigators suggest raises the possibility that LRP6-mediated signaling may regulate some of the catabolic effects of Wnt/β-catenin signaling in opposition to the anabolic effects mediated by LRP5.

Lrp5$^{-/-}$ mice have a 50% reduction in mineral apposition rate (MAR) (Kato *et al.*, 2002). This could be because of a reduction in the ability of the osteoblast to mineralize or reduced numbers of osteoblasts. Because Lrp5$^{-/-}$ mice have reduced osteoblast numbers in their long bones, which was correlated with a 50% decrease in calvarial osteoblast proliferation, and osteoblast apoptosis and differentiation were not altered, this suggests that the reduced MAR in these mice is likely caused solely by the reduction in osteoblast proliferation rather than a functional deficit in the osteoblast. The HBM transgenic mouse display decreased osteoblast and osteocyte apoptosis (Babij *et al.*, 2003), which also argues in favor of increased osteoblast number as the underlying mechanism behind the increased bone mineral density and strength (Akhter *et al.*, 2004) observed in these mice. As discussed previously, the role of Lrp6 in bone cell function is less understood, but clearly some increment of bone mass is being contributed by the pathway through activation of this coreceptor. In both the Lrp5$^{-/-}$ and HBM transgenic mice osteoclast number appeared to be unaffected. Thus, Wnt/β-catenin signaling clearly plays an important role in osteoblast differentiation, proliferation, and apoptosis.

Activity through the Wnt/β-catenin signaling pathway is controlled by a number of regulatory proteins and these are clearly involved in the regulation of bone cell function. The Dickkopf (Dkk) family of proteins (Krupnik *et al.*, 1999), designated Dkk1, 2, 3, and 4, play important roles in development, bone disease, cancer, and Alzheimer's disease (Niehrs, 2006). Dkks 1, 2, and 4 have been shown to bind to Lrp5/6 (Bafico *et al.*, 2001; Mao *et al.*, 2001; Semënov *et al.*, 2001) and another protein called Kremen (Mao *et al.*, 2002), which is a single-pass transmembrane protein. Formation of the Dkk-Lrp5/6-Kremen complex results in internalization of Lrp5/6 and its subsequent degradation (Mao and Niehrs, 2003; Mao *et al.*, 2002; Rothbacher and Lemaire, 2002). Thus, Dkk removes functional Lrp5/6 from the cell surface. The *LRP5*^{G171V} mutation was initially shown to alter the ability of DKK1 to inhibit the pathway (Boyden *et al.*, 2002). *In vitro*, Dkk1 overexpression has been shown to decrease osteoblast differentiation and maturation, which is Runx2 dependent, and conversely loss of a single *Dkk1* allele in mice results in a high-bone-mass phenotype (Morvan *et al.*, 2006). Deletion of Dkk-2 resulted in decreased osteoblast terminal differentiation and reduced mineralization (Li *et al.*, 2005a). Thus both Dkk1 and Dkk2 play roles in bone formation.

Dkk1 has been shown to be overexpressed in patients who develop lytic bone lesions associated with multiple myeloma (Tian *et al.*, 2003). Overexpression of Dkk1 by myeloma cells is proposed to inhibit osteoblast differentiation and reduced viability of osteoblast stem cells, which in combination with increased RANKL production by the myeloma cells would favor osteoclastogenesis and the development of the lytic bone lesions (Glass *et al.*, 2003; Tian *et al.*, 2003). TNF-α has been shown to regulate Dkk1 as a mechanism for maintaining the equilibrium between bone formation and bone resorption that is disrupted in various joint diseases (Diarra *et al.*, 2007).

Emerging data suggest that the protein product of the *SOST* gene called sclerostin (Brunkow *et al.*, 2001) is an important inhibitor of the Wnt/β-catenin signaling pathway in bone. Sclerostin is produced by osteocytes (Poole *et al.*, 2005; van Bezooijen *et al.*, 2005). The Lrp5^{G171V} mutation has also been shown to interfere with the ability of sclerostin to bind Lrp5 and thereby inhibit Wnt/β-catenin signaling (Ellies *et al.*, 2006; Semenov and He, 2006). The high levels of sclerostin produced by osteocytes implies that the Wnt/β-catenin signaling pathway is held in the "off" position in bone and therefore activation of the pathway requires either a by-pass of the inhibition of Lrp5/6 by sclerostin or a decrease in the levels of sclerostin, thereby permitting Wnt ligand-mediated activation of the pathway. In this regard, it has been proposed that PTH stimulation of bone formation involves a reduction in Sost gene expression (Bellido *et al.*, 2005; Keller and Kneissel, 2005). Mechanical loading has also been shown to decrease sclerostin levels in bone (Robling *et al.*, 2006). Reduction of sclerostin levels by use of a neutralizing antibody has been shown to result in increased bone mass in rats and monkeys, further supporting the concept that the pathway is held in the "off" position by sclerostin and that removal of this "brake" will lead to increased bone formation (Ominsky *et al.*, 2006a, 2006b). This identifies sclerostin as another potential target for the design of anabolic therapies aimed at manipulating the Wnt/β-catenin signaling pathway (see further discussion in subsequent text).

Another class of extracellular regulatory molecules are the secreted frizzled related proteins (sFRPs). The sFRPs have a cysteine rich domain that is highly homologous to the Wnt-binding domain of the frizzled coreceptor and are capable of binding Wnts (Rattner *et al.*, 1997).

sFRPs can act as competitive inhibitors of Wnt-mediated activation of the Wnt/β-catenin pathway (Moon *et al.*, 1997). sFRP-1 has been shown to bind RANKL ligand and therefore is capable of acting as a decoy receptor like OPG and inhibit osteoclastogenesis (Hausler *et al.*, 2001). Studies with a human osteoblast cell line (HOB) demonstrated that sFRP-1 is a negative regulator of osteoblast and osteocyte survival (Mount *et al.*, 2006). Deletion of sFRP-1 results in an increased bone mass phenotype, but unlike the HBM transgenic mice, the effects of sFRP-1 deletion are restricted to trabecular bone and are not manifest in cortical bone (Bodine *et al.*, 2004b). sFRP-1$^{-/-}$ mice have decreased osteoblast and osteocyte apoptosis and increase osteoblast proliferation and differentiation. Unlike the Lrp5$^{-/-}$ mice, the sFRP-1$^{-/-}$ mice had elevated expression of *Runx2* mRNA (Gaur *et al.*, 2005). Deletion of sFRP-1 also has effects on chondrogenesis and endochondral bone formation (Gaur *et al.*, 2006).

Activation of the Wnt/β-catenin signaling pathway leads to changes in the expression of a number of genes. The protein products of many of these genes can act as feedback regulators of the pathway. For example, activation of this pathway is linked to new bone formation in response to mechanical loading, which results in changes in the expression of a number of β-catenin target genes such as Wnt10b, Wnt 1, Wnt 3a, Axin, WISP2, sFRP1, and sFRP4 (Lau *et al.*, 2006; Robinson *et al.*, 2006). Whereas the Wnts would function as stimulators of the pathway, the sFRPs and Axin could serve to inhibit the pathway. Thus, activation of the pathway in response to loading initiates feedback loops that can further activate or inhibit the pathway.

Given this large repertoire of modulators understanding how the pathway is regulated in a coordinated and temporal fashion by these multiple regulators it is somewhat remarkable that mutations in any one of these modulators can have such a dramatic effect on the skeleton. Clearly, we have much more to understand about the complex interplay of the various proteins that regulate the Wnt/β-catenin signaling pathway in bone cells. The function of these regulators of the pathway clearly is often restricted to some aspect of bone cell function and how this occurs is not understood. For example, loss of Axin2 results in increased osteoblast proliferation and differentiation (Yu *et al.*, 2005). However, osteoblast apoptosis was not altered in these mice. In the HBM transgenic mice (Babij *et al.*, 2003), the G171V mutation was associated with decreased osteoblast and osteocyte apoptosis. The G171V mutation has also been shown to reduce the ability of sclerostin (Ellies *et al.*, 2006; Li *et al.*, 2005b; Semenov *et al.*, 2005) and Dkk1 (Boyden *et al.*, 2002) to bind and inhibit Lrp5. It is not known how or whether these might be connected. At still another level, the Wise protein has been shown to be a context-specific activator or inhibitor of Wnt/β-catenin signaling and seems to be more important during embryonic skeletal development (Itasaki *et al.*, 2003). Thus, the picture that is emerging is that different modulators of the pathway may selectively regulate different aspects of bone cell biology and/or specific bone cell types and function during different developmental windows.

INTERACTIONS BETWEEN Wnt/β-CATENIN SIGNALING AND OTHER PATHWAYS IMPORTANT IN BONE MASS REGULATION

The Wnt/β-catenin signaling pathway is not the only pathway that is known to be important in the regulation of bone cell activities and considerable effort is now being focused on understanding how all of these various pathways interact to regulate bone mass. As already discussed, GSK-3β is the critical intracellular enzyme controlling β-catenin levels. Therefore any pathway that can regulate GSK-3β activity has the potential to interact with the Wnt/β-catenin signaling pathway independent of the Lrp5/6-Frizzled coreceptors and any modulators that function at that level.

One set of the well known pathways that have the potential to crosstalk with the Wnt/β-catenin signaling pathway are those pathways that signal through Akt/PKB. Akt/PKB is activated by phosphatidylinositol-3-kinase whose activity is opposed by the PTEN tumor suppressor (Datta *et al.*, 1999; Kandel and Hay, 1999). Akt/PKB has been shown to be stimulated by Wnt and in association with Dsh to phosphorylate GSK-3β (Fukumoto *et al.*, 2001). This raises the potential for several growth factors to potentially crosstalk with the Wnt/β-catenin signaling pathway. Recently it has been shown that the growth of colon cancer cells induced by PGE_2 is partially the result of activation of the Wnt/β-catenin pathway through a dual mechanism involving Akt-mediated phosphorylation of GSK-3β and the $G\alpha_s$ subunit trimeric G-proteins associated with the PGE_2 EP2 receptor binding to axin and thereby promoting dissociation of the degradation complex (Castellone *et al.*, 2005). PGE_2 release/production in bone cells is a well known early response to mechanical loading. Fluid flow shear stress has been shown to increase the phosphorylation of both Akt and GSK-3β in osteoblasts (Norvell *et al.*, 2004). Our laboratory has presented preliminary evidence that in response to fluid flow shear stress there is an initial activation of the β-catenin signaling that is Lrp5 independent and likely mediated through crosstalk with PGE_2 signaling (Kamel *et al.*, 2006). We are currently testing a model in which this initial activation results in a feedback amplification loop that functions at the level of Lrp5 and leads to new bone formation. When Lrp5 is not present, then the amplification loop does not occur and no new bone formation can occur as was observed in the loading studies in the Lrp5$^{-/-}$ mouse (Sawakami *et al.*, 2006). It has also been shown that integrin-linked kinase (ILK) can regulate Akt/PKB and GSK-3β (Delcommenne *et al.*, 1998), which potentially connects the important role of integrins in the response of bone cells to mechanical loading (Pavalko *et al.*, 1998; Wozniak *et al.*, 2000) with the Wnt/β-catenin signaling pathway through a crosstalk mechanism.

Additionally, signaling pathways regulate the expression of activators or inhibitors (discussed previously) of the Wnt/β-catenin pathway and thereby interact at this level. For example, results of studies with PTH suggest that PTH acts in part through a complementary pathway and not entirely through the Lrp5 Wnt/β-catenin signaling pathway. Continuous PTH treatment of rats *in vivo* or UMR 106 cells in culture results in a downregulation of *Lrp5* and *Dkk1* and an upregulation of *Lrp6* and *Frz*-1 (Kulkarni *et al.*, 2005) and, based on these studies, it was suggested that the effects of PTH are in part mediated by a cAMP-PKA pathway. In studies with the sFRP-1 knockout mouse, it was concluded that PTH and Wnt signaling may share some common components, but PTH action appears to extend beyond the Wnt pathway (Bodine *et al.*, 2004a). However, studies with the Lrp5$^{-/-}$ mouse have shown that Lrp5 is not required for the bone anabolic effects of PTH on bone (Iwaniec *et al.*, 2004; Sawakami *et al.*, 2006). Recently, evidence has been presented that PTH infusion in mice decreases *Sost* mRNA expression and sclerostin levels in osteocytes (Bellido *et al.*, 2005). This finding implies a negative feedback control mechanism in which sclerostin production by osteocytes opposes the actions of Wnts and/or BMPs on osteoblast precursors and PTH by decreasing sclerostin levels indirectly, therefore stimulating osteoblast differentiation and bone formation. Thus the picture that emerges with PTH is complex.

Likewise, several groups have examined potential crosstalk with the BMP signaling pathway and Wnt/β-catenin signaling (Gaur *et al.*, 2006; He *et al.*, 2004b; Liu *et al.*, 2006; Nakashima *et al.*, 2005; Rawadi *et al.*, 2003; Tian *et al.*, 2005; van den Brink, 2004). One model that has been proposed (Liu *et al.*, 2006) for crosstalk between these two pathways in bone marrow stromal cells involves a Dsh-Smad1 interaction in the unstimulated state that becomes disrupted when Wnt is present. When both Wnt and BMP-2 are present the phosphorylation of Smad1 stabilizes the interaction and thereby inhibits Wnt/β-catenin signaling (Liu *et al.*, 2006). In intestinal crypt stem cell self-renewal, BMP inhibitory effects have been proposed to be mediated through suppression of Wnt/β-catenin signaling through a mechanism involving PTEN, PI3-K, and Akt (He *et al.*, 2004b). In bone it has been proposed that BMP-2 increases the expression of Wnt proteins that then activates the Wnt/β-catenin signaling pathway in an autocrine/paracrine feedback loop (Rawadi *et al.*, 2003). It has also been proposed that Wnts induce the expression of BMPs and this induction is necessary for expression of alkaline phosphatase (Winkler *et al.*, 2005). This model also implicated sclerostin as imposing a level of control preferentially at the level of BMP signaling versus Wnt signaling. However, there is now clear evidence for sclerostin being a negative regulator of the Wnt/β-catenin signaling through binding to Lrp5 (Ellies *et al.*, 2006; Semenov and He, 2006). Thus, although it is clear that these two pathways are somehow intertwined, the exact nature of their interaction(s) is not yet fully understood.

As mentioned previously there is now compelling evidence for the involvement of Lrp5 and the Wnt/β-catenin signaling pathway in the formation of new bone induced by mechanical loading of bone. Evidence also indicates that the LRP5^{G171V} mutation may also protect against bone loss related to disuse, but that bone loss owing to estrogen withdrawal is not affected by this mutation (Bex *et al.*, 2003). However, it has been shown that the estrogen receptor, ERα, and β-catenin can/do form a functional interaction and potentially regulate gene expression (Kousmenko *et al.*, 2004). Recently, it has also been suggested that in bone there is a convergence of the ER, kinases, BMP, and Wnt signaling pathways that regulates the differentiation of osteoblasts (Kousteni *et al.*, 2007).

What other pathways might also interact with the Wnt/β-catenin signaling pathway? At present this is an open question, but it is likely that only by understanding the complex interplay of these various pathways will the control, differentiation, proliferation, and function of bone cells be fully appreciated.

THE Wnt SIGNALING PATHWAY AS A TARGET FOR ANABOLIC THERAPY IN BONE

Given the major role of Wnt/β-catenin signaling in skeletal development and adult bone mass regulation it is not surprising that considerable attention is being focused on using key components of the pathway as targets for new drug development (Baron and Rawadi, 2007; Janssens *et al.*, 2006). At the same time we must be cognizant that misregulation of the pathway can lead to numerous diseases (Johnson and Rajamannan, 2006; Moon *et al.*, 2002; Nusse, 2005; Polakis, 2000; Prunier, Hocevar *et al.*, 2004). What hope exists that a bone-specific anabolic agent can be developed without undesirable side effects? Some evidence already exists in the literature that this might be possible, but considerably more study is needed. Another question is what is the best target to focus on for drug development? The majority of pharmaceutical agents target either receptors or specific enzymes. In the Wnt/β-catenin pathway the obvious targets in this regard are the coreceptors Lrp5 and frizzled and GSK-3β, which is the key enzyme controlling intracellular β-catenin levels.

The LRP5^{G171V} mutation results in a high-bone-mass phenotype and a skeleton that is resistant to fracture (Johnson *et al.*, 1997). The affected members of this kindred seem to have no other health consequences related to the presence of this mutation, although in a second kindred with the LRP5^{G171V} mutation affected members all had torus lesions in the oral cavity to varying degrees (Boyden *et al.*, 2002). It has also been reported that some mutations in *LRP5* may be associated with pathology (Whyte *et al.*, 2004). The findings that this mutation is associated with increased sensitivity to mechanical loading (Johnson *et al.*, 2002; Robinson *et al.*, 2006) suggests a potentially novel pharmaceutical approach. Perhaps one strategy would be to use a pharmaceutical agent that can alter regulation of the pathway (mimicking the effect of the LRP^{G171V} mutation) in combination with an exercise regimen to produce an optimal bone anabolic effect. It might even be possible to use such a combination-therapy approach with a drug dose that by itself has little consequence on bone mass and thus perhaps avoid undesirable side effects. One approach that is currently being tested with success is the use of anti-sclerostin antibodies to overcome the inhibitory effects of sclerostin on Lrp5 (Ominsky *et al.*, 2006a, 2006b). Other inhibitors of the pathway such as Dkk1 (Wang *et al.*, 2007; Yaccoby *et al.*, 2007) and members of the sFRP family (Bodine *et al.*, 2004b; Nakanishi *et al.*, 2006) are potential targets.

Regulation of GSK-3β activity has already been reported to produce a bone anabolic effect. LiCl treatment, which inhibits GSK-3β, restores bone mass in the Lrp5$^{-/-}$ mouse (Clement-Lacroix *et al.*, 2005) and in human users of lithium there appears to be a decreased fracture risk for any fracture and a trend for decreased risk of osteoporotic fractures of the spine and wrist (Colles' fracture) (Vestergaard *et al.*, 2005). GSK-3β inhibitors have also been shown to produce a bone anabolic effect *in vivo* and increase expression of bone formation markers and induce osteoblast cell differentiation *in vitro* (Kulkarni *et al.*, 2006; Robinson *et al.*, 2006). In one study changes in gene expression profile that were obtained from treatment with these agents was virtually identical to changes observed in the HBMtg mouse bones, in bone cells after mechanical loading, and in primary bone cells from affected members of HBM kindred (Robinson *et al.*, 2006). What these studies suggest is that as we learn more about how the Wnt/β-catenin signaling pathway functions in bone, it will be possible to design better and more specific pharmaceutical agents and approaches.

Given the crosstalk between other signaling pathways and the Wnt/β-catenin signaling pathway, it may be that, as we understand more about the nature of these complex interactions, new targets will be revealed. PTH has been proposed to decrease levels of sclerostin produced by osteocytes and thus releases the inhibition of osteoblast differentiation (Bellido *et al.*, 2005). Could it be possible to regulate *Sost* gene expression through other means and thereby influence a bone anabolic effect? BMPs appear to induce the expression of Wnts (and perhaps vice versa) and so understanding what regulates Wnt gene expression in bone may provide a key to new pharmaceutical approaches. The result of β-catenin nuclear translocation is a change in expression of a number of genes, many of which function in a feedback loop as inhibitors or activators of the pathway. As we understand more about the nature of these genes/proteins and how they are regulated it may be possible to design drugs that can alter the activity of these proteins and thereby tip the balance between bone formation and bone resorption in favor of formation and either increase bone mass or prevent bone loss.

Although many of these ideas may be considered fanciful speculation at this point, if we have learned anything in the past 5 years since the discovery of the mutations in *LRP5* that give rise to low and high bone mass, it may simply be that the discoveries of the next 5 years may be even more enlightening in terms of our understanding of the role of Wnt/β-catenin signaling in bone.

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Chapter 7

SOST/Sclerostin: An Osteocyte-Derived Inhibitor of Bone Formation that Antagonizes Canonical Wnt Signaling

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INTRODUCTION

Over the past few years, there has been mounting interest in the pathophysiology of the rare sclerosing bone disorders sclerosteosis, van Buchem disease, and high-bone-mass (HBM) phenotype as human models of an imbalance in bone remodeling in favor of increased bone formation.

Identification of the molecular defects at the source of these sclerosing disorders has led to the recognition of the critical role of the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) and of canonical Wnt (Wingless and INT-1) signaling in the regulation of bone formation. Increased bone formation occurs as the result of inactivating mutations in the SOST gene in sclerosteosis and as a result of a large genomic deletion downstream of this gene in van Buchem disease, respectively. HBM phenotype is caused by activating mutations in LRP5 that inhibit the binding and function of antagonists to canonical Wnt signaling, such as dickkopf-1 (Dkk1) and/or the SOST gene protein sclerostin. The known genetic defects in SOST and LRP5 could not be documented, however, in a number of patients reported to have a very similar phenotype, suggesting further as yet unknown mutations in the Wnt signaling pathway.

In this chapter, we focus on two of the known sclerosing disorders: sclerosteosis and van Buchem disease with specific emphasis on the associated genetic defects. We describe the restricted expression pattern and regulation of the SOST gene product sclerostin and the molecular mechanism by which it acts as a negative regulator of bone formation. Finally, we address the possible clinical implications that the discovery of this protein may have for the management of patients with osteoporosis.

SCLEROSTEOSIS AND VAN BUCHEM DISEASE

Sclerosteosis (OMIM 269500) and van Buchem disease (OMIM 239100) are two rare sclerosing bone disorders, first described in the 1950s as distinct clinical entities despite their closely related phenotypes (Truswell, 1958; van Buchem *et al.*, 1955). Sclerosteosis has been reported mainly in Afrikaners of Dutch descent living in South Africa. Although the diagnosis has been established so far in only 70 patients (H. Hamersma, personal communication), the estimated carrier rate among Afrikaners is high, being one in one hundred individuals (Beighton *et al.*, 2004; Hamersma *et al.*, 2003). The vast majority of the patients with a diagnosis of van Buchem disease come from the small isolated fishing village on an island in the IJsselmeer (former Zuiderzee) Urk in The Netherlands. The population remained isolated for centuries until the island was connected to the main land by land reclamation in 1939. About twenty patients have been diagnosed so far, the majority of whom are descendants of a couple who married in 1751 (Vanhoenacker *et al.*, 2003). The carrier rate of van Buchem disease has not been investigated. A few affected individuals and families with the sclerosteosis or van Buchem disease phenotype have been reported in other parts of the world, including Spain, Brazil, United States, Germany, Japan, Switzerland, and Senegal (Hamersma *et al.*, 2003).

The skeletal manifestations of sclerosteosis and van Buchem disease are the result of endosteal hyperostosis and are characterized by progressive generalized osteosclerosis

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(Beighton *et al.*, 1984; Beighton, 1988; Hamersma *et al.*, 2003; van Bezooijen *et al.*, 2005). The manifestations are most pronounced in mandible and skull, with characteristic enlargement of the jaw and facial bones leading to facial distortion, entrapment of cranial nerves often associated with facial palsy, and also eventually potential hearing loss, loss of smell, and increased intracranial pressure. Patients with sclerosteosis present, however, with the additional clinical features of syndactyly, have overall the worst prognosis. Sudden death owing to impaction of the brainstem in the foramen magnum has been described so far only in patients with sclerosteosis (Beighton, 1988; van Bezooijen *et al.*, 2005).

Biochemical and histological data suggest that bone formation is dramatically increased in both sclerosteosis and van Buchem disease (Hill *et al.*, 1986; Stein *et al.*, 1983; van Bezooijen *et al.*, 2004; Wergedal *et al.*, 2003). The newly laid bone is lamellar with no mineralization defect. Data on bone resorption are limited, however, and increase, decrease, or no change in bone resorption has been variably reported in the few patients studied. Bone is overall of very good quality as suggested by increased calculated bone volume and polar moment of inertia in patients with van Buchem disease (Wergedal *et al.*, 2003), increased bone mineral density (BMD) in patients with sclerosteosis (Gardner *et al.*, 2005), and no reported fractures in either disease (Beighton *et al.*, 2004).

Genetic Defects in Sclerosteosis and van Buchem Disease

The search for the gene defect that leads to sclerosteosis succeeded in 2001 with two groups simultaneously identifying mutations in a new gene that was named SOST (sclerosteosis) (Balemans *et al.*, 2001; Brunkow *et al.*, 2001). The SOST gene consists of two exons located at chromosome 17q12-21 and encodes for the protein sclerostin. The coding sequence consists of 642 base pairs that translate into a 213-amino-acid protein of which the first 23 amino acid residues provide a signal peptide for secretion (Fig. 1). Five mutations of the SOST gene have been identified so far in patients with sclerosteosis: the first introduces a premature termination codon in exon 1 (C70T/Gln24X), the second and third introduce premature termination codons in exon 2 (G372A/Trp124X and C376T/Arg126X), and the last two consist of base substitutions in the intron at positions +1 (G→C) and +3 (A→T), resulting in improper splicing of the coded message (Balemans *et al.*, 2001, 2005; Brunkow *et al.*, 2001).

None of these mutations in the SOST gene could be identified in the Dutch patients with van Buchem disease, but the same two groups did identify a 52-kb deletion 35 kb downstream of the SOST gene in these patients (Balemans *et al.*, 2002; Staehling-Hampton *et al.*, 2002). The deleted region was later found to contain regulatory elements for SOST transcription, thus explaining its ability to induce a phenotype closely resembling the sclerosteosis of South African patients, although of a less severe form and with no syndactyly. Genetic differences between the Dutch and South African populations and an incomplete absence of sclerostin in patients with van Buchem disease may explain the difference in severity of clinical symptoms between sclerosteosis and van Buchem disease.

Identification of the different genetic defects underlying the pathophysiology of the endosteal hyperosteosis in

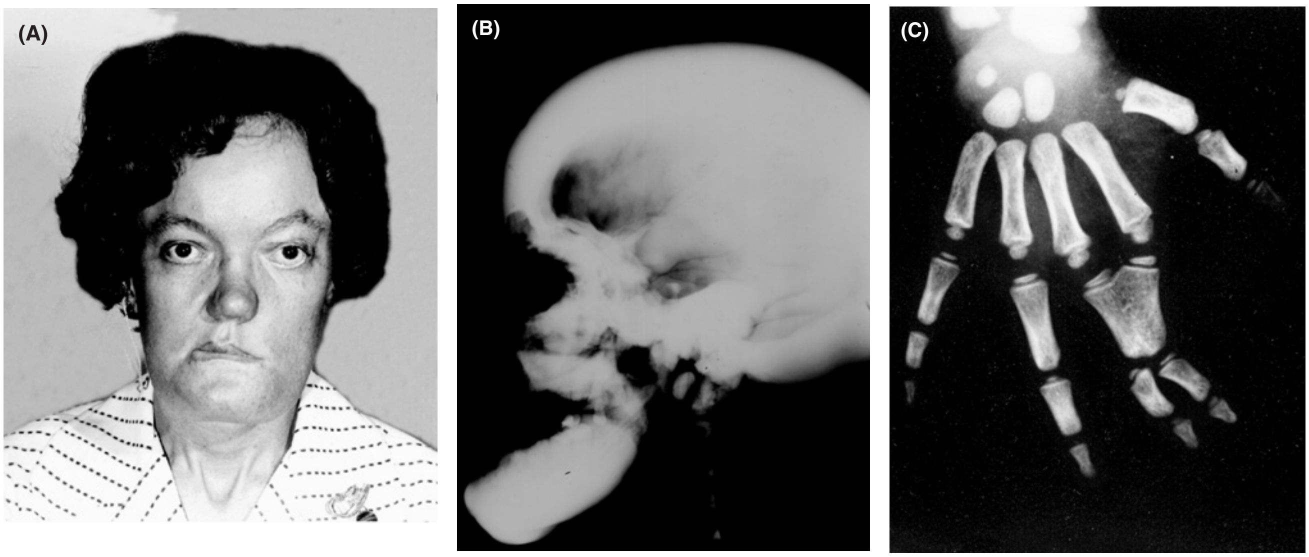

FIGURE 1 Clinical manifestations of sclerosteosis. (**A**) Patient with sclerosteosis with characteristic mandibular overgrowth, facial palsy, tall forehead, and deafness. (**B**) X-ray of the skull of an adult patient with sclerosteosis showing thickening of the skull and mandible. (**C**) X-ray of the hand of a child with sclerosteosis showing syndactyly of the second and third finger (Reprinted from van Bezooijen *et al.*, SOST/sclerostin, an osteocyte-derived negative regulator of bone formation. Cytokine & Growth Factor Reviews 2005 **16**(3) pp. 319–327 with permission from Elsevier).

sclerosteosis, van Buchem disease, and HBM phenotype (mutations in SOST, the van Buchem deletion, and mutations in LRP5, respectively), calls for a reclassification of previously identified patients on the basis of their genetic profile. For example, a patient previously described as having van Buchem disease was found to have a mutation in LRP5 (Van Wesenbeeck *et al.*, 2003), belonging thus to the HBM phenotype group.

SOST/Sclerostin Expression

SOST mRNA is expressed in many tissues, especially during embryogenesis, whereas sclerostin protein is expressed only postnatally in terminally differentiated cells embedded within a mineralized matrix, i.e., osteocytes, mineralized hypertrophic chondrocytes, and cementocytes.

SOST/Sclerostin Expression in Nonmineralized Tissues

SOST mRNA expression can be detected in several tissues other than bone during embryogenesis. Embryonic day 10.5 (E10) of mouse development is the earliest time point for detecting SOST expression that can be demonstrated in the otic vesicle (Ellies *et al.*, 2006). SOST mRNA expression is also found at E13 in the peridigital or interdigital regions of limb buds and it has been speculated that this may be implicated in the pathogenesis of syndactyly in patients with sclerosteosis (Ohyama *et al.*, 2004). A role for SOST in digit formation is further supported by abnormal digit formation in mice overexpressing SOST under its own promoter (Loots *et al.*, 2005).

From E15.5 up to the neonatal period, SOST is expressed within the medial vessel wall of the great arteries containing smooth muscle cells, more specifically in that of the ascending aorta, aortic arch, brachiocephalic artery, common carotids, and pulmonary trunk (van Bezooijen *et al.*, 2007b). In adult mice, however, SOST expression is no longer detected in the great arteries, suggesting that SOST expression is limited to the embryonic and early postnatal stages of cardiovascular development. In contrast, SOST/sclerostin remains expressed in mineralized bone well into adulthood (van Bezooijen *et al.*, 2004; Bellido *et al.*, 2005; Silvestrini *et al.*, 2007). In humans, SOST is expressed in heart and aorta as evaluated by real-time PCR analysis (Balemans *et al.*, 2001; Brunkow *et al.*, 2001). Our group could not detect, however, sclerostin expression in valves or great arteries of human adults (R. L. van Bezooijen, S. E. Papapoulos, N. A. T. Hamdy, and C. W. G. M. Löwik, unpublished observations). In a recent whole-genome expression profiling study, SOST mRNA expression was found to be downregulated in patients with abdominal aortic aneurysms compared with controls (Lenk *et al.*, 2007). Furthermore, high SOST mRNA levels have been reported in human kidney (Balemans *et al.*, 2001; Brunkow *et al.*, 2001) and SOST mRNA expression is expressed in mouse kidney (R. L. van Bezooijen *et al.*, unpublished observations). We did not find, however, any sclerostin protein in the few human renal biopsy specimens analyzed so far, including one previously reported to express SOST mRNA in glomeruli by *in situ* hybridization (Balemans and Van Hul, 2002). SOST mRNA expression has also been reported in fetal and adult human liver biopsies (Brunkow *et al.*, 2001; Kusu *et al.*, 2003). Patients with sclerosteosis or van Buchem disease do not demonstrate kidney abnormalities or clinical or radiological evidence for cardiovascular abnormalities (Hamersma *et al.*, 2003).

SOST/Sclerostin Expression in Mineralized Tissues

In vitro studies of SOST/sclerostin expression are technically difficult because of the current inability to isolate osteocytes from mammalian bones, with osteogenic cell cultures that form mineralized bone nodules being the only available method of generating osteocyte-like cells *in vitro* (Pockwinse *et al.*, 1992). In mouse primary osteogenic bone marrow and mouse mesenchymal KS483 cell cultures, SOST mRNA expression is induced at low levels after onset of bone nodule mineralization. This suggests that SOST is only expressed by a few cells, possibly osteocytes, within the bone nodules (van Bezooijen *et al.*, 2004). However, in mouse primary osteoblastic cultures of embryonic calvaria-derived cells, SOST is already expressed in undifferentiated cells and expression increases with differentiation (Ohyama *et al.*, 2004). In human primary osteogenic cultures that do not form bone nodules, SOST expression has been detected at the undifferentiated stage and increased upon mineralization (Balemans *et al.*, 2001; Sutherland *et al.*, 2004; van Bezooijen *et al.*, 2004; Winkler *et al.*, 2003).

SOST expression in undifferentiated osteoblastic cell lines is cell line dependent and may vary from absent/near absent (C2C12, C3H10T1/2, KS483, MC3T3-E1, OP9, ROS17/2.8, MG63, and TE85 cells) to moderate (Saos-2 and U2-OS cells) or high (UMR106 cells) (Keller and Kneissel, 2005; van Bezooijen *et al.*, 2004; Sevetson *et al.*, 2004). In the murine long-bone osteocyte (MLO) cell lines, reported to represent different stages of osteocyte differentiation (Kato *et al.*, 2001), SOST expression is variable and different from the observed induction of SOST expression upon mineralization. In MLO-Y4 cells that represent mature osteocytes in a mineralized matrix, SOST expression was either low (Bellido *et al.*, 2005) or absent (Keller and Kneissel, 2005), whereas in MLO-A5 cells that represent preosteocytes in osteoid, SOST mRNA levels were high (Bellido *et al.*, 2005).

During mouse embryogenesis, SOST mRNA is expressed in mineralized bones (Ellies *et al.*, 2006; Kusu *et al.*, 2003; van Bezooijen *et al.*, 2004; Winkler *et al.*, 2003; Ohyama *et al.*, 2004). At this early stage of bone development, it is difficult to determine whether these cells are mineralizing osteoblasts or osteocytes. Sclerostin protein expression has not been reported at the embryonic stage, and we were unable to detect it using several monoclonal antibodies known to act on adult bone tissue (R. L. van Bezooijen *et al.*, unpublished observations). In contrast to the previously described expression of SOST mRNA at the embryonic stage, the earliest time point at which it was possible to detect sclerostin protein expression in mice was 5 days postnatally. In human embryonic bone, sclerostin expression could be detected in osteocytes, but the validity of these data is questionable because samples tested were from embryos with accelerated endochondral and intramembranous ossification in Blomstrand lethal osteochondrodysplasia. In normal human bone, the earliest detection of sclerostin expression so far is in osteocytes of a toe bone biopsy of a one-year-old boy (van Bezooijen *et al.*, 2005). In adult mouse and human bone, sclerostin expression is restricted to osteocytes with diffuse staining, representing dendrites in osteocytic canaliculi (Bellido *et al.*, 2005; Poole *et al.*, 2005; van Bezooijen *et al.*, 2004; Winkler *et al.*, 2003). Similar to the regulation of SOST mRNA *in vitro*, newly embedded osteocytes within unmineralized osteoid do not express sclerostin, but become positive for the protein at, or shortly after, primary mineralization (Poole *et al.*, 2005). The majority of osteocytes in mineralized cortical and cancellous bone are positive for sclerostin, and sclerostin-negative cells are located significantly closer to the surface than sclerostin-positive cells. Osteoclasts, osteoblasts, and bone lining cells do not express sclerostin. As expected, sclerostin is not expressed by osteocytes in bone biopsies of patients with sclerosteosis (van Bezooijen *et al.*, 2004). In addition, no sclerostin expression was found in bone biopsies from patients with van Buchem disease, supporting the notion of the presence of essential regulatory elements for sclerostin expression in bone within the deleted van Buchem region (van Bezooijen *et al.*, 2007a).

During mouse embryogenesis, SOST expression is also observed in odontoblasts that give rise to alveolar bone (Ellies *et al.*, 2006). In contrast, sclerostin expression is restricted to cementocytes in teeth of adult mice and humans (van Bezooijen *et al.*, 2007a). Sclerostin expression was absent in teeth of two patients with van Buchem disease and only a low signal was found in a third patient. It is possible, therefore, that sclerostin is involved in a similar negative feedback mechanism in teeth as in bone and that the protein inhibits cementum formation by cementoblasts. Lack of sclerostin may in turn result in hypercementosis and in a tighter junction between teeth and alveolar bone. No overt changes in cementum thickness were observed, however, by plain radiology of the jaw in patients with sclerosteosis or van Buchem disease.

At birth (P0), SOST mRNA expression can be detected in hypertrophic chondrocytes in mice (Ellies *et al.*, 2006). In human bone, sclerostin protein expression was found in hypertrophic chondrocytes (Winkler *et al.*, 2003), more specifically in mineralized hypertrophic chondrocytes in the growth plate (R. L. van Bezooijen *et al.*, unpublished observations), but it was absent in articular cartilage (van Bezooijen *et al.*, 2004). Although the role of sclerostin in the growth plate remains as yet to be elucidated, it may be responsible for a similar negative feedback mechanism on chondrocyte differentiation and function as on that of osteoblasts. Were this to be confirmed, the lack of sclerostin expression in mineralized hypertrophic chondrocytes may explain the reported tall stature observed in patients with sclerosteosis. Because tall stature is not a feature of Dutch patients with van Buchem disease, it would be very interesting, although technically difficult, to determine whether sclerostin is expressed in the growth plates of these patients.

MECHANISM OF ACTION OF SCLEROSTIN

Sclerostin Inhibits Bone Formation

In patients with sclerosteosis the combination of high bone mass owing to increased bone formation with premature termination codons in the SOST gene suggested an inhibitory effect of the gene product sclerostin on bone formation. Indeed, addition of exogenous sclerostin to osteogenic cultures inhibited proliferation and differentiation of mouse and human osteoblastic cells (Fig. 2) (Sutherland *et al.*, 2004; van Bezooijen *et al.*, 2004; Winkler *et al.*, 2003). It has also been shown that sclerostin may limit the life span of osteoblasts by stimulating their apoptosis (Sutherland *et al.*, 2004). Overexpression of sclerostin, using either the osteocalcin promoter or BAC recombination, induced osteopenia in mice by suppressing bone formation whereas bone resorption was reported to be unaffected (Loots *et al.*, 2005; Winkler *et al.*, 2003).

Sclerostin is a Member of the DAN Family of Glycoproteins

The amino acid sequence of sclerostin contains eight conserved cysteine and one glycine residue suggesting a cystine knot structure (Avsian-Kretchmer and Hsueh, 2004; Balemans *et al.*, 2001; Brunkow *et al.*, 2001; van Bezooijen *et al.*, 2005). Based on the finding of characteristic cysteine and glycine spacing, sclerostin belongs to the DAN (differential screening-selected gene aberrant in neuroblastoma) family of glycoproteins. This family consists of a group of secreted proteins that include wise, cerberus, DAN, coco, caronte, gremlin, dante, and protein related to DAN and cerberus (PRDC), sharing the ability to antagonize bone morphogenetic protein (BMP) activity. Of the BMP antagonists described so far, chordin and noggin have been shown to antagonize BMP signaling by blocking binding of BMPs to their receptors (Piccolo *et al.*, 1999;

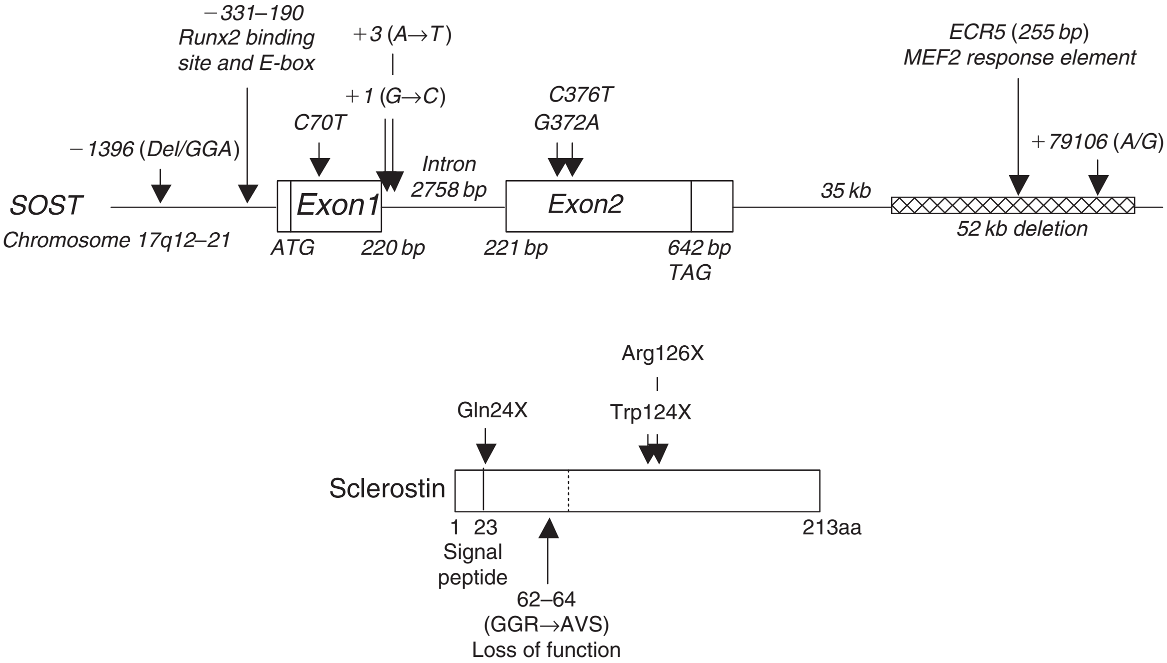

FIGURE 2 Schematic representation of SOST gene and sclerostin protein. The SOST gene is located at chromosome 17q12-21 and consists of 2 exons. In patients with sclerosteosis, three mutations (C70T, G372A, and C376T) have been found in the exons and two mutations (+1 G→C and +3 A→T) in the intron. In Dutch patients with van Buchem disease a 52-kb deletion is found 35 kb downstream of the SOST gene. Two polymorphisms surrounding the SOST gene have been associated with BMD (−1396 del/GGA and +79106 A/G). Regulatory elements of transcription have been identified upstream of the SOST gene (−331–190) and within the region deleted in van Buchem disease (ECR5). MEF2 transcription factors have been shown to bind and stimulate activity of this element. The SOST gene encodes for a 213-amino-acid protein of which the first 23 residues provide a signal peptide for secretion. The three mutations within the exons result in a premature stop of translation at Gln24X, Trp124X, and Arg126X. Amino acids 62–64 are essential for sclerostin function.

Zimmerman *et al.*, 1996). A similar mechanism has been proposed for members of the DAN family.

BMPs are secreted cytokines, originally identified by their ability to induce ectopic bone and cartilage formation (Urist, 1965). BMPs exert their effects through two distinct types of serine/threonine kinase receptors, i.e., type I and type II receptors (Canalis *et al.*, 2003; van Bezooijen *et al.*, 2005). Although having a relative low binding affinity for BMPs, sclerostin was shown to antagonize BMP-stimulated bone formation *in vitro* and *in vivo* (Kusu *et al.*, 2003; van Bezooijen *et al.*, 2004, 2007c; Winkler *et al.*, 2003). The mechanism by which sclerostin antagonizes BMP-stimulated bone formation is different from that of classical BMP antagonists, because it neither antagonizes early BMP responses such as Smad1/5/8 phosphorylation and BMP reporter construct activation nor inhibits stimulation of direct BMP target genes in several osteoblastic cell lines (van Bezooijen *et al.*, 2004, 2007c).

Sclerostin Antagonizes LRP5/6-Mediated Canonical Wnt Signaling

At the turn of the century, human genetic studies provided compelling evidence for a role of gain-of-function mutations in LRP5 in patients with the HBM phenotype (Boyden *et al.*, 2002; Little *et al.*, 2002) and loss-of-function mutations in LRP5 in the osteoporosis pseudoglioma syndrome (OPPG) (Gong *et al.*, 2001). LRP5 is a coreceptor of the canonical Wnt signaling pathway that has been shown to play a pivotal role in bone formation (Baron *et al.*, 2006; Glass and Karsenty, 2007). Wnts are secreted cytokines with pivotal roles in a variety of cellular activities, including cell fate determination, proliferation, migration, polarity, and differentiation (Clevers, 2006). Wnts are distinguished into Wnts that involve β-catenin signaling (canonical pathway) and Wnts that do not (noncanonical pathway). The canonical pathway involves the formation of complexes of Wnts with Frizzled receptors and coreceptors LRP5/6. Upon stimulation, the intracellular signaling molecule β-catenin accumulates and translocates into the nucleus, where it initiates transcription of target genes via complex formation with TCF/Lef1 transcription factors (Fig. 3). Conversely, in the absence of Wnt ligand, β-catenin forms a complex with the tumor suppressor proteins APC and Axin, and the kinases glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1), which facilitates phosphorylation and proteosomal degradation of β-catenin.

Wnt signaling is extracellularly regulated by antagonists of which Dkk1 is the best characterized. Dkk1 exerts its

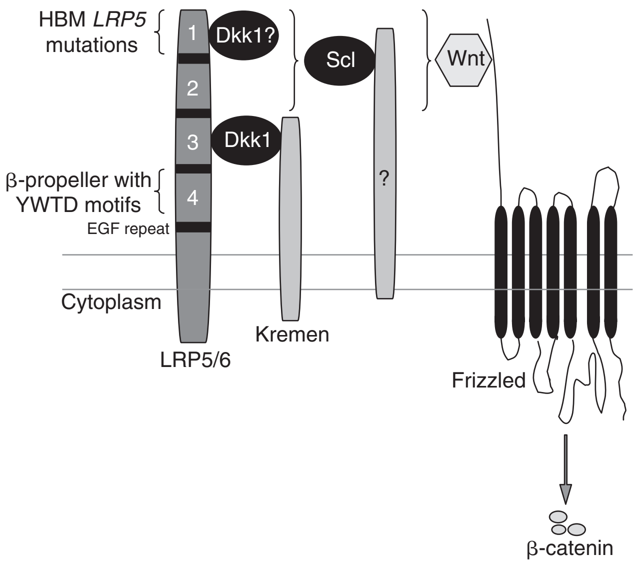

FIGURE 3 Schematic model of antagonized canonical Wnt signaling. Canonical Wnt signaling involves the formation of complexes of Wnts with Frizzled receptors and LRP5/6 coreceptors, resulting in the accumulation of β-catenin in the cytoplasm and translocation into the nucleus. The antagonist Dkk1 inhibits canonical Wnt signaling by the formation of complexes with LRP5/6 and Kremen, resulting in the removal of LRP5/6 from the membrane. Dkk1 binds to the third and probably also the first β-propeller of LRP5/6. The antagonist sclerostin inhibits canonical Wnt signaling by binding to probably the first β-propeller of LRP5/6. Whether sclerostin requires a cofactor like Kremen for Dkk1 to exert its antagonistic effect remains to be established.

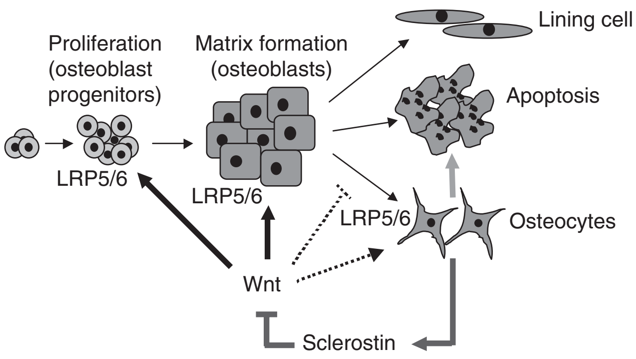
Lining cell
Proliferation (osteoblast progenitors)
Matrix formation (osteoblasts)
Apoptosis
LRP5/6
LRP5/6
LRP5/6
Osteocytes
Wnt
Sclerostin

FIGURE 4 Simplified scheme of the inhibitory effect of sclerostin on Wnt-mediated osteoblast development and osteoblast survival. Sclerostin produced and secreted by osteocytes antagonizes LRP5/6-mediated Wnt signaling and inhibits, thereby, the stimulatory effect of Wnts on osteoblast development. In addition, sclerostin may stimulate osteoblast apoptosis.

antagonistic effect by forming a complex with Kremen and LRP5/6 that triggers clearance of these Wnt coreceptors from the membrane by endocytosis (Fig. 4) (Mao *et al.*, 2002). The extracellular part of LRP5/6 consists of four β-propellers that contain YWTD motifs and are separated by EGF-like repeats. The third β-propeller of LRP5/6 was found to be crucial for binding to and for the inhibition by Dkk-1 (Mao *et al.*, 2001; Zhang *et al.*, 2004). However, all ten currently described mutations in patients with HBM are located within the first β-propeller of LRP5 (Balemans and Van Hul, 2007) and have been shown to exhibit reduced binding to and decreased inhibition by Dkk-1, suggesting an additional role for this region in Dkk-1 function (Li *et al.*, 2005; Balemans *et al.*, 2007; Ai *et al.*, 2005; Boyden *et al.*, 2002; Zhang *et al.*, 2004; Bhat *et al.*, 2007).

Three members of the DAN family, i.e., *Xenopus* cerberus, coco, and wise, have been found to antagonize Wnt activity (Bell *et al.*, 2003; Itasaki *et al.*, 2003; Piccolo *et al.*, 1999). Of these antagonists, wise (also known as USAG-1 and ectodin) has the highest amino acid similarity (38%) with sclerostin (Avsian-Kretchmer and Hsueh, 2004). Both genes are missing in the fly and the nematode and a single orthologue is found in *Fugu rubripes* and *Ciona intestinalis*. Both proteins lack a cysteine residue, present in other DAN family members, which is potentially involved in dimerization. Wise appears to be a context-dependent inhibitor and activator of Wnt signaling (Itasaki *et al.*, 2003). In the absence of Wnts, wise binds to the coreceptor LRP6 and may act as a weak agonist to Wnt signaling. In the presence of Wnts, wise competes, however, with Wnt for binding to LRP6 and antagonizes Wnt signaling.

Sclerostin also binds to LRP5/6 and antagonizes Wnt1, Wnt3, Wnt3a, Wnt6, and Wnt10b-stimulated activation of canonical Wnt reporter constructs in human embryonic kidney and mouse osteoblastic cell lines (see Fig. 4) (Li *et al.*, 2005; Semenov *et al.*, 2005; van Bezooijen *et al.*, 2007c; Ellies *et al.*, 2006; Semenov and He, 2006). It was reported that sclerostin did not antagonize Wnt3A-induced β-catenin stabilization in mouse mesenchymal C3H10T1/2 cells (Winkler *et al.*, 2005). We found, however, that sclerostin overexpression decreased Wnt-induced β-catenin nuclear translocation in human embryonic kidney cells (R. L. van Bezooijen *et al.*, unpublished observation). *In vivo*, SOST overexpression inhibits Wnt8-induced duplication of the body axis, and expression of the direct target genes siamois and Xnr3 in *Xenopus* embryos (Ellies *et al.*, 2006; Semenov *et al.*, 2005).

Sclerostin binds to the first two β-propeller domains of LRP5/6 that include the HBM LRP5 mutations (see Fig. 4) (Li *et al.*, 2005; Semenov *et al.*, 2005). Subsequent studies have demonstrated that the binding of sclerostin to some of these HBM LRP5 mutations (G171V, D111Y, R154M, N198S, A214T, and T253I) and the analogous change to LRP5G171V in LRP6 (G158V) is decreased, leading to decreased inhibition of canonical Wnt signaling (Ellies *et al.*, 2006; Semenov and He 2006; Li *et al.*, 2005).

The mechanism by which sclerostin binding to LRP5/6 antagonizes Wnt signaling is intriguing. Although sclerostin and Wnts both bind to the first two β-propellers of LRP5/6, neither appears to compete for LRP5/6 binding (Li *et al.*, 2005). In a Wnt reporter assay, site-directed mutagenesis of amino acids 62–64 of sclerostin by similarly charged amino acids (GGR → AVS) reduced binding of sclerostin to LRP6 and reduced its inhibitory effect, demonstrating the importance of this conserved domain for the function of sclerostin (see Fig. 1) (Ellies *et al.*, 2006). Whether sclerostin requires a coreceptor like Kremen for Dkk1 to induce internalization of LRP5/6 remains to be established. It is, nevertheless, attractive to speculate that mutations in such a cofactor may explain the sclerosteosis-like phenotype in patients who do not demonstrate mutations in SOST or in conserved surrounding regions or in patients who do not have a deletion of the van Buchem region {Balemans, 2007; R. L. van Bezooijen *et al.*, unpublished observations}.

LRP5-mediated canonical Wnt signaling occurs in a wide range of tissues. Nevertheless, patients with activating mutations in LRP5 have a phenotype that is restricted to the skeleton. This can be explained by decreased binding to LRP5 by the antagonist sclerostin of which expression is largely restricted to bone in adults. However, it is currently unclear how decreased binding of the widely expressed antagonist Dkk1 could result in such a restricted skeletal phenotype.

REGULATION OF SOST/SCLEROSTIN EXPRESSION

Regulation of SOST/Sclerostin Expression by Local and Systemic Factors

Osterix and runx-2 are two essential transcription factors in osteoblast differentiation that have been shown to stimulate SOST expression. In primary mouse osteoblastic cultures, osterix expression was found to precede SOST induction and to colocalize in the limb buds, calvaria, and mandibles of mouse embryos, and RNAi silencing of osterix expression downregulated SOST expression (Ohyama *et al.*, 2004). This effect may have been indirect via inhibition of osteoblastic differentiation, because osteocalcin expression was also downregulated. BMPs, BMP-2, 4, and 6, induce SOST expression in mouse and human osteogenic cells, suggesting a protective mechanism to prevent excessive bone formation owing to BMP stimulation (Ohyama *et al.*, 2004; Sutherland *et al.*, 2004).

Parathyroid hormone (PTH) is currently the only available therapy able to stimulate bone formation in patients with osteoporosis (Neer *et al.*, 2001; Shoback, 2007). Although its anabolic effect is well established, the molecular mechanism responsible for this action is not yet fully understood. PTH has recently been shown to inhibit SOST/sclerostin expression *in vitro* and *in vivo*. *In vitro*, PTH decreased SOST transcription within 4 hours and this was not affected by the protein synthesis inhibitor cycloheximide (Keller and Kneissel, 2005; Bellido *et al.*, 2005). SOST mRNA degradation was not affected by PTH. PTH-inhibited SOST expression may involve the cAMP pathway, because addition of the cAMP inducer forskolin also decreased SOST expression. This downregulation is probably not regulated at the proximal SOST promoter site, but involves inhibition of a response element for myocyte enhancer factor 2 (MEF2) transcription factors within the van Buchem deletion (see Fig. 1) (Leupin *et al.*, 2007).

Interpretation of *in vivo* data is more complicated. Consistent with the hypothesis that PTH stimulates bone formation by inhibiting sclerostin expression, daily injections of PTH decreased SOST/sclerostin expression in mice and rats (Keller and Kneissel, 2005; Silvestrini *et al.*, 2007). However, daily PTH injections for 4 days did no affect SOST/sclerostin expression in another study in mice, but a bone catabolic schedule of 4 days of continuous PTH infusion did (Bellido *et al.*, 2005). This difference in response may be because of differences in the time points at which SOST/sclerostin expression analysis was performed, i.e., 15 minutes and 24 hours after the last injection.

Continuous PTH excess and intermittent elevated PTH levels both increase osteoblast (and osteoclast) numbers, but are associated with bone catabolism and anabolism, respectively. SOST/sclerostin downregulation may thus be involved in the stimulation of bone formation in both conditions, whereas other effects of PTH may determine the effect of the hormone on bone resorption. The balance between all these effects will eventually determine the absolute effect on bone mass. Further studies are required to elucidate the precise role of sclerostin in the bone-forming action of PTH.

1,25-Dihydroxyvitamin D3 and glucocorticoids are two other systemic factors that may affect SOST expression. In the human osteosarcoma cell line Saos-2, endogenously expressed SOST is increased by the addition of 1,25-dihydroxyvitamin D3 (Sevetson *et al.*, 2004). In human mesenchymal stem cells, 1,25-dihydroxyvitamin D3 and retinoic acid further stimulated BMP-induced SOST expression (Sutherland *et al.*, 2004). In contrast, dexamethasone abolishes SOST expression in these cells. Because dexamethasone is required to induce human osteogenic cells to differentiate into mature mineralizing cells, this may be another explanation for the low levels of SOST expression in these cultures.

The location of osteocytes and their extensive connected cellular network renders these cells ideal for sensing and translating mechanical signals into cellular adaptive responses (Knothe Tate *et al.*, 2004; Han *et al.*, 2004). Sclerostin's specific expression in osteocytes and its negative regulatory effect on bone formation renders it an attractive candidate for a mechanically modulated osteocyte-derived factor that translates mechanical signals into cellular responses. Preliminary data indicate that mechanical loading decreases both the number and the staining intensity of sclerostin-positive osteocytes in the ulnar diaphysis of mice (Robling *et al.*, 2006). This suggests that stimulation of bone formation by mechanical loading may involve downregulation of sclerostin expression in osteocytes.

SOST Promoter and Regulatory Elements

Alignment of human and mouse SOST nucleotide sequences showed a more than 50% homology within the first 2000 bp upstream of the SOST gene, and this region was found to drive transcription in human and rat osteosarcoma in human embryonic kidney cell lines (Loots *et al.*, 2005; Sevetson *et al.*, 2004). A 142-bp element was identified within this region between nucleotides −331 and −190 relative to the initiation methionine of the SOST coding gene, which was enough to drive SOST transcription in human osteosarcoma Saos-2 cells (see Fig. 1) (Sevetson *et al.*, 2004). Within this element, a runx-2-binding site was essential for its transcriptional activity and runx-2 overexpression stimulated it. In addition, an E-box motif within this element was involved in its transcriptional activity and MyoD overexpression may stimulate it. A C/EBP consensus site, also located within this region, did not appear to regulate transcription. This juxtaposition of Cbfa-1, E-box, and C/EBP within the SOST promoter was suggested to have an intriguing resemblance with the osteocalcin promoter. Osteocalcin is also differentially expressed in the course of osteoblastic differentiation and its deficiency in knockout mice is associated with high bone mass (Ducy *et al.*, 1996).

Analysis of BAC SOST transgenic mice lacking the van Buchem region showed that this region contains regulatory elements essential for SOST expression in bone (Loots *et al.*, 2005). More detailed analysis by stringent alignment between human and mouse sequences (= 80% identity over a 200-bp window) identified seven evolutionarily conserved regions within the van Buchem deletion. One of these regions (ECR5) stimulated transcription specifically in osteoblastic cells and in the skeleton of E14.5 mouse embryos (see Fig. 1). Existence of key regulatory elements within the van Buchem deletion is further supported by the presence of a polymorphism within this region (SRP9, +79106; A/G) that is associated with BMD in elderly men (Uitterlinden *et al.*, 2004). This polymorphism is not within a human–mouse conserved region, but may be in linkage disequilibrium with ECR5. In elderly women, another polymorphism associated with BMD (SRP3, −1396;Del/GGA) is located within the direct upstream promoter region of the SOST gene.

SCLEROSTIN FUNCTION IN BONE MODELING AND REMODELING

Two processes are responsible for construction and reconstruction of the skeleton throughout life, bone modeling and remodeling, respectively. In the process of modeling, bones are shaped and reshaped by the independent actions of osteoblasts and osteoclasts during skeletal development or in response to changes in mechanical loading and aging in adults. Bone remodeling is the process that enables constant renewal of the skeleton. It begins *in utero* and continues throughout life. In this process, bone resorption by osteoclasts and formation by osteoblasts are tightly coupled within a basic multicellular unit (BMU) in which distinct phases of activation, resorption, reversal, and formation can be distinguished.

In the process of remodeling, bone lining cells may be involved in the initiation of bone resorption (Teitelbaum, 2000). The limited data available on patients with sclerostin deficiency indicate, however, that bone resorption is not consistently altered (Hill *et al.*, 1986; Stein *et al.*, 1983; Wergedal *et al.*, 2003), suggesting that an effect of sclerostin on bone resorption is unlikely. It is conceivable that induction of sclerostin expression by newly embedded osteocytes at the onset of mineralization of osteoid may serve as a negative feedback mechanism that would inhibit bone formation by osteoblasts and prevent overfilling of the BMU and uncontrolled bone growth (Fig. 5A) (Poole *et al.*, 2005; van Bezooijen *et al.*, 2004). In this case, absence or downregulation of sclerostin would increase osteoblast numbers and/or prolong the active bone-forming phase of osteoblasts and thereby increase the amount of bone formed.

In the process of bone modeling, it could be hypothesized that sclerostin expression by osteocytes would keep bone lining cells in a state of quiescence (Poole *et al.*, 2005) and may thereby prevent initiation of *de novo* bone

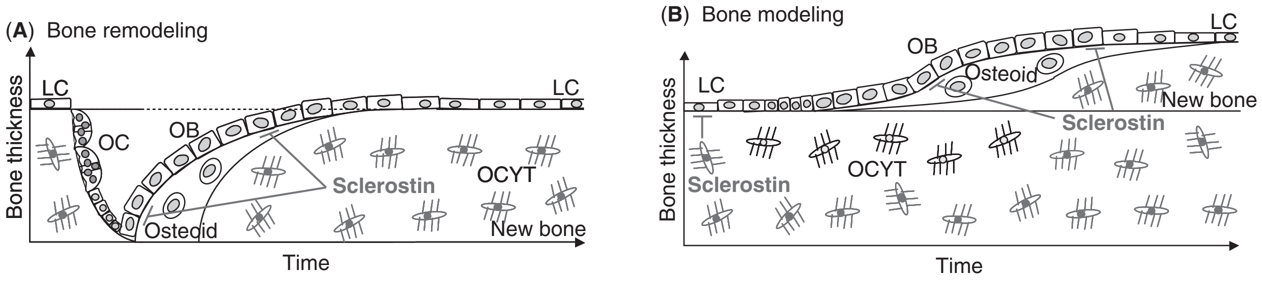

FIGURE 5 Schematic model of the mechanism of action of sclerostin in bone remodeling and modeling. (**A**) In remodeling, sclerostin produced and secreted by newly embedded osteocytes may be transported to the bone surface where it inhibits osteoblastic bone formation and prevents overfilling of the BMU. (**B**) In modeling, sclerostin may serve two actions. First, it may keep bone lining cells in a state of quiescence and prevent, thereby, initiation of *de novo* bone formation. In addition, sclerostin produced and secreted by newly embedded osteocytes may inhibit osteoblastic bone formation similarly to a BMU.

formation (see Fig. 5B). In this model, downregulation of sclerostin expression in osteocytes would induce activation of osteoblasts and stimulate bone formation without previous bone resorption. In addition, sclerostin expressed by osteocytes embedded in new bone formed by bone modeling may demonstrate a similar negative feedback mechanism on bone formation as in a BMU.

Sclerostin produced and secreted by osteocytes may be transported to the bone surface via the canaliculi where it may antagonize LRP5/6 mediated canonical Wnt signaling in osteoblasts, thereby, inhibiting Wnt-stimulated bone formation. Alternatively, sclerostin may antagonize canonical Wnt signaling in osteocytes and regulate another signal that is transported to osteoblasts and inhibits bone formation. In support of this latter view is the observation that Wnt signaling is present in osteocytes in bones of embryonic and adult mice (Hens *et al.*, 2005).

Of the canonical Wnts so far reported to be antagonized by sclerostin, Wnt1, Wnt3, Wnt3a, Wnt6, and Wnt10b, only the last one is expressed in bone. Other Wnts reported to be expressed in bone are Wnt4, Wnt7b, and Wnt9a (formally Wnt14), but the effect of sclerostin on these Wnts is unknown. Interestingly, Wnt10b overexpression, using the adipocyte-specific FABP4 promoter, increased bone mass in mice, whereas mice deficient in Wnt10b had a lower bone mass (Bennett *et al.*, 2005).

Mechanical loading and PTH therapy, two important stimulatory pathways for bone formation, have both been reported to mediate their bone anabolic effect, at least in part, by downregulating SOST/sclerostin expression in osteocytes (Bellido *et al.*, 2005; Keller and Kneissel, 2005; Robling *et al.*, 2006; Silvestrini *et al.*, 2007). Both pathways have been shown to affect bone modeling and remodeling and could, therefore, stimulate bone formation by downregulating sclerostin expression (see Fig. 5A and B).

RELATIONSHIP BETWEEN INHIBITION OF CANONICAL WNT SIGNALING AND THE CLINICAL SPECTRUM OF ENDOSTEAL HYPEROSTOSIS DISORDERS

HBM phenotype is an autosomal dominant condition that resembles sclerosteosis and van Buchem disease and is characterized by increased bone mass owing to enhanced bone formation (Johnson *et al.*, 1997). Although sclerosteosis, van Buchem disease, and the HBM phenotype have similar bone phenotypes, two distinct molecular mechanisms, increased BMP and Wnt signaling, were initially thought to be involved in the pathogenesis of these disorders. The recent discovery that sclerostin antagonizes Wnt signaling rather than BMP signaling suggests, however, that these skeletal disorders are more likely to be caused by increased activity of one and the same signaling pathway, i.e., the canonical Wnt signaling pathway. In the HBM phenotype, mutations within the first propeller of LRP5 result in increased canonical Wnt signaling, resulting in increased bone formation. Whether this results from the decreased ability of Dkk1 and/or sclerostin to inhibit canonical Wnt signaling is as yet unclear. In sclerosteosis and van Buchem disease, absence of sclerostin may result in increased LPR5/6-mediated Wnt signaling and increased bone formation.

The difference in the severity of clinical features between sclerosteosis, van Buchem disease, and HBM phenotype suggests a possible relationship with the degree of inhibition of canonical Wnt signaling (Fig. 6). Within the Afrikaner population in South Africa heterozygote carriers of sclerosteosis have BMD values consistently higher than healthy subjects but have no skeletal manifestations of hyperostosis (Gardner *et al.*, 2005). This suggests that loss of one functional SOST allele may be sufficient to lead to increased canonical Wnt signaling, resulting in increased bone formation and bone mass. Patients with the HBM phenotype have increased bone mass and variable symptomatology, ranging from complete absence of symptoms and signs to jaw enlargement, torus palatinus, cranial nerve entrapment, craniosynostosis, and developmental delay (Balemans and Van Hul, 2007; Levasseur *et al.*, 2005). This variance may be related to the extent by which mutations in LPR5 may affect the negative control of canonical Wnt signaling. Absence of sclerostin staining in bone biopsies of patients with van Buchem disease (van Bezooijen *et al.*, 2007a) and the essential role of the van Buchem region in sclerostin expression in bone (Loots *et al.*, 2005) suggest that in these patients increased bone formation is caused by increased LRP5-mediated Wnt signaling resulting from absence of the canonical Wnt antagonist sclerostin. Sclerostin also binds to LRP6, the other coreceptor involved in canonical Wnt signaling. Absence of sclerostin may, therefore, increase canonical Wnt signaling mediated through both coreceptors. Patients with sclerosteosis have the most severe phenotype. This may be because of the differences in genetic background between patients with van Buchem disease and sclerosteosis, but it may also be because of residual sclerostin in patients with

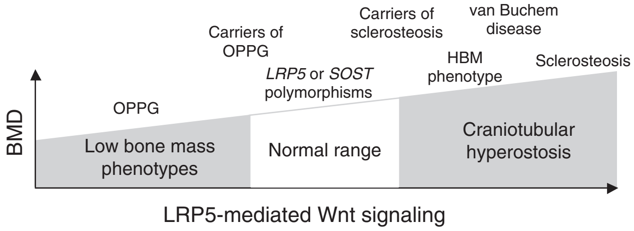
Carriers of OPPG
Carriers of sclerosteosis
van Buchem disease
LRP5 or SOST polymorphisms
HBM phenotype
Sclerosteosis
OPPG
BMD
Low bone mass phenotypes
Normal range
Craniotubular hyperostosis
LRP5-mediated Wnt signaling

FIGURE 6 Relationship between inhibition of canonical Wnt signaling and clinical spectrum and variations in BMD. Decreased and increased LRP5-mediated canonical Wnt signaling is associated with decreased and increased BMD in humans, respectively.

van Buchem disease. Whereas no sclerostin expression was found in bone biopsies of all six patients with sclerosteosis analyzed so far (van Bezooijen *et al.*, 2004), we found very low levels of sclerostin expression in cementocytes from one of three patients with van Buchem disease (van Bezooijen *et al.*, 2007a). This may indicate that deletion of the van Buchem region does not result in complete absence of sclerostin expression and that the remaining sclerostin levels may still regulate canonical Wnt signaling, thereby, potentially explaining the less severe phenotype of patients with van Buchem disease.

Mutations in LRP6 have not been reported in patients with the HBM phenotype. Although this might be just a matter of time, there may also be a biological explanation for the absence of these mutations. For instance, contribution of LRP6 to the determination of bone mass may be less than that of LRP5. However, LRP5 and LRP6 heterozygote knockout mice demonstrate similar changes in bone mass and in biochemical markers of bone metabolism (Holmen *et al.*, 2004). Alternatively, mutations in LRP6 that decrease sclerostin function may have lethal effects that are not induced by LRP5 HBM mutations.

Mutations in LRP5 other than those in the HBM phenotype have been associated with a decrease in bone mass. Forty-eight mutations in LRP5 have been reported in OPPG, a rare autosomal recessive disorder characterized by severe juvenile-onset osteoporosis, congenital or infancy-onset blindness, and a variable degree of mental retardation, muscular hypotonia, ligamentous laxity, obesity, abnormal hair, seizures, and ventricular septum defects (Balemans and Van Hul, 2007; Levasseur *et al.*, 2005). These mutations are either deleterious (nonsense, frameshift, or splice site mutations) or consist of missense variants shown or expected to result in decreased canonical Wnt signaling. Similar to the allele dose-dependent effect of SOST on BMD in heterozygous carriers of sclerosteosis and homozygotes (Gardner *et al.*, 2005), heterozygous carriers of OPPG are considered phenotypically normal but have decreased BMD and are more susceptible to osteoporotic fractures (Gong *et al.*, 2001).

Variations in BMD within the normal range have been correlated with polymorphisms in both SOST and LRP5. Two polymorphisms in SOST, a 3-bp insertion at position −1396 of the SOST gene and a G-variant in the van Buchem region, have been associated with BMD in elderly whites aged 55 years (Uitterlinden *et al.*, 2004) and several studies have reported an association between polymorphisms in LRP5 and BMD in children, adolescents, and adults (Balemans and Van Hul, 2007). Interestingly, two genetic variations in exons 10 and 18 of LRP5 that are associated with BMD also affect Wnt signaling (Kiel *et al.*, 2007). In addition, these variations affected the relationship between physical activity and BMD in men, suggesting that differences in Wnt signaling owing to variations in LRP5 may play a role in adaptation of bone to mechanical loading, subsequently determining bone mass.

THERAPEUTIC POTENTIAL OF TARGETING SCLEROSTIN

Skeletal disorders are prevalent and associated with significant morbidity and mortality. Independent of etiology, disturbances of bone remodeling constitute the pathophysiological basis of most of the known skeletal diseases. A typical example is osteoporosis in which there is an imbalance between bone resorption and bone formation, in favor of the former, leading to bone loss, disturbed bone microarchitecture, and increased risk of fracture. Correction of the imbalance in bone remodeling either by suppressing bone resorption by using antiresorptive agents such as bisphosphonates or stimulating bone formation by using anabolic agents such as PTH have been shown to significantly reduce the risk of fractures. Antiresorptive agents do not restore already lost bone, however. For this, new therapeutic strategies that stimulate bone formation and induce a positive bone balance are required. In patients with sclerosteosis and van Buchem disease the positive bone balance observed results from the increased formation of bone of excellent quality. Therefore, inhibition of sclerostin production and/or activity may thus provide a therapeutic target for the development of specific bone-forming agents. Moreover, the restricted pattern of sclerostin expression suggests that such agents would be devoid of any extraskeletal effects and thus bear limited risks for side effects.

Inhibition of sclerostin activity by humanized neutralizing monoclonal antibodies has been shown to stimulate bone formation and restore bone mass in preliminary studies in rodents and primates (Ominsky *et al.*, 2006a, 2006b). Recently, preliminary data from a blinded, placebo-controlled, dose-escalating single-dose study in 48 healthy postmenopausal women demonstrated that a single injection of a monoclonal antibody to sclerostin significantly increased the bone formation markers osteocalcin, BSAP, and P1NP, and that therapy was well tolerated (Padhi *et al.*, 2007).

PERSPECTIVES

Identification of sclerostin deficiency as the underlying cause of the two rare skeletal disorders sclerosteosis and van Buchem disease has opened an exciting new field in bone research. Sclerostin's highly restricted expression pattern and its negative regulatory effect on bone formation has identified it as a secreted factor produced by osteocytes that inhibits osteoblast activity and prevents excessive bone formation. Regulating sclerostin expression is likely to represent a key mechanism in the determination of bone mass. Furthermore, inhibition of sclerostin activity has been shown to stimulate bone formation and increase bone mass in rodents and primates and serum bone formation markers in humans.

Data accumulated during the past 4 years demonstrated that sclerostin antagonizes canonical Wnt signaling through binding to the coreceptors LRP5/6. The precise mechanism by which binding of sclerostin to LRP5/6 interferes with canonical Wnt signaling, however, remains to be elucidated. It also remains unclear whether HBM LRP5 mutations result in increased canonical Wnt signaling owing to decreased Dkk-1 and/or sclerostin function. Nevertheless, the discovery of the mechanism underlying the increased bone formation observed in sclerosteosis and van Buchem disease has revealed increased canonical Wnt signaling as the common affected pathway in these skeletal disorders of high bone mass owing to increased bone formation.

An important challenge will be the identification of regulators of sclerostin/SOST expression that in turn may determine sclerostin's highly restricted expression in pattern. An essential regulatory element within the van Buchem region and its control by MEF2 transcription factors has already been identified.

Characterization of sclerosteosis and van Buchem disease, in combination with the HBM phenotype, has also opened a new research area for therapeutic interventions for the most prevalent of skeletal disorders, osteoporosis. Of particular interest are the consistently higher BMD values of carriers of sclerosteosis without any clinical complications, suggesting that production and/or activity of sclerostin may be titrated without any side effects.

In addition to providing a new basis for the management of individuals with osteoporosis, better understanding of the sclerosing skeletal disorders sclerosteosis and van Buchem disease and of the molecular mechanism of sclerostin action may help in the management of this small group of patients, for whom the only currently available treatment is the hazardous and technically difficult surgical removal of excessive bone in the skull.

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Chapter 8

The Osteocyte

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INTRODUCTION

The osteocyte is the most abundant cell type of bone. There are approximately 10 times as many osteocytes as osteoblasts in adult human bone (Parfitt, 1977), and the number of osteoclasts is only a fraction of the number of osteoblasts. Our current knowledge of osteocytes lags behind what we know of the properties and functions of both osteoblasts and osteoclasts. However, the striking structural design of bone predicts an important role for osteocytes.

Considering that osteocytes have a very particular location in bone, not on the bone surface but spaced regularly throughout the mineralized matrix, and considering their typical morphology of stellate cells, which are connected with each other via long, slender cell processes, a parallel with the nerve system springs to one's mind. Are the osteocytes the "nerve cells" of the mineralized bone matrix, and if so, what are the stimuli that "excite" the cells? The answers to these questions may very well come from studies in which biomechanical concepts and techniques are applied to bone cell biology. Both theoretical considerations and experimental results have strengthened the notion that osteocytes are the pivotal cells in the biomechanical regulation of bone mass and structure (Cowin *et al*., 1991; Mullender and Huiskes, 1994, 1995; Klein-Nulend *et al*., 1995b). This idea poses many new questions that must be answered. By which mechanism(s) are loading stimuli on bone translated into biochemical stimuli that regulate bone (re)modeling, and what is the nature of these signaling molecules? How and where do the mechanical and hormonal regulatory systems of bone interact? Are osteocytes mere signaling cells, or do they contribute actively to bone metabolic processes such as mineralization, a process that takes place around newly incorporated osteocytes at some distance of the bone matrix formation front?

The development of osteocyte isolation techniques, the use of highly sensitive (immuno)cytochemical and *in situ* hybridization procedures, and the usefulness of molecular biological methods even when only small numbers of cells are available, have rapidly increased our knowledge about this least understood cell type of bone in the recent past and will certainly continue to do so in the future. This chapter compiles and analyzes the most recent findings, and it is hoped that it contributes to the development of new ideas and thoughts about the role of osteocytes in the physiology of bone.

THE OSTEOCYTIC PHENOTYPE

The Osteocyte Network

Mature osteocytes are stellate shaped or dendritic cells enclosed within the lacunocanalicular network of bone. The lacunae contain the cell bodies. From these cell bodies, long, slender cytoplasmic processes radiate in all directions, but with the highest density perpendicular to the bone surface (Fig. 1). They pass through the bone matrix via small canals, the canaliculi. Processes and their canaliculi may be branched. The more mature osteocytes are connected by these cell processes to neighboring osteocytes, the most recently incorporated osteocytes to neighboring osteocytes and to the cells lining the bone surface. Some of the processes oriented to the bone surface, however, appear not to connect with the lining cells, but to pass through this cell layer, thereby establishing a direct contact between the osteocyte syncytium and the extraosseus space. This intriguing observation by Kamioka *et al*. (2001) suggests the existence of a signaling system between the osteocyte and the bone marrow compartment without intervention

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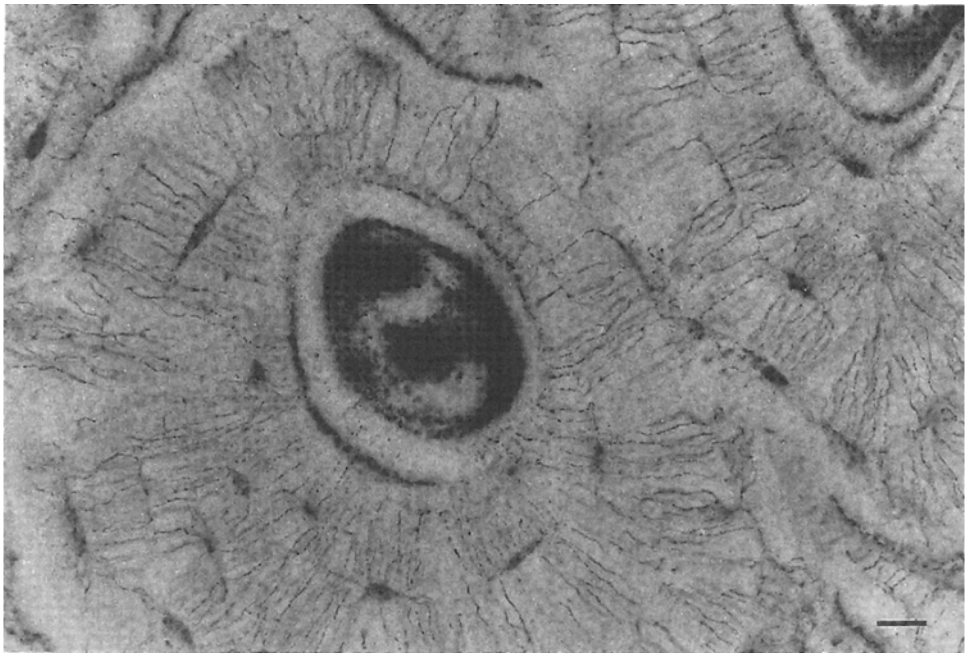

FIGURE 1 Osteon in mature human bone. Osteocytes are arranged in concentric circles around the central haversian channel. Note the many cell processes, radiating from the osteocyte cell bodies, in particular in the perpendicular directions. Schmorl staining. (Original magnification: ×390; bar: 25 μm.)

of the osteoblasts/lining cells. Osteocytes also appear to be able to retract and extend their dendritic processes, not only between cells in the bone matrix, but also into marrow spaces as shown by dynamic imaging (Veno *et al.*, 2006). This has implications with regards to osteocytes making and breaking communication between cells.

Dramatic changes occur in the distribution of actin-binding proteins during terminal differentiation of osteoblasts to osteocytes (Kamioka *et al.*, 2004). The typical morphology of the osteocyte was originally thought to be enforced on differentiating osteoblasts during their incorporation in the bone matrix. Osteocytes must remain in contact with other cells and ultimately with the bone surface to ensure the access of oxygen and nutrients. Culture experiments with isolated osteocytes have shown, however, that although the cells lose their stellate shape in suspension, they re-express this morphology as soon as they settle on a support (Van der Plas and Nijweide, 1992) (Fig. 2). Apparently, the typical stellate morphology and the need to establish a cellular network are intrinsic characteristics of terminal osteocyte differentiation.

In bone, gap junctions are present between the tips of the cell processes of connecting osteocytes (Doty, 1981). Within each osteon or hemiosteon (on bone surfaces), therefore, osteocytes form a network of gap junction-coupled cells. As the lacunae are connected via the canaliculi, the osteocyte network represents two network systems: an intracellular one and an extracellular one. Gap junctions are transmembrane channels connecting the cytoplasm of two adjacent cells and regulating the passage of molecules less than 1 kD_a (Goodenough *et al.*, 1966; Bennett *et al.*, 1978). Gap junction channels are formed by members of a family of proteins known as connexins. One of these members, Cx43, appears to play an important role in bone cells as Cx43-null mice have delayed ossification, craniofacial abnormalities, and osteoblast dysfunction (Lecanda *et al.*, 2000). It has been proposed that gap junctions function through the propagation of intracellular signals contributing to mechanotransduction in bone, thereby regulating bone cell differentiation (Donahue *et al.*, 2000). A dominant negative mutant of Cx43 diminishes fluid flow-induced release of PGE_2, but not Ca^{2+} responses (Saunders *et al.*, 2001). Fluid flow-induced shear stress stimulates gap junction-mediated intercellular communication and increases Cx43 expression (Cheng *et al.*, 2001), while oscillating fluid flow has been shown to upregulate gap junction communication by an ERK1/2 MAP kinase-dependent mechanism (Alford *et al.*, 2003) in MLO-Y4 osteocyte-like cells.

Recently, hemichannels, unapposed halves of gap junction channels, have been identified in osteocytes localizing at the cell surface, independent of physical contact with adjacent cells (Goodenough *et al.*, 2003). Primary osteocytes and MLO-Y4 osteocyte-like cells (Kato *et al.*, 1997) express very large amounts of Cx43 compared to other cell types such as osteoblasts, yet these cells are only in contact through the tips of their dendritic processes, raising the question regarding function of Cx43 on the rest of the cell

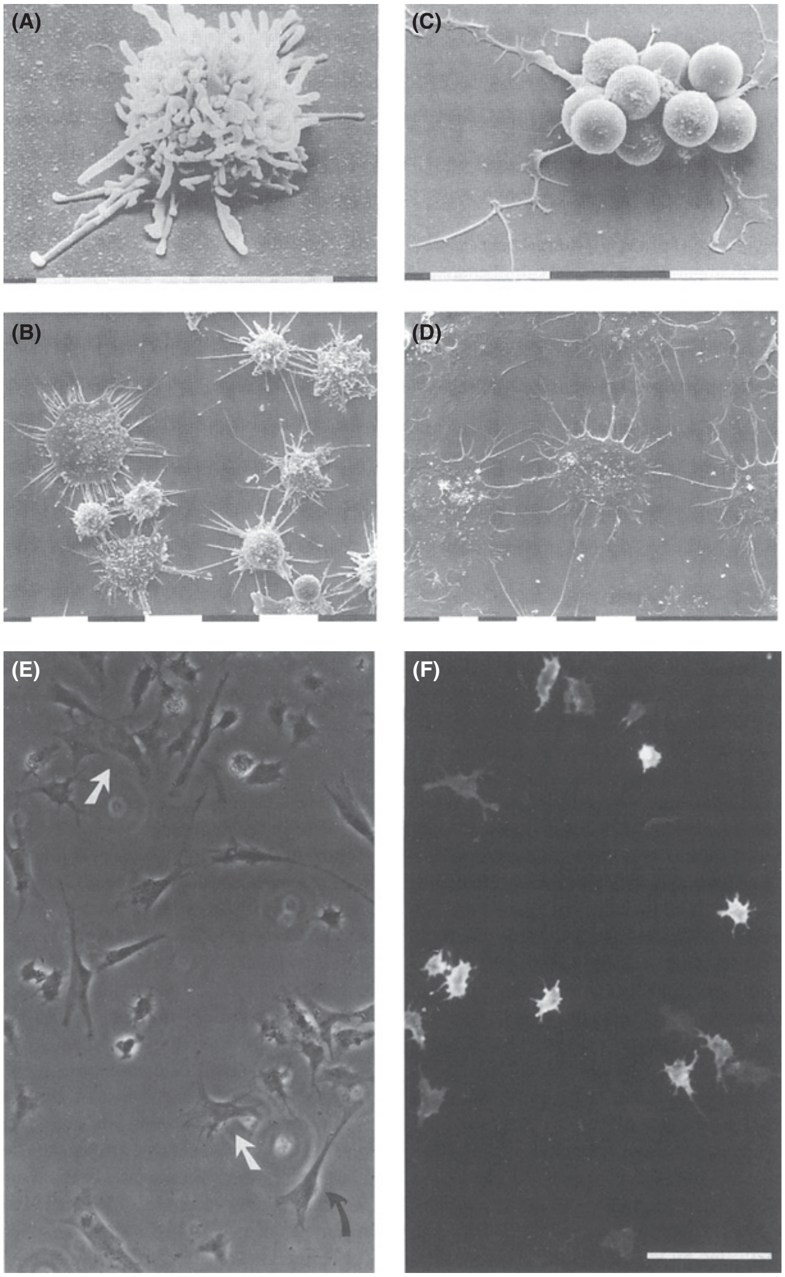

FIGURE 2 Isolated osteocytes in culture. Osteocytes were isolated by an immunodissection method using MAb OB7.3-coated magnetic beads. After isolation the cells were seeded on a glass support, cultured for 5 min **(A)**, 30 min **(B)**, or 24 hr (**C** and **D**) and studied with a scanning electron microscope. Immediately after attachment, osteocytes form cytoplasmic extrusions in all directions **(A)**. During subsequent culture, the cell processes perpendicular on the support disappear, while the processes in the plane of the support elongate **(B)** and ultimately form smooth connections between neighboring cells **(D)**. In **A**, **B**, and **D** the immunobeads were removed from the cells before seeding; in **C** the beads were left on the cells. (Original magnification: **(A)** ×7200, **(B)** ×1400, **(C)** ×2900, and **(D)** ×940; bar: 10 µm). Cells were also isolated from periosteum-free 18-day-old chicken calvariae by collagenase digestion, seeded, and cultured for 24 hr. Subsequently the osteocytes in the mixed population were specifically stained with MAb OB7.3 in combination with biotinylated horse-antimouse IgG and streptavidin-Cy3. **(E)** Phase contrast. **(F)** Immunofluorescence. Black arrows, fibroblast-like cells; white arrows, osteoblast-like cells. (Original magnification: ×300; bar: 100 µm).

membrane. The opening of hemichannels results in ATP and NAD^+ release, which in turn raises intracellular Ca^{2+} levels and wave propagation of Ca^{2+}. It has been shown that oscillating fluid flow activates hemichannels in MLO-Y4 osteocyte-like cells, but not in MC3T3-E1 osteoblast-like cells. This activation involved protein kinase C, and resulted in ATP and PGE_2 release (Genetos *et al.*, 2007). Hemichannels expressed in bone cells such as MLO-Y4 cells appear to function as essential transducers of the anti-apoptotic effects of bisphosphonates (Plotkin *et al.*, 2002) and serve as a portal for the exit of elevated intracellular PGE_2 in osteocytes induced by fluid flow shear stress (Cherian *et al.*, 2005). Therefore, gap junctions at the tip of dendrites mediate intracellular communication while hemichannels along the dendrite and the cell body mediate extracellular communication within the osteocyte network.

Osteocyte Formation and Death

Osteogenic cells arise from multipotential mesenchymal stem cells (see Chapter 4). These stem cells have the capacity to also differentiate into other lineages, including those of chondroblasts, fibroblasts, adipocytes, and myoblasts (Aubin *et al.*, 1995; Chapter 4). By analogy with hemopoietic differentiation, each of these differentiation lineages is thought to originate from a different committed progenitor, which for the osteogenic lineage is called the osteoprogenitor. Osteodifferentiation progresses via a number of progenitor and precursor stages to the mature osteoblast. An osteoblast has one of three fates: it can embed in its own osteoid and differentiate into an osteocyte, quiescence into a lining cell, or undergo apoptosis (for review see Manalogas (2000). The mechanism by which osteoblasts differentiate into osteocytes is, however, still unknown. Imai *et al.* (1998) found evidence that osteocytes may stimulate osteoblast recruitment and differentiation by expressing osteoblast stimulating factor-1 (OSF-1) (Tezuka *et al.*, 1990). The osteoblasts further differentiate to osteocytes being surrounded by the osteoid matrix that they produce, and they then may become a new source of OSF-1 for the next round of osteoblast recruitment. The expression of OSF-1 in osteocytes may be activated by local damage to bone or local mechanical stress (Imai *et al.*, 1998). Marotti (1996) has postulated that a newly formed osteocyte starts to produce an osteoblast inhibitory signal when its cytoplasmic processes connecting the cell with the osteoblast layer have reached their maximal length. The osteoid production of the most adjacent, most intimately connected osteoblast will be relatively more inhibited by that signal than that of its neighbors. The inactivated osteoblast then spreads over a larger bone surface area, thereby reducing its linear appositional rate of matrix production even further. A second consequence of the widening and flattening of the cell is that it may intercept more osteocytic processes carrying the inhibitory signal. This positive feedback mechanism results in the embedding of the cell in matrix produced by the neighboring osteoblasts. Ultimately, the cell will acquire the typical osteocyte morphology and the surrounding matrix will become calcified. The theory of Marotti is based entirely on morphologic observations. There is no biochemical evidence about the nature or even the existence of the proposed inhibitory factor. Martin (2000) has, however, used the concept successfully in explaining mathematically the changing rates of matrix formation during bone remodeling.

Osteoid-osteocytes were described by Palumbo (1986) to be cells actively making matrix and calcifying this matrix while the cell body reduces in size in parallel with the formation of cytoplasmic processes. Bordier and co-workers (1976) and Nijweide and co-workers (1981) proposed that osteoid-osteocytes play an important role in the initiation and control of mineralization of the bone matrix. During the time an osteoblast has become an osteocyte, the cell has manufactured three times its own volume in matrix (Owen, 1995). Franz-Odendaal and co-workers (2006) proposed that once a cell is surrounded by osteoid that the differentiation process has not ended, but continues.

An enzyme that is produced in high amounts by embedding osteoid-osteocytes and not by osteoblasts, casein kinase II, appears to be responsible for phosphorylation of matrix proteins essential for mineralization (Mikuni-Takagaki *et al.*, 1995). Phosphoproteins appear to be essential for bone mineralization as evidenced by *in vitro* crystal nucleation assays (Boskey, 1996) and *in vivo* by osteomalacia in animal models with deletion of (osteocyte-specific) genes such as dentin matrix protein-1 (DMP1) and PHEX (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) (Strom *et al.*, 1997). The role of these proteins in mineral homeostasis will be discussed later in this chapter. Recently an osteocyte-selective promoter, the 8 kb dentin matrix protein-1 (DMP1), driving GFP has been generated (Kalajzic *et al.*, 2004). With the identification of other markers selective for osteocytes such as E11/gp38 for early osteocytes (Zhang *et al.*, 2006) and sclerostin for late osteocytes (Poole *et al.*, 2006), new tools are on the horizon for the study of osteocyte formation.

The life span of osteocytes is probably largely determined by bone turnover, when osteoclasts resorb bone and either "liberate" or destroy osteocytes. Osteocytes may have a half-life of decades if the particular bone they reside in has a slow turnover rate. The fate of living osteocytes that are liberated by osteoclast action is presently unknown. There is little evidence that osteocytes may reverse their differentiation back into the osteoblastic state (Van der Plas *et al.*, 1994). Some of them, only half released by osteoclastic activity, may be reembedded during new bone formation that follows the resorption process (Suzuki *et al.*, 2000). These osteocytes are then the cells that cross the cement lines between individual osteons, sometimes seen in cross sections of osteonal bone. Most of the osteocytes, however, will probably die by apoptosis and become phagocytosed. Phagocytosis of osteocytes by osteoclasts as part of the bone resorption process has been documented in several reports (Bronckers *et al.*, 1996; Elmardi *et al.*, 1990).

Apoptosis of osteocytes in their lacunae is attracting growing attention because of its expected consequence of decreased bone mechanoregulation, which may lead to osteclastic bone resorption. Osteocyte apoptosis can occur as a result of immobilization, microdamage, estrogen deprivation, elevated cytokines, glucocorticoid treatment, osteoporosis, osteoarthritis, and aging. The resulting fragility is considered to be due to loss of the ability of osteocytes to signal other bone cells for repair because of a loss of capacity to sense microdamage (Manolagas, 2000; Noble *et al.*, 2003). Apoptotic regions around microcracks were found to be surrounded by surviving osteocytes expressing Bcl-2, whereas dying osteocytes appeared to be the target of resorbing osteoclasts (Verborgt *et al.*, 2000; 2002). Apoptotic changes in osteocytes were shown to be associated with high bone turnover (Noble *et al.*, 1997). However, fatigue-related microdamage in bone may cause decreased osteocyte accessibility for nutrients and oxygen inducing osteocyte apoptosis and subsequent bone remodeling (Burger and Klein-Nulend, 1999; Verborgt *et al.*, 2000). Lack of oxygen elevates hypoxia-inducing factor alpha, HIFa, a transcription activator by inactivation of prolyl hydroxylase leading to apoptosis and induction of the osteoclastogenic factor TNFα and VEGF (Gross *et al.*, 2001) and osteopontin, a mediator of environmental stress and a potential chemoattractant for osteoclasts (Gross *et al.*, 2005).

In contrast to overloading, which induces microdamage, physiological mechanical loads might prevent osteocyte apoptosis *in vivo*. Mechanical stimulation of osteocytes *in vitro*, by means of a pulsating fluid flow, affects TNFα-induced apoptosis. One-hour pulsating fluid flow (0.70 ± 0.30 Pa, 5 Hz) inhibited (25%) TNFα-induced apoptosis in osteocytes, but not in osteoblasts or periosteal fibroblasts (Tan *et al.*, 2006). Although the exact mechanism is not clear, loading-induced nitric oxide production by the osteocytes might be involved in the antiapoptotic effects of mechanical loading.

Also, loss of estrogen (Tomkinson *et al.*, 1998) and chronic glucocorticoid treatment (Weinstein *et al.*, 1998) were demonstrated to induce osteocyte apoptosis, which may, at least in part, explain the bone-deleterious effects of these conditions. Several agents, such as bisphosphonates and calcitonin (Plotkin *et al.*, 1999), CD40 ligand (Ahuja *et al.*, 2003), calbindin-D28 k (Liu *et al.*, 2004), and estrogen and selective estrogen receptor modulators (Kousteni *et al.*, 2001), have been found to reduce or inhibit osteoblast and osteocyte apoptosis. Bisphosphonates inhibit apoptosis through interaction with hemichannels and the ERK pathway (Plotkin *et al.*, 2001). Interestingly, the two antiapoptotic agents PTH and MCP-3 have been shown to be selective for apoptosis induced by one particular agent. Unlike the agents listed above, both PTH (Jilka *et al.*, 1999) and MCP-3 will only inhibit glucocorticoid-induced apoptosis, but not TNFα induced apoptosis of MLO-Y4 osteocyte-like cells (Kitase *et al.*, 2006).

Osteocyte viability may play a significant role in the maintenance of bone homeostasis and integrity, yet agents that block apoptosis may exacerbate conditions that require repair and, in addition, might have potentially detrimental effects on tumor formation.

Osteocyte Isolation

Analysis of osteocyte properties and functions has long been hampered by the fact that they are embedded in a mineralized matrix. Although sensitive methods are now available, such as immunocytochemistry and *in situ* hybridization, by which osteocytes can be studied in the tissue in some detail, osteocyte isolation and culture offer a major step forward. This approach became possible by the development of osteocyte-specific antibodies (Fig. 3) directed to antigenic sites on the outside of the cytoplasmic membrane (Bruder and Caplan, 1990; Nijweide and Mulder, 1986). Using an immunodissection method, Van der Plas and Nijweide (1992) subsequently succeeded in the isolation and purification of chicken osteocytes from mixed bone cell populations isolated from fetal bones by enzymatic digestion. Isolated osteocytes appeared to behave *in vitro* like they do *in vivo* in that they reacquired their stellate morphology and, when seeded sparsely, formed a network of cells coupled to one another by long, slender, often branched cell processes (Fig. 2). The cells retained this morphology in culture throughout the time studied (5–7 days) and even reexpressed it when passaged for a second time (Van der Plas and Nijweide, 1992).

Mikuni-Takagaki *et al.* (1995) have isolated seven cell fractions from rat calvariae by sequential digestion. They

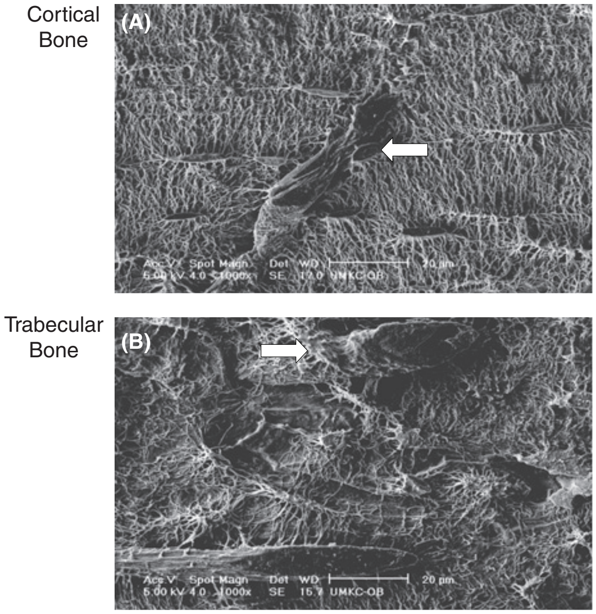

FIGURE 3 Images of acid-etched resin embedded murine cortical **(A)** and trabecular **(B)** bone visualized by scanning electron microscopy showing the complexity of the osteocyte lacuno-canalicular system and the intimate relationship between the lacuno-canalicular system and blood vessels. In the cortical bone, note the linear alignment of the lacunae and the complexity of the canaliculi. In the trabecular bone, the lacunae are not as organized as in the cortical bone. In both sections, note the close relationship of some osteocyte lacunae with blood vessels (arrows).

claimed that the last fraction consisted of osteocytic cells. The cells displayed dendritic cell processes, were negative for alkaline phosphatase, had high extracellular activities of casein kinase II and ecto-5′nucleotidase, and produced large amounts of osteocalcin. After a few days of little change in cell numbers, the cells of fraction VII proliferated, however, equally fast as those of fraction III, the osteoblastic cells, in culture. With the identification of new osteocyte selective markers such as Sost, E11/gp38, Dmp1, Phex and Mepe, it is becoming easier to identify isolated osteocytes (see below).

Osteocyte Markers

In bone, osteocytes are fully defined by their location within the bone matrix and their stellate morphology. One marker for isolated osteocytes is therefore their typical morphology, which they reacquire in culture (Mikuni-Takagaki *et al*., 1995; Van der Plas and Nijweide, 1992). Related to this stellate morphology, osteocytes have a typical cytoskeletal organization, which is important for the osteocyte's response to loading (McGarry *et al*., 2005). The prominent actin bundles in the osteocytic processes, together with the abundant presence of the actin-bundling protein fimbrin, are exemplary for osteocytes and are retained after isolation (Tanaka-Kamioka *et al*., 1998). In addition, osteocytes are generally found to express osteocalcin, osteonectin, and osteopontin, but show little alkaline phosphatase activity, particularly the more mature cells (Aarden *et al*., 1996b). As stated previously, these metabolic markers have, however, little discriminating value in mixtures of isolated cells. Franz-Odendaal and co-authors (2006) provide a list of molecular markers for the preosteoblast to the osteocyte.

Until recently, no osteocyte-specific markers were known, and only few osteocyte-specific antibodies were available. Examples are the monoclonal antibodies MAb OB7.3 (Nijweide and Mulder, 1986) (Fig. 2), MAb OB37.11 (Nijweide *et al*., 1988), and MAb SB5 (Bruder and Caplan, 1990). All three are specific for avian osteocytes and do not cross-react with mammalian cells. The identities of the three antigens involved have not been reported, although that of OB7.3 has been elucidated and found to be the avian homologue of mammalian Phex (Westbroek *et al*., 2002). Using an antibody to Phex allowed purification of avian osteocytes from enzymatically isolated bone cells.

Osteoblast/osteocyte factor 45 (OF45) also known as MEPE (matrix extracellular phosphoglycoprotein) is also highly expressed in osteocytes as compared to osteoblasts. Messenger RNA expression for OF45/MEPE begins at E20 in more differentiated osteoblasts that have become encapsulated by bone matrix (Igarashi *et al*., 2002). *Mepe* was isolated and cloned from a TIO tumor cDNA library (Rowe *et al*., 2000). Cathepsin D or B can cleave MEPE, releasing the highly phosphorylated C-terminal ASARM region that is a potent inhibitor of mineralization *in vitro* (Bresler *et al*., 2004; Rowe *et al*., 2005). OF45/MEPE null mice have increased bone formation, bone mass, and resistance to age-associated trabecular bone loss (Gowen *et al*., 2003). The authors speculate that osteocytes act directly on osteoblasts through OF45 to inhibit their bone forming activity.

Both Toyosawa (2001) and Feng and colleagues (2006) found dentin matrix protein 1 to be highly expressed in osteocytes with very low expression in osteoblasts. DMP1 is specifically expressed along and in the canaliculi of osteocytes within the bone matrix (Feng *et al*., 2006). Deletion of this gene in mice results in a phenotype similar if not identical to the hyp-phenotype, suggesting that Dmp1 and Phex are interactive and essential for phosphate metabolism. Potential roles for DMP1 in osteocytes may be related to the post-translational processing and modification resulting in a highly phosphorylated protein and regulator of hydroxyapatite formation. Interestingly, Dmp1 and OF45/MEPE belong to the SIBLING (small, integrin-binding ligand, N-linked glycoprotein) family that also includes bone sialoprotein, osteopontin, and sialophosphoprotein (Fisher, 2003). This family of proteins may function differently in osteocytes compared to other cell types especially upon phosphorylation with casein kinase II, a marker of the osteoblast to osteocyte transition (Mikuni-Takagaki *et al*., 1995).

Confusion exists regarding the expression of E11/gp38 in bone because Wetterwald (1996) and others (Aubin and Turksen, 1996) have described expression in osteoblasts. However, Schultze (1999) and Zhang (2006) have described expression exclusively in osteocytes and not in osteoblasts *in vivo*. A punctate antibody reaction is observed at the interface between osteoblasts and uncalcified osteoid at the tips and along dendritic processes with less reactivity in osteocytes deeper in the bone matrix. E11/gp38 appears to be responsible for the formation of dendritic processes as reduction in protein expression using an siRNA approach led to a decrease in dendrite extension in MLO-Y4 osteocyte-like cells in response to shear stress (Zhang *et al*., 2006). E11/gp38 colocalizes with ezrin, radixin, and moesin, ERMs (Scholl *et al*., 1999), that are concentrated in cell-surface projections where they link the actin cytoskeleton to plasma membrane proteins and are involved in cell motility (Mangeat *et al*., 1999). CD44 is highly expressed in osteocytes compared to osteoblasts (Hughes *et al*., 1994), and as E11 is physically associated with CD44 in tumor vascular endothelial cells (Ohizumi *et al*., 2000), this association most likely occurs in osteocytes to regulate the formation of dendritic processes.

Sclerostin appears to be highly expressed in the mature but not the early osteocyte (Poole *et al*., 2005). Transgenic mice lacking sclerostin have increased bone mass, and the human condition of sclerostosis is due to a premature termination of the SOST gene (Balemans *et al.*, 2001). Sclerostin clearly functions as a Wnt antagonist by binding Lrp5 (van Bezooijen *et al.*, 2004). Lrp5 is shown to be an important positive regulator of bone mass (Li *et al.*, 2001). Sclerostin protein may be transported through canaliculi to the bone surface to inhibit bone-forming osteoblasts. Sclerostin is down regulated by mechanical loading (Robling *et al.*, 2006). It has also been proposed that the anabolic effects of PTH are through inhibition of SOST expression (Bellido *et al.*, 2005).

In summary, it is becoming easier to define cells as osteocytic. Even though the E11/gp38 molecule is expressed in other tissues, it is only expressed in osteocytes, not osteoblasts, in bone (Zhang *et al.*, 2006). While E11/gp38 appears to be a marker for the early osteocyte, Sost/sclerostin is a specific marker for the late osteocyte (Poole *et al.*, 2005). Dmp1, Phex, and MEPE, while expressed in low levels in osteoblasts and other tissues, are highly elevated in osteocytes. In addition, a panel of markers for osteocytes is being developed (Franz-Odendaal *et al.*, 2006). Combining these markers with other properties such as dendricity and low or no alkaline phosphatase (see later) can be useful to define not only isolated primary cells, but also cell lines.

Osteocytic Cell Lines

Because the number of primary osteocytes that can be isolated from chickens each time (Van der Plas and Nijweide, 1992) is limited, several groups have tried to establish osteocytic cell lines. Basically, an osteocytic cell line is a contradiction in terms. Osteocytes are postmitotic. However, a cell line of proliferating precursor cells that would differentiate to osteocytes under specific circumstances could prove to be very valuable in the study of osteocyte properties and functions. HOB-01-C1 (Bodine *et al.*, 1996) may meet these requirements. It is a temperature-sensitive cell line, prepared from immortalized, cloned human adult bone cells. It proliferates at 34°C but stops dividing at 39°C. HOB-01-C1 cells display putative osteocytic markers, such as cellular processes, low alkaline phosphatase activity, high osteocalcin production, and the expression of CD44.

MLO-A5 cells, a postosteoblast/preosteocyte-like cell line established from the long bones of 14-day-old mice expressing the large T-antigen driven by the osteocalcin promoter, differentiate into osteoid osteocyte-like cells (Kato *et al.*, 2001). MLO-A5 cells express all of the markers of the late osteoblast such as high alkaline phosphatase, bone sialoprotein, PTH type 1 receptor, and osteocalcin, but begin to express markers of osteocytes such as E11/gp38 as they generate cell processes. MLO-A5 cells begin to express E11/gp38 in culture while generating dendritic processes and generating spherical structures that bud from and mineralize on their developing cellular processes. As the cellular process narrows in diameter, these mineralized structures become associated with and initiate collagen mediated mineralization (Barragan-Adjemian *et al.*, 2006). PTH has been shown to reduce Sost/sclerostin expression in these cells (Bellido *et al.*, 2006).

MLO-Y4 (Kato *et al.*, 1997) is another osteocyte-like cell line in that the cells, when seeded at low density, display complex dendritic processes. They produce high amounts of E11/gp38 (Zhang *et al.*, 2006), CD44, osteocalcin, and osteopontin, and have low alkaline phosphatase activity. Numerous investigators have used these cells to examine gap junctions, hemichannels, and apoptosis and other potential functions of osteocytes (Genetos *et al.*, 2007; Plotkin *et al*, 2005; Vatsa *et al.*, 2006; Vatsa *et al.*, in press; Xiao *et al.*, 2006; Zaman *et al.*, 2006; Zhang *et al.*, 2006; Cherian *et al.*, 2005; Liu *et al.*, 2004; Alford *et al.*, 2003; Ahuja *et al.*, 2003; Zhao *et al.*, 2002; Heino *et al.*, 2002, 2004; Cheng *et al.*, 2001; Yellowley *et al.*, 2000). These cells will support osteoclast formation and activation in the absence of any osteotropic factors (Zhao *et al.*, 2002), and estrogen will reduce support of osteoclast formation by an increase in TGFβ3 (Heino *et al.*, 2002) and, conversely, will also support osteoblast mesenchymal stem cell differentiation (Heino *et al.*, 2004), supporting the hypothesis that osteocytes are orchestrators of both bone resorption and bone formation.

Osteocytic cell lines have also been generated from mice lacking the type 1 PTH/PTHrp receptor (Divieti *et al.*, 2001) and have proved useful to determine the effects of PTH on osteoblasts and osteocytes and for the discovery of a receptor that binds to the carboxy terminus of PTH, CTPH-R (Divieti *et al.*, 2005). CPTH-R is more highly expressed on osteocytes than on other bone cells. Functional studies of CPTH-Rs suggest a role in cell survival and intracellular communication, and in proapoptotic and antiresorptive actions. Investigators using these cell lines cannot assume the cells to be representative of normal osteocytes as they lack the type 1PTH/PTHrp receptor.

Matrix Synthesis

The subcellular morphology of osteocytes and the fact that they are encased in mineralized matrix do not suggest that osteocytes partake to a large extent in matrix production. Osteocytes, especially the more mature, have relatively few organelles necessary for matrix production and secretion. Nevertheless, a limited secretion of specific matrix proteins may be essential for osteocyte function and survival. Several arguments are in favor of such limited matrix production. First, as the mineralization front lags behind the osteoid formation front in areas of new bone formation, osteocytes may be involved in the maturation and mineralization of the osteoid matrix by secreting specific matrix molecules. It is, however, also possible that osteocytes enable the osteoid matrix to be mineralized by phosphorylating certain matrix constituents, as was suggested by Mikuni-Takagaki *et al.* (1995). Mikuni-Takagaki and colleagues proposed that casein kinase II, produced in high amounts by embedding osteoid osteocytes and not by osteoblasts, is responsible for phosphorylation of matrix proteins essential for mineralization. Therefore, the embedding osteoid cell and the osteocyte probably play roles in the mineralization process and potentially in phosphate metabolism (see later). Osteocytes must inhibit mineralization of the matrix directly surrounding them to ensure the diffusion of oxygen, nutrients, and waste products through the lacunocanalicular system. Osteocalcin, which is expressed to a relative high extent by osteocytes, may play an important role here (Aarden *et al.*, 1996b; Ducy *et al.*, 1996; Mikuni-Takagaki *et al.*, 1995), as does OF45/MEPE and Sost. Osteocytes have been found to be positive for osteocalcin and osteonectin (Aarden *et al.*, 1996b), molecules that are probably involved in the regulation of calcification. Osteopontin, fibronectin, and collagen type I (Aarden *et al.*, 1996b) have also been demonstrated in and immediately around (isolated) osteocytes. These proteins may be involved in osteocyte attachment to the bone matrix (see later). Finally, if osteocytes are the mechanosensor cells of bone (see later), the attachment of osteocytes to matrix molecules is likely of major importance for the transduction of stress signals into cellular signals. Production and secretion of specific matrix molecules offer a possibility for the cells to regulate their own adhesion and, thereby, sensitivity for stress signals.

In addition to collagenous and noncollagenous proteins, the bone matrix contains proteoglycans. These macromolecules consist of a core protein to which one or more glycosaminoglycan (GAG) side chains are covalently bound. Early electron microscopic studies (Jande, 1971) already showed that the osteocyte body, as well as its cell processes, is surrounded by a thin layer of unmineralized matrix containing collagen fibrils and proteoglycans. The proteoglycans were shown to consist of chondroitin 4-sulfate, dermatan sulfate, and keratan sulfate with immunocytochemical methods (Maeno *et al.*, 1992; Smith *et al.*, 1997; Takagi *et al.*, 1997). These observations are supported by the findings of Sauren *et al.* (1992), who demonstrated an increased presence of proteglycans in the pericellular matrix by staining with the cationic dye cuprolinic blue. Of special interest is the reported presence of hyaluronan in osteocyte lacunae (Noonan *et al.*, 1996). CD44, which is highly expressed on the osteocyte membrane, is a hyaluronan-binding protein and also binds to collagen, fibronectin, and osteopontin (Nakamura and Ozawa, 1996; Yamazaki *et al.*, 1999).

The Osteocyte Cytoskeleton and Cell–Matrix Adhesion

As mentioned earlier, the cell–matrix adhesion of osteocytes is of importance for the translation of biomechanical signals produced by loading of bone into chemical signals. Study of the adhesion of osteocytes to extracellular matrix molecules became feasible with the development of osteocyte isolation and culture methods (Van der Plas and Nijweide, 1992). These studies found little difference between the adhesive properties of osteocytes and osteoblasts, although the pattern of adhesion plaques (osteocytes, many small focal contacts, osteoblasts, larger adhesion plaques) was quite different (Aarden *et al.*, 1996a). Both cell types adhered equally well to collagen type I, osteopontin, vitronectin, fibronectin, and thrombospondin. Integrin receptors are involved, as is shown by the inhibiting effects of small peptides containing a RGD sequence on the adhesion to some of these proteins. Adhesion to all aforementioned matrix molecules was blocked by an antibody reacting with the β_1-integrin subunit (Aarden *et al.*, 1996a). The identity of the a units involved is yet unknown.

Deformation of the bone matrix upon loading may cause a physical "twisting" of integrins at sites where osteocytes adhere to the matrix. Integrins are coupled to the cytoskeleton via molecules such as vinculin, talin, and a-actinin. In osteocytes, especially in the osteocytic cell processes, the actin-bundling protein fimbrin appears to play a prominent role (Tanaka-Kamioka *et al.*, 1998). Mechanical twisting of the cell membrane via integrin-bound beads has been demonstrated to induce cytoskeletal rearrangements in cultured endothelial cells (Wang and Ingber, 1994). The integrin-cytoskeleton complex may therefore play a role as an intracellular signal transducer for stress signals. In addition to the integrins, the nonintegrin adhesion receptor CD44 may attribute to the attachment of osteocytes to the surrounding matrix. CD44 is present abundantly on the osteocyte surface (Hughes *et al.*, 1994; Nakamura and Ozawa, 1995) and is also linked to the cytoskeleton.

Evidence has accumulated recently that underscores the crucial role of the cytoskeleton in a multitude of cellular processes. The cytoskeleton, just like our bony skeleton, provides structure and support for the cell, is actively adapted, and is highly responsive to external physical and chemical stimuli. The cytoskeleton is strongly involved in processes such as migration, differentiation, mechanosensing, and even cell death, and largely determines the material properties of the cell (i.e., stiffness).

For bone cells it has been shown that the production of signaling molecules in response to an *in vitro* fluid shear stress (at 5 and 9 Hz) and vibration stress (5–100 Hz) correlated with the applied stress rate (Bacabac *et al.*, 2004; Bacabac *et al.*, 2006; Mullender *et al.*, 2006). The faster the stress was applied, the stronger the observed response of the cells. Interestingly, high-rate stimuli were found to condition bone cells to be more sensitive for high-frequency, low-amplitude loads (Bacabac *et al.*, 2005). From the field of physics it is known that the effect of stresses applied at different rates at an object are largely determined by the material properties of that object. This implies that bone cellular metabolic activity (e.g., the production of signaling molecules) and mechanical properties of the cell are related. Bacabac *et al.* (2006b) developed a novel application of two-particle microrheology, for which he devised a three-dimensional *in vitro* system using optical traps to quantify the forces induced by cells on attached fibronectin-coated probes (4 microns). The frequency at which the cells generate forces on the beads is related to the metabolic activity of the cell. This system can also be used to apply controlled forces on the cell using the beads, and enables the study of cells under controlled three-dimensional morphologic configuration, with possibilities for a variety of probe coatings for simulating cell-ECM attachment. Using this setup, the generation of forces by different cells was probed in order to understand the relation between cellular metabolic activities and material properties of the cell. It was shown that, at 37°C, CCL-224 fibroblasts exhibited higher force fluctuation magnitude compared to MLO-Y4 osteocytes (Bacabac *et al.*, 2006b). The force fluctuations on the attached probes reflect intracellular movement, which might include actin (and microtubule) polymerization, as well as motor and crosslinker dynamics. Because cell migration involves these dynamic processes, the lower magnitude of force fluctuation might reflect a lower capacity of osteocytes for motility compared to fibroblasts.

Using the optical trap device, the material properties of round suspended MLO-Y4 osteocytes and flat adherent MLO-Y4 osteocytes were characterized. In addition, the cells were loaded with a nitric oxide (NO) sensitive fluorescent dye (Vatsa *et al.*, 2006) and the NO response of the cells to forces applied on the cell using the attached probes was studied. Osteocytes under round-suspended morphology required lower force stimulation in order to show a NO response, even though they were an order of magnitude more elastic compared to flat-adherent cells (Bacabac *et al.*, 2006b). Apparently, elastic osteocytes seem to require less mechanical forces in order to respond than stiffer cells. On the other hand, flat adherent MLO-Y4 cells, primary chicken osteocytes, MC3T3-E1 osteoblasts, and primary chicken osteoblasts all showed a similar elastic modulus of less than 1 kPa (Bacabac *et al.*, 2006b), even though osteocytes are known to be more responsive to mechanical stress than osteoblasts (Klein-Nulend *et al.*, 1995a). This indicates that differences in mechanosensitivity between cells might not be directly related to the elasticity of the cell, but might be more related to other cell-specific properties (i.e., the presence of receptors or ion-channels in the membrane). Alternatively, the mechanosensitivity of a cell might be related to how cells change their material properties in relation to deformation.

Simultaneous with the increased NO release in response to mechanical stimulation, MLO-Y4 osteocytes showed increased force traction on the attached beads. In other words, the cells started to "pull harder" on the beads and generated a force up to nearly 30 pN, which interestingly is within the order of forces necessary for activating integrins. Whether there was a causal link between loading-induced NO production by the cells and force generation is currently under investigation. Since force generation and cell elasticity are (indirectly) related, these results might indicate that osteocytes adapt their elasticity in response to a mechanical stimulus. Indeed, experiments with an atomic force microscope and optical tweezers have shown that osteocytes become "stiffer" after mechanical loading (Bacabac *et al.*, 2006b; and unpublished observations). This stiffening response was related to actual changes in material properties of the cell, suggesting that the cells actively change their cytoskeleton in response to a mechanical load.

Considering the role of the osteocytes as the professional mechanosensors of bone, and the importance of the cytoskeleton for the response of osteocytes to mechanical loading, much is to be expected from research focusing on the cytoskeletal components of the osteocyte.

Hormone Receptors in Osteocytes

Parathyroid hormone (PTH) receptors have been demonstrated on rat osteocytes *in situ* (Fermor and Skerry, 1995) and on isolated chicken osteocytes (Van der Plas *et al.*, 1994). Administered *in vitro*, PTH was reported to increase cAMP levels in isolated chicken osteocytes (Van der Plas *et al.*, 1994; Miyauchi *et al.*, 2000) and, administered *in vivo*, to increase fos protein (Takeda *et al.*, 1999) and the mRNAs of c-fos, c-jun, and I1-6 in rat osteocytes (Liang *et al.*, 1999). Therefore, although the original theory of osteocytic osteolysis and its regulation by calciotropic hormones such as PTH (Bélanger, 1969) has been abandoned, the presence of PTH receptors on osteocytes and their short-term responses to PTH suggest a role for PTH in osteocyte function. As it is now generally accepted that osteocytes are involved in the transduction of mechanical signals into chemical signals regulating bone (re)modeling, PTH might modulate the osteocytic response to mechanical strain. Injection of PTH in rats was shown to augment the osteogenic response of bone to mechanical stimulation *in vivo*, whereas thyroparathyroidectomy abrogated the mechanical responsiveness of bone (Chow *et al.*, 1998). However, such an approach cannot separate an effect at the level of osteocyte mechanosensing from one at the level of osteoprogenitor recruitment. One mechanism by which PTH may act on osteocytes was suggested by the reports of Schiller *et al.* (1992) and Donahue *et al.* (1995). These authors found that PTH increased Cx43 gene expression and gap junctional communication in osteoblastic cells. In osteocytes, where cell-to-cell communication is so important, a similar effect might lead to more efficient communication within the osteocyte network. Osteocytes also express the receptor for the carboxy-terminal region of PTH that may play a role in osteocyte viability (Divieti *et al.*, 2001). Activation of receptors for 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), which were also shown to be present in osteocytes by immunocytochemistry (Boivin *et al.*, 1987) and by *in situ* hybridization (Davideau *et al.*, 1996), may have similar effects.

Another important hormone involved in bone metabolism is estrogen. Numerous studies have demonstrated that a decrease in blood estrogen levels is accompanied by a loss of bone mass. One explanation for this phenomenon is that estrogen regulates the set point for the mechanical responsiveness of bone (Frost, 1992), i.e., that lowering the ambient estrogen level increases the level of strain in bone necessary for the bone to respond with increased bone formation. If osteocytes are the main mechanosensors of bone, it is reasonable to suppose that osteocytes are the site of set point regulation by estrogen. Estrogen receptors (ER*a*) were demonstrated in osteocytes with immunocytochemistry and *in situ* hybridization (Braidman *et al.*, 1995; Hoyland *et al.*, 1999) in tissue sections. In addition, Westbroek *et al.* (2000b) found higher levels of ER*a* in isolated osteocytes than in osteoblasts or osteoblast precursors. It has been shown that the anabolic response of bone to mechanical loading requires ER*a* (Lee *et al.*, 2003). Recent studies suggested that osteocytes indeed use ER*a* to respond to strain, although the ER*a* content is regulated by estrogen (Zaman *et al.*, 2006).

Receptors for PTH, 1,25$(OH)_2D_3$, and estrogen, as well as the androgen receptor (Abu *et al.*, 1997), the glucocorticoid receptor *a* (Abu *et al.*, 2000; Silvestrini *et al.*, 1999), and various prostaglandin receptors (Lean *et al.*, 1995; Sabbieti *et al.*, 1999) have been described in osteocytes. As outlined earlier, glucocorticoids clearly induce osteocyte apoptosis but may have additional effects. Glucocorticoid treatment causes mature osteocytes to enlarge their lacunae and remove mineral from their microenvironment (Lane *et al.*, 2006). Therefore, osteocytes appear to be able to modify their microenvironment in response to certain factors. The prostaglandin receptors, in addition to viability, may be important for communication within the osteocyte network during mechanotransduction (see later).

OSTEOCYTE FUNCTION

Blood–Calcium/Phosphate Homeostasis

The organization of osteocytes as a network of gap junction-coupled cells in each osteon represents such a unique structure that one expects it to have an important function in the metabolism and maintenance of bone. The network offers two advantages that may be exploited by the tissue.

1. A tremendous cell–bone surface contact area, about two orders of magnitude larger than the contact area the osteoblasts and lining cells have (Johnson, 1966).
2. An extensive intracellular and an extracellular communication system between sites within the bone and the bone surface.

The first consideration has led Bélanger (1969) and others to propose the hypothesis that osteocytes are capable of local bone remodeling or osteocytic osteolysis. According to this hypothesis, osteocytes are coresponsible for blood–calcium homeostasis. Later studies (Boyde, 1980; Marotti *et al.*, 1990) supplied alternative explanations for the observations that appeared to support the osteocytic osteolysis theory. The possibility remains, however, that osteocytes are involved in the facilitation of calcium diffusion in and out of the bone (Bonucci, 1990). Although the bulk of calcium transport in and out of the bone is apparently taken care of by osteoblasts and osteoclasts (Boyde, 1980; Marotti *et al.*, 1990), osteocytes may have a function in the fine regulation of blood–calcium homeostasis. The major emphasis of present day thinking is, however, on the role of the osteocyte network as a three-dimensional sensor and communication system in bone.

Osteocytes may also play a major role in phosphate homeostasis. Phex is a metalloendoproteinase found on the plasma membrane of osteoblasts and osteocytes (Ruchon *et al.*, 2000) whose substrate is not known. The precise function of Phex is unclear but it clearly plays a role in phosphate homeostasis and bone mineralization. Pex deletion or loss of function results in X-linked hypophosphatemic rickets (The Hyp Consortium 1995). Nijweide and co-workers (Westbroek *et al.*, 2002) were one of the first to propose that the osteocyte network could be considered a gland that regulates bone phosphate metabolism through expression of Phex.

Other proteins known to regulate mineralization and mineral homeostasis are also highly expressed in osteocytes, such as DMP1, Mepe, and sclerostin. Deletion or mutation of DMP1, a gene that is highly expressed in embedding osteocytes and mature osteocytes, results in hypophosphatemic rickets (Feng *et al.*, 2006), similar to the deletion of Phex. DMP1 is expressed along the canalicular wall, while Phex is expressed on the membrane surface of dendrites and the cell body. Other players in mineral metabolism include MEPE and FGF23, which are also highly expressed in osteocytes (Liu *et al.*, 2006). FGF23 is highly elevated in osteocytes in DMP1 null mice, which results in hypophosphatemia in these animals. The osteocyte network might be viewed as an endocrine gland regulating mineral metabolism. Another fascinating hypothesis is that mineral metabolism is regulated by mechanical loading. DMP1 and Pex gene expression are increased in response to load (Gluhak-Heinrich *et al.*, 2003), Sost expression is inhibited (Robling *et al.*, 2006), while a biphasic response is observed for Mepe expression (Gluhak-Heinrich *et al.*, 2005). Whether FGF23 expression is also regulated by loading is currently unknown.

Functional Adaptation: Wolff's Law

Functional adaptation is the term used to describe the ability of organisms to increase their capacity to accomplish a specific function with increased demand and to decrease this capacity with lesser demand. In the 19th century, the anatomist Julius Wolff proposed that mechanical stress is responsible for determining the architecture of bone and that bone tissue is able to adapt its mass and three-dimensional structure to the prevailing mechanical usage to obtain a higher efficiency of load bearing (Wolff, 1892). For the past century, Wolff's law has become widely accepted. Adaptation will improve an individual animal's survival chance because bone is not only hard but also heavy. Too much of it is probably as bad as too little, leading either to uneconomic energy consumption during movement (for too high bone mass) or to an enhanced fracture risk (for too low bone mass). This readily explains the usefulness of mechanical adaptation as an evolutionary driver, even if we do not understand how it is performed.

Osteocytes as Mechanosensory Cells

In principle, all cells of bone may be involved in mechanosensing, as eukaryotic cells in general are sensitive to mechanical stress (Oster, 1989). However, several features argue in favor of osteocytes as the mechanosensory cells *par excellence* of bone as discussed earlier in this chapter. In virtually all types of bone, osteocytes are dispersed throughout the mineralized matrix and are connected with their neighbor osteocytes via long, slender cell processes that run in slightly wider canaliculi of unmineralized matrix. The cell processes contact each other via gap junctions (Doty, 1981; Donahue *et al.*, 1995), thereby allowing direct cell-to-cell coupling. The superficial osteocytes are connected with the lining cells covering most bone surfaces, as well as the osteoblasts that cover (much-less abundant) surfaces where new bone is formed. From a cell biological viewpoint therefore, bone tissue is a three-dimensional network of cells, most of which are surrounded by a very narrow sheath of unmineralized matrix, followed by a much wider layer of mineralized matrix. The sheath of unmineralized matrix is penetrated easily by macromolecules such as albumin and peroxidase (McKee *et al.*, 1993; Tanaka and Sakano, 1985). However, others have shown that although small tracers (<6 nm) readily pass through the lacunar-canalicular porosity in the absence of mechanical loading, there appears to be an upper limit of size between 6 and 10 nm for molecular movement from bone capillaries to osteocytic lacunae in rat long bone. It was suggested that this range of pore size represents the fiber spacing that has been proposed for the annular space based on the presence of a proteoglycan fiber matrix surrounding the osteocytes (Wang *et al.*, 2004). Therefore, there is an intracellular as well as an extracellular route for the rapid passage of ions and signal molecules. This allows for several manners of cellular signaling from osteocytes lying deep within the bone tissue to surface-lining cells and vice versa (Cowin *et al.*, 1995).

Experimental studies indicate that osteocytes are indeed sensitive to stress applied to intact bone tissue. *In vivo* experiments using the functionally isolated turkey ulna have shown that immediately following a 6-min period of intermittent (1 Hz) loading, the number of osteocytes expressing glucose-6-phosphate dehydrogenase (G6PD) activity was increased in relation to local strain magnitude (Skerry *et al.*, 1989). The tissue strain magnitude varied between 0.05 and 0.2% (500–2000 microstrain) in line with *in vivo* peak strains in bone during vigorous exercise. Other models, including strained cores of adult dog cancellous bone, embryonic chicken tibiotarsi, mouse ulnae, rat caudal vertebrae, and rat tibiae, as well as experimental tooth movement in rats, have demonstrated that osteocytes in intact bone change their enzyme activity and RNA synthesis rapidly after mechanical loading (El Haj *et al.*, 1990; Dallas *et al.*, 1993; Lee *et al.*, 2006; Lean *et al.*, 1995; Forwood *et al.*, 1998; Terai *et al.*, 1999). These studies show that intermittent loading produces rapid changes of metabolic activity in osteocytes and suggest that osteocytes may indeed function as mechanosensors in bone. The mechanical environment of the stress-sensitive osteocyte varies with the geometry of the osteocyte lacuna (McCreadie *et al.*, 2004). Computer simulation studies of bone remodeling, assuming this to be a self-organizational control process, predict a role for osteocytes, rather than lining cells and osteoblasts, as stress sensors of bone (Mullender and Huiskes, 1995, 1997; Huiskes *et al.*, 2000; Ruimerman *et al.*, 2005). A regulating role of strain-sensitive osteocytes in basic multicellular unit (BMU) coupling has been postulated by Smit and Burger (2000). Using finite-element analysis, the subsequent activation of osteoclasts and osteoblasts during coupled bone remodeling was shown to relate to opposite strain distributions in the surrounding bone tissue. In front of the cutting cone of a forming secondary osteon, an area of *decreased* bone strain was demonstrated, whereas a layer of *increased* strain occurs around the closing cone (Smit and Burger, 2000). Osteoclasts therefore attack an area of bone where the osteocytes are underloaded, whereas osteoblasts are recruited in a bone area where the osteocytes are overloaded. Hemiosteonic remodeling of trabecular bone showed a similar strain pattern (Smit and Burger, 2000). Thus, bone remodeling regulated by load-sensitive osteocytes can explain the maintenance of osteonic and trabecular architecture as an optimal mechanical structure, as well as adaptation to alternative external loads (Huiskes *et al.*, 2000; Smit and Burger, 2000).

If osteocytes are the mechanosensors of bone, how do they sense mechanical loading? This key question is, unfortunately, still open because it has not yet been established unequivocally how the loading of intact bone is transduced into a signal for the osteocytes. The application of force to bone during movement results in several potential cell stimuli. These include changes in hydrostatic pressure, direct cell strain, fluid flow, and electric fields resulting from electrokinetic effects accompanying fluid flow (Pienkowski and Pollack, 1983). Evidence has been increasing steadily for the flow of canalicular interstitial fluid as the likely stress-derived factor that informs the osteocytes about the level of bone loading (Cowin *et al.*, 1991, 1995; Cowin, 1999; Weinbaum *et al.*, 1994; Klein-Nulend *et al.*, 1995b; Knothe-Tate, 2000; Burger and Klein-Nulend, 1999; You *et al.*, 2000). In this view, canaliculi are the bone porosity of interest, and the osteocytes the mechanosensor cells.

Canalicular Fluid Flow and Osteocyte Mechanosensing

In healthy, adequately adapted bone, strains as a result of physiological loads (e.g., resulting from normal locomotion) are quite small. Quantitative studies of the strain in bones of performing animals (e.g., galloping horses, fast-flying birds, even a running human volunteer) found a maximal strain not higher than 0.2–0.3% (Rubin, 1984; Burr *et al.*, 1996). This poses a problem in interpreting the results of *in vitro* studies of strained bone cells, where much higher deformations, in the order of 1–10%, were needed to obtain a cellular response (for a review, Burger and Veldhuijzen, 1993). In these studies, isolated bone cells were usually grown on a flexible substratum, which is then strained by stretching or bending. For instance, unidirectional cell stretching of 0.7% was required to activate prostaglandin E_2 production in primary bone cell cultures (Murray and Rushton, 1990). However, in intact bone, a 0.15% bending strain was already sufficient to activate prostaglandin-dependent adaptive bone formation *in vivo* (Turner *et al.*, 1994; Forwood, 1996). If we assume that bone organ strain is somehow involved in bone cell mechanosensing, then bone tissue seems to possess a lever system whereby small matrix strains are transduced into a larger signal that is detected easily by osteocytes. The canalicular flow hypothesis proposes such a lever system. Indeed, *in vitro* experiments that relate the effects of fluid flow and substrate straining have shown that fluid flow-induced shear stress induces higher release of signaling molecules (McGarry *et al.*, 2005). A numerical study showed that the deformation of cells on a two-dimensional substrate caused by fluid flow is fundamentally different from that induced by substrate straining (McGarry *et al.*, 2005). Fluid shear stress had a larger overturning effect on the bone cells, while substrate strain predominantly affected cell-substrate attachments. Whether these observations can be extrapolated towards osteocytes that are embedded in a three-dimensional matrix is a matter of debate, but they clearly indicate that substrate deformation and a flow of interstitial fluid could have differential effects on cells.

The flow of extracellular tissue fluid through the lacuno-canalicular network as a result of bone tissue strains was made plausible by the theoretical study of Piekarski and Munro (1977) and has been shown experimentally by Knothe-Tate and colleagues (1998, 2000). This strain-derived extracellular fluid flow may help keep osteocytes healthy, particularly the deeper ones, by facilitating the exchange of nutrients and waste products between the Haversian channel and the osteocyte network of an osteon (Kufahl and Saha, 1990). However, a second function of this strain-derived interstitial fluid flow could be the transmission of "mechanical information" (Fig. 4). The magnitude of interstitial fluid flow through the lacunocanalicular network is directly related to the amount of strain of the bone organ (Cowin *et al.*, 1991). Because of the narrow diameter of the canaliculi, bulk bone strains of about 0.1% will produce a fluid shear stress in the canaliculi of roughly 1 Pa (Weinbaum *et al.*, 1994), enough to produce a rapid response in endothelial cells (Frangos *et al.*, 1985; Kamiya and Ando, 1996).

Experimental studies *in vitro* have demonstrated that osteocytes are indeed quite sensitive to the fluid shear stress of such a magnitude compared to osteoblasts and osteo-progenitor cells (Klein-Nulend *et al.*, 1995a,b; Ajubi *et al.*, 1996; Westbroek *et al.*, 2000; Westbroek *et al.*, 2001). These results suggest that the combination of the cellular three-dimensional network of osteocytes and the accompanying porous network of lacunae and canaliculi acts as the mechanosensory organ of bone. Different loading-induced canalicular flow patterns around cutting cone and reversal zone during remodeling could, for instance, explain the typical alignment of haversian canals (Burger *et al.*, 2003). Volumetric strain in the bone around a BMU cutting cone has been related to canalicular fluid flow (Smit *et al.*, 2002), and the predicted area of low canalicular flow around the tip of the cutting cone was proposed to induce local osteocyte apoptosis. Unloading-induced osteocytes apoptosis has been shown in rat bone, and was highly associated with osteoclastic bone resorption (Basso *et al.*, 2006). Osteocyte apoptosis at the tip of the cutting cone would attract osteoclasts, leading to further excavation of bone in the direction of loading. Importantly, mechanical loading by fluid shear stress have been shown to promote osteocyte survival (Bakker *et al.*, 2004). The model by Smit *et al.* (2002) further predicts that at the base of the cutting cone and further down the reversal zone, osteocytes receive enhanced fluid shear stress during loading. This could prevent osteocyte apoptosis, but may also promote the retraction and detachment of osteoclasts from the bone surface. These two mechanisms, attraction of osteoclasts to the cutting cone tip and induction of osteoclast detachment from the cutting cone base, together explain the mechanically meaningful behaviour of osteoclasts during remodeling.

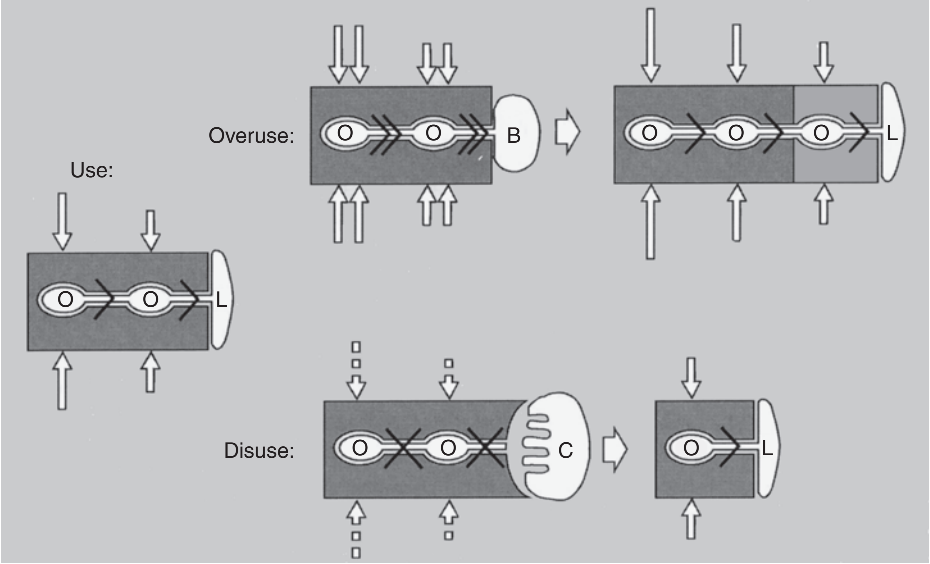

FIGURE 4 Schematic representation of how the osteocyte network may regulate bone modeling. In the steady state (USE), normal mechanical use ensures a basal level of fluid flow through the lacunocanalicular porosity, indicated by an arrowhead through the canaliculi. This basal flow keeps the osteocytes viable and also ensures basal osteocyte activation and signaling, thereby suppressing osteoblastic activity as well as osteoclastic attack. During (local) overuse (OVERUSE), osteocytes are overactivated by enhanced fluid flow (indicated by double arrowheads), leading to the release of osteoblast-recruiting signals. Subsequent osteoblastic bone formation reduces the overuse until normal mechanical use is reestablished, thereby reestablishing the steady state of basal fluid flow. During (local) disuse (DISUSE), osteocytes are inactivated by lack of fluid flow (indicated by crosses through canaliculi). Inactivation leads to a release of osteoclast-recruiting signals, to a lack of osteoclast-suppressing signals, or both. Subsequent osteoclastic bone resorption reestablishes normal mechanical use (or loading) and basal fluid flow. O, osteocyte; L, lining cell; B, osteoblast; C, osteoclast; dark-gray area, mineralized bone matrix; light-gray area, newly formed bone matrix; white arrows represent direction and magnitude of loading. Adapted from Burger and Klein-Nulend (1999).

The flow of interstitial fluid through the bone canaliculi will have two effects: a mechanical one derived from the fluid shear stress and an electrokinetic one derived from streaming potentials (Pollack *et al.*, 1984; Salzstein and Pollack, 1987). Either of the two, or both in combination, might activate the osteocyte. For instance, streaming potentials might modulate the movement of ions such as calcium across the cell membrane (Hung *et al.*, 1995, 1996), whereas shear stress will pull at the macromolecular attachments between the cell and its surrounding matrix (Wang and Ingber, 1994). Both ion fluxes and cellular attachment are powerful modulators of cell behavior and therefore good conveyors of physical information (Sachs, 1989; Ingber, 1991). *In vitro* experiments using bone cells subjected to a flow of fluid of increasing viscosity suggest that fluid shear stress is the major activator of the bone cell response to loading (Bakker *et al.*, 2001). However, care should be taken when extrapolating results obtained with cells seeded on a two-dimensional surface to the three-dimensional *in vivo* situation.

Mechanosensation and the resulting mechanotransduction of biochemical signals may be a complex event (Bonewald, 2006). Perturbation of dendrites, the cell body, and even primary cilia could be involved in combination or sequentially in this response. The osteocytes' response to different kinds of mechanical loading has predominantly been studied in cell cultures or entire bone, while knowledge of mechanosensing in osteocytes single-cell level is essential for understanding the complex process of bone adaptation. For instance, the mechanosensitive part of the osteocytes, the cell body or the cell processes, has not yet been determined. More information is needed on the single osteocyte's response to a localized mechanical stimulation.

It has been suggested that fluid flow over dendrites in the lacunar-canalicular porosity can induce strains in the actin filament bundles of the cytoskeleton that are more than an order of magnitude larger than tissue level strains. Using the latest ultrastructural data for the cell process cytoskeleton and the tethering elements that attach the process to the canalicular wall, a realistic three-dimensional model was created for the osteocyte process. Using this model, the deformed shape of the tethering elements and the hoop strain on the central actin bundle as a result of loading induced fluid flow was predicted. It was found that tissue-level strains of >1,000 microstrain at 1 Hz resulted in a hoop strain of >0.5%. The tethering elements of the osteocyte process can thus act as a strain-amplifier (Han *et al.*, 200).

Recent publications have shown that osteocytes express a single primary cilia (Xiao *et al.*, 2006). These authors also showed that PKD1/PC1, a known mechanosensory protein in the kidney, does play a role in normal bone structure. Primary cilia in other cells clearly function as sensors of odors, light, and movement (Singla and Reiter, 2005). It remains to be determined whether the bone defect in animals with defective PKD1 function is due to defective function of PKD1 in cilia, or whether PKD1 has a function in another location in the cell. Very recent, early data suggest that loss of cilia results in decreased sensitivity to fluid flow shear stress (Malone *et al.*, 2006). Determining the function of PKD1 and/or cilia should lend insight into mechanotransduction in osteocytes.

The osteocyte has either been viewed as a quiescent cell acting as a "placeholder" in bone or as a mechanosensory cell as outlined in this chapter. Most observations using static two-dimensional imaging suggest that the dendritic processes are somewhat permanently anchored to the lacunar wall. Dallas and co-workers have shown cell body movement and the extension and retraction of dendritic processes over time using dynamic imaging of living osteocytes within their lacunae (Veno *et al.*, 2006). Calvarial explants from transgenic mice with green fluorescent protein (GFP) expression targeted to osteocytes revealed that, far from being a static cell, the osteocyte is highly dynamic. Therefore dendrites, rather than being permanent connections between osteocytes and with bone surface cells, may have the capacity to connect and disconnect and reconnect. With this new information, theories regarding shear stress within lacunae and osteocyte signaling will require modification.

Response of Osteocytes to Fluid Flow *in Vitro*

The technique of immunodissection, as discussed earlier in this chapter, made it possible to test the canalicular flow hypothesis by comparing the responsiveness of osteocytes, osteoblasts, and osteoprogenitor cells to fluid flow. The strength of the immunodissection technique is that three separate cell populations with a high (90%) degree of homogeneity are prepared, representing (1) osteocytes with the typical "spider-like" osteocyte morphology and little matrix synthesis, (2) osteoblasts with a high synthetic activity of bone matrix-specific proteins, and (3) (from the periosteum) osteoprogenitor cells with a fibroblast-like morphology and very high proliferative capacity (Nijweide *et al.*, 1986). Because the cells are used within 2 days after isolation from the bone tissue, they may well represent the three differentiation steps of osteoprogenitor cell, osteoblast, and osteocyte. In contrast, mixed cell cultures derived from bone that are generally used to represent "osteoblastic" cells likely contain cells in various stages of differentiation. Therefore, changes in mechanosensitivity related to progressive cell differentiation cannot be studied in such cultures. The development of differentiation stage specific bone-cell lines have facilitated the comparison in cell-type specific responses to mechanical loading. Recently Ponik *et al.* (2007) have described significant differences in the response to fluid shear between MC3T3-E1 osteoblasts and MLO-Y4 osteocytes. Stress fibers were formed and aligned within osteoblasts after 1 h of unidirectional fluid flow, but this response was not observed until greater than 5 h of oscillatory fluid flow (Ponik *et al.*, 2007). However, owing to their clonal selection, cell lines do not necessarily express the whole range of bone-specific genes characteristic of primary bone cells. Therefore, the use of primary bone cells can be preferred over the use of cell lines when comparing the behavior of osteoblasts and osteocytes.

Using immunoseparated primary cell populations, osteocytes were found to respond far stronger to fluid flow than osteoblasts and these stronger than osteoprogenitor cells (Klein-Nulend *et al.*, 1995a,b; Ajubi *et al.*, 1996; Westbroek *et al.*, 2000a). Osteocytes also appeared to be more responsive to fluid flow than osteoblasts or osteoprogenitor cells with respect to the production of soluble signaling molecules affecting osteoblast proliferation and differentiation (Vezeridis *et al.*, 2005). The release of these soluble factors was at least partially dependent on the activation of a nitric oxide (NO) pathway in osteocytes in response to fluid flow. Pulsating fluid flow (PFF) with a mean shear stress of 0.5 Pa (5 dynes/cm^2) with a cyclic variation of plus or minus 0.2 Pa at 5 Hz stimulated the release of NO and prostaglandin E_2 and I_2 (PGE_2 and PGI_2) rapidly from osteocytes within minutes (Klein-Nulend *et al.*, 1995a; Ajubi *et al.*, 1996). Osteoblasts showed less response, and osteoprogenitor cells (periosteal fibroblasts) still less. Intermittent hydrostatic compression (IHC) of 13,000 Pa peak pressure at 0.3 Hz (1 sec compression followed by 2 sec relaxation) needed more than a 1-hr application before prostaglandin production was increased, again more in osteocytes than in osteoblasts, suggesting that mechanical stimulation via fluid flow is more effective than hydrostatic compression (Klein-Nulend *et al.*, 1995b). A 1-hr treatment with PFF also induced a sustained release of PGE_2 from the osteocytes in the hour following PFF treatment (Klein-Nulend *et al.*, 1995b). This sustained PGE_2 release, continuing after PFF treatment had been stopped, could be ascribed to the induction of prostaglandin G/H synthase-2 (or cyclooxygenase 2, COX-2) expression (Westbroek *et al.*, 2000a). Again, osteocytes were much more responsive than osteoblasts and osteoprogenitor cells, as only a 15-min treatment with PFF increased COX-2 mRNA expression by threefold in osteocytes but not in the other two cell populations (Westbroek *et al.*, 2000). Upregulation of COX-2 but not COX-1 by PFF had been shown earlier in a mixed population of mouse calvarial cells (Klein-Nulend *et al.*, 1997) and was also demonstrated in primary bone cells from elderly women (Joldersma *et al.*, 2000), whereas the expression of COX-1 and -2 in osteocytes and osteoblasts in intact rat bone has been documented (Forwood *et al.*, 1998). These *in vitro* experiments on immunoseparated cells suggest that as bone cells mature, they increase their capacity to produce prostaglandins in response to fluid flow (Burger and Klein-Nulend, 1999). First, their immediate production of PGE_2, PGI_2, and probably $PGF_{2}a$ (Klein-Nulend *et al.*, 1997), in response to flow, increases as they develop from osteoprogenitor cell, via the osteoblastic stage into osteocytes. Second, their capacity to increase expression of COX-2 in response to flow, and thereby to continue to produce PGE_2 even after the shear stress has stopped (Westbroek *et al.*, 2000a), increases as they reach terminal differentiation. Because induction of COX-2 is a crucial step in the induction of bone formation by mechanical loading *in vivo* (Forwood, 1996), these results provide direct experimental support for the concept that osteocytes, the long-living terminal differentiation stage of osteoblasts, function as the "professional" mechanosensors in bone tissue.

Pulsating fluid flow also rapidly induced the release of NO in osteocytes but not in osteoprogenitor cells (Klein-Nulend *et al.*, 1995a). Rapid release of NO was also found when whole rat bone rudiments were mechanically strained in organ culture (Pitsillides *et al.*, 1995) and in human bone cells submitted to fluid flow (Sterck *et al.*, 1998). In line with these *in vitro* observations, inhibition of NO production inhibited mechanically induced bone formation in animal studies (Turner *et al.*, 1996; Fox *et al.*, 1996). NO is a ubiquitous messenger molecule for intercellular communication and is involved in many tissue reactions where cells must collaborate and communicate with each other (Koprowski and Maeda, 1995). The intracellular upregulation of NO after mechanical stimulation has been shown in single bone cells using DAR-4M AM chromophore (Vatsa *et al.*, in press). It was shown that a single osteocyte can disseminate a mechanical stimulus to its surrounding osteocytes via extracellular soluble signaling factors, which reinforces the putative mechanosensory role of osteocytes, and demonstrates a possible mechanism by which a single mechanically stimulated osteocyte can communicate with other cells in a bone multicellular unit, which might help to better understand the intricacies of intercellular interactions in BMUs and thus bone remodeling (Vatsa *et al.*, in press). Another interesting example of the involvement of NO in tissue reactions where cells must collaborate and communicate with each other is the adaptation of blood vessels to changes in blood flow. In blood vessels, enhanced blood flow, e.g., during exercise, leads to widening of the vessel to ensure a constant blood pressure. This response depends on the endothelial cells, which sense the increased blood flow, and produce intercellular messengers such as NO and prostaglandins. In response to these messengers, the smooth muscle cells around the vessel relax to allow the vessel to increase in diameter (Kamiya and Ando, 1996). The capacity of endothelial cells to produce NO in response to fluid flow is related to a specific enzyme, endothelial NO synthase or ecNOS. Interestingly, this enzyme was found in rat bone lining cells and osteocytes (Helfrich *et al.*, 1997; Zaman *et al.*, 1999) and in cultured bone cells derived from human bone (Klein-Nulend *et al.*, 1998). Both endothelial and neuronal NOS isoforms are present in osteocytes (Cabbalero-alias *et al.*, 2004). The rapid release by mechanically stressed bone cells makes NO an interesting candidate for intercellular communication within the three-dimensional network of bone cells. Treatment with pulsatile fluid flow increased the level of ecNOS RNA transcripts in the bone cell cultures (Klein-Nulend *et al.*, 1998), a response also described in endothelial cells (Busse and Fleming, 1998; Uematsu *et al.*, 1995). Enhanced production of prostaglandins is also a well-described response of endothelial cells to fluid flow (Busse and Fleming, 1998; Kamiya and Ando, 1996). It seems, therefore, that endothelial cells and osteocytes possess a similar sensor system for fluid flow and that both cell types are "professional" sensors of fluid flow.

Mechanotransduction starts by the conversion of physical loading-derived stimuli into cellular signals. Several studies suggest that the attachment complex between intracellular actin cytoskeleton and extracellular matrix macromolecules, via integrins and CD44 receptors in the cell membrane, provides the site of mechanotransduction (Wang *et al.*, 1993; Watson, 1991; Ajubi *et al.*, 1996, 1999; Pavalko *et al.*, 1998). An important early response is the influx of calcium ions through mechanosensitive ion channels in the plasma membrane and the release of calcium from internal stores (Hung *et al.*, 1995, 1996; Ajubi *et al.*, 1999; Chen *et al.*, 2000; You *et al.*, 2000). The rise in intracellular calcium concentration activates many downstream signaling cascades such as protein kinase C and phospholipase A_2 and is necessary for activation of calcium/calmodulin dependent proteins such as NOS. The activation of phospholipase A_2 results among others in the activation of arachidonic acid production and PGE_2 release (Ajubi *et al.*, 1999). Other genes whose expression in osteocytes is modified by mechanical loading include c-fos, MEPE and IGF-I (Lean *et al.*, 1995; 1996).

Many steps in the mechanosignaling cascade are still unknown in osteocytes as well as other mechanosensory cells. Recently, it has been shown that the Wnt family of proteins strongly modulate the anabolic response of bone to mechanical loading (Robinson *et al.*, 2006). More research will need to be conducted to elucidate the physiological role of Wnts in bone cell mechanotransduction. Undoubtedly, in the mean time new players in the field of bone cell mechanotransduction will be identified.

SUMMARY AND CONCLUSION

Tremendous progress has been made in discerning the function of osteocytes since this chapter was first written about seven or eight years ago. One only has to search the term "osteocytes" on *PubMed* to see the dramatic increase in references to and studies of this elusive bone cell. Markers for osteocytes have been expanded to delineate early to late stages of osteocyte differentiation and the critical role of mineralization in this process. Genomic profiling of osteocytes is a storehouse of information regarding the potential functions of these cells and their response to strain.

Molecular mechanisms and pathways involved in osteocyte mechanosensation have been identified and expanded significantly. Although some investigators hold to the concept of a single mechanoreceptor initiating a linear cascade of events, new theories suggesting simultaneous triggering events in response to load are being investigated. It remains to be determined what makes these cells more responsive to shear stress than to osteoblasts and what role the cell body, dendrites, and even cilia may play in this response.

These cells make up 90–95% of all bone cells in the adult skeleton. Collectively, any minor modulation of the entire population could have significant local and systemic effects, not only on bone, but also on other organs. Although it was postulated several decades ago that osteocytes could potentially play a role in calcium metabolism, it appears that this cell may have a more important role in phosphate metabolism. It remains to be determined how factors produced by osteocytes can enter the circulation suggesting an intimate connection between the lacuno-canalicular system and the blood supply.

One reason for the dramatic expansion in knowledge in the field of osteocyte biology is the generation of new tools and the advancement of technology. No longer is this cell safe within its mineralized cave from "prying eyes." Transgenic technology has targeted the osteocyte with fluorescent markers and generated conditional deletions. Cell lines, osteocyte selective and specific antibodies, and probes have been generated. Advanced instrumentation such as Raman spectroscopy and atomic force microscopy can probe and characterize the osteocyte microenvironment. Dynamic imaging has revealed osteocyte movement within their lacuna and canaliculi.

Even with these advances, we may still only be seeing "the tip of the iceberg" with regards to osteocyte function. It has been proposed that the osteocyte could be the orchestrator of bone remodeling in the adult skeleton that directs both osteoblast and osteoclast function. The future looks exciting and full of new discoveries with regards to the biology of the osteocyte and its role in mechanosensation.

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Chapter 9

Osteoclast Generation

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INTRODUCTION

Osteoclasts, the multinucleated giant cells that resorb bone, develop from hematopoietic cells of the monocyte-macrophage lineage. We developed a mouse coculture system of osteoblasts/stromal cells and hematopoietic cells in which osteoclasts are formed in response to bone-resorbing factors such as 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], parathyroid hormone (PTH), prostaglandin E_2 (PGE_2), and interleukin 11 (IL-11). A series of experiments using this coculture system established the concept that osteoblasts/stromal cells are crucially involved in osteoclast development. Cell-to-cell contact between osteoblasts/stromal cells and osteoclast progenitors is necessary for inducing osteoclast differentiation in the coculture system. Studies on macrophage colony-stimulating factor (M-CSF, also called CSF-1)-deficient *op/op* mice have shown that M-CSF produced by osteoblasts/stromal cells is an essential factor for inducing osteoclast differentiation from monocyte-macrophage lineage cells. Subsequently, another essential factor for osteoclastogenesis, receptor activator of nuclear factor κB ligand (RANKL) was molecularly cloned in 1998. RANKL [also known as osteoclast differentiation factor (ODF)/osteoprotegerin ligand (OPGL)/tumor necrosis factor (TNF)-related activation-induced cytokine (TRANCE)] is a member of the TNF-ligand family, which is expressed as a membrane-associated protein in osteoblasts/stromal cells in response to many bone-resorbing factors. Osteoclast precursors that possess RANK (receptor activator of nuclear factor κB), a TNF receptor family member, recognize RANKL through cell–cell interaction with osteoblasts/stromal cells and differentiate into osteoclasts in the presence of M-CSF. Mature osteoclasts also possess RANK, and RANKL induces their bone-resorbing activity. Osteoprotegerin [OPG, also called osteoclastogenesis inhibitory factor (OCIF)] mainly produced by osteoblasts/stromal cells is a soluble decoy receptor for RANKL. OPG has been shown to function as an inhibitory factor for osteoclastogenesis *in vivo* and *in vitro*. Mutations of OPG, RANK, and RANKL have been detected in humans. TNFα and IL-1 directly induce differentiation and function of osteoclasts, respectively, by a mechanism independent of the RANKL–RANK interaction. This chapter describes the history of the discovery of RANKL and its development afterward.

ROLE OF OSTEOBLASTS/STROMAL CELLS IN OSTEOCLAST DIFFERENTIATION AND FUNCTION

Role of Osteoblasts/Stromal Cells in Osteoclast Differentiation

Development of osteoclasts proceeds within a local microenvironmental milieu of bone. This process can be reproduced *ex vivo* in a coculture of mouse calvarial osteoblasts and hematopoietic cells (Takahashi *et al.*, 1988; Suda *et al.*, 1992; Chambers *et al.*, 1993). Multinucleated cells formed

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TABLE I Osteoclast Formation in Cocultures with hIL-6R Transgenic Mice or Mice Carrying the Disrupted Genes of VDR, PTHR1, or EP4

	Coculture system[a]			
Osteotropic factor	Osteoblasts	Hematopoietic cells	Osteoclast formation	Reference
hIL-6	wt	hIL-6R tg	−	Udagawa *et al.* (1995)
	hIL-6R tg	wt	+	
PTH	PTHR1(−/−)	wt	−	Liu *et al.* (1998)
	wt	PTHR1(−/−)	+	
PGE_2	EP4(−/−)	wt	−	Sakuma *et al.* (2000)
	wt	EP4(−/−)	+	
$1\alpha,25(OH)_2D_3$	VDR(−/−)	wt	−	Takeda *et al.* (1999)
	wt	VDR(−/−)	+	

[a] *Osteoblasts or hematopoietic cells obtained from human* IL-6R (*h*IL-6R) *transgenic* (*tg*) *mice*, VDR *knockout* [VDR(−/−)] *mice*, PTHR1 *knockout* [PTHR1(−/−)] *mice, or* EP4 *knockout* [EP4(−/−)] *mice were cocultured with their counterparts* (*osteoblasts or hematopoietic cells*) *obtained from wild-type* (*wt*) *mice.*

in the coculture exhibit major characteristics of osteoclasts, including tartrate-resistant acid phosphatase (TRAP) activity, expression of calcitonin receptors, c-Src (p60c-Src), vitronectin receptors ($\alpha_v\beta_3$), and the ability to form resorption pits on bone and dentine slices (Suda *et al.*, 1992). Some mouse stromal cell lines, such as MC3T3-G2/PA6 and ST2, are able to support osteoclastogenesis when cultured with mouse spleen cells (Udagawa *et al.*, 1989). In this coculture, osteoclasts are formed in response to various osteotropic factors, including $1\alpha,25(OH)_2D_3$, PTH, PGE_2, and IL-11. Cell-to-cell contact between osteoblasts/stromal cells and osteoclast progenitors is required to induce osteoclastogenesis. Subsequent experiments established that the target cells of osteotropic factors for inducing osteoclast formation *in vitro* are osteoblasts/stromal cells (Table I).

IL-6 exerts its activity via a cell surface receptor that consists of two components: a ligand-binding IL-6 receptor (IL-6R) and a non-ligand-binding but signal-transducing protein gp130. The genetically engineered soluble IL-6R (sIL-6R), which lacks both transmembrane and cytoplasmic domains, has been shown to mediate IL-6 signals through gp130 in response to IL-6. Neither recombinant IL-6 nor sIL-6R alone induced osteoclast formation in coculture, but osteoclasts were formed in response to IL-6 in the presence of sIL-6R (Tamura *et al.*, 1993). This suggests that a signal(s) mediated by gp130 is involved in osteoclast development, and that osteoblasts/stromal cells do not have enough membrane-bound IL-6R. Using transgenic mice constitutively expressing human IL-6R, it was shown that the expression of human IL-6R in osteoblasts was indispensable for inducing osteoclast recruitment (Udagawa *et al.*, 1995) (see Table I). When osteoblasts obtained from human IL-6R transgenic mice were cocultured with normal spleen cells, osteoclast formation was induced in response to human IL-6 without adding human sIL-6R. Indeed, cytokines such as IL-11, oncostatin M, and leukemia inhibitory factor (LIF), which transduce their signals through gp130 in osteoblasts/stromal cells, induced osteoclast formation in the coculture. These results established for the first time the concept that bone-resorbing cytokines using gp130 as a common signal transducer act directly on osteoblasts/stromal cells but not on osteoclast progenitors to induce osteoclast formation.

Requirement of PTH/PTHrP receptors (PTHR1) in the osteoblast was confirmed by using cocultures of osteoblasts and spleen cells obtained from PTHR1 knockout mice (Liu *et al.*, 1998). Osteoblasts obtained from PTHR1(−/−) mice failed to support osteoclast development in cocultures with normal spleen cells in response to PTH (see Table I). Osteoclasts were formed in response to PTH in cocultures of spleen cells obtained from PTHR1(−/−) mice and normal calvarial osteoblasts. This suggests that the expression of PTHR1 in osteoblasts/stromal cells is critical for PTH-induced osteoclast formation *in vitro*.

PGE_2 exerts its effects through PGE receptors (EPs) that consist of four subtypes (EP1, EP2, EP3, and EP4). Intracellular signaling differs among the receptor subtypes: EP1 is coupled to Ca^{2+} mobilization and EP3 inhibits adenylate cyclase activity, whereas both EP2 and EP4 stimulate adenylate cyclase activity. It was reported that 11-deoxy-PGE_1 (an EP4 and EP2 agonist) stimulated osteoclast formation more effectively than butaprost (an EP2 agonist) and other EP agonists in the coculture of primary osteoblasts and bone marrow cells, suggesting that EP4 is the main receptor responsible for PGE_2-induced osteoclast formation (Sakuma *et al.*, 2000). Furthermore, the PGE_2-induced osteoclast formation was not observed in the coculture of osteoblasts from EP4(−/−) mice and spleen cells from wild-type mice, whereas osteoclasts were formed in the coculture of wild-type osteoblasts and EP4(−/−) spleen cells (Sakuma *et al.*, 2000) (see Table 1). These results indicate that PGE_2 enhances osteoclast formation through the receptor subtype EP4 on osteoblasts. Li *et al.* (2000b) used cells from mice in which the EP2 receptor had been disrupted to test the role of EP2 in osteoclast formation. The response to PGE_2 for osteoclast formation was also reduced in cultures of bone marrow cells obtained from EP2(−/−) mice. In mouse calvarial organ cultures, the EP4 agonist stimulated bone resorption markedly, but its maximal stimulation was less than that induced by PGE_2 (Suzawa *et al.*, 2000). The EP2 agonist also stimulated bone resorption, but only slightly. EP1 and EP3 agonists showed no effect on bone resorption. These findings suggest that PGE_2 stimulates bone resorption by a mechanism involving cAMP production in osteoblasts/stromal cells, mediated mainly by EP4 and partially by EP2.

The other known pathway used for osteoclast formation is that stimulated by $1\alpha,25(OH)_2D_3$. Using $1\alpha,25(OH)_2D_3$ receptor (VDR) knockout mice, Takeda *et al.* (1999) clearly showed that the target cells of $1\alpha,25(OH)_2D_3$ for inducing osteoclasts in the coculture were also osteoblasts/stromal cells but not osteoclast progenitors (see Table 1). Spleen cells from VDR(−/−) mice differentiated into osteoclasts when cultured with normal osteoblasts in response to $1\alpha,25(OH)_2D_3$. In contrast, osteoblasts obtained from VDR(−/−) mice failed to support osteoclast development in coculture with wild-type spleen cells in response to $1\alpha,25(OH)_2D_3$. These results suggest that the signals mediated by VDR are also transduced into osteoblasts/stromal cells to induce osteoclast formation in the coculture.

Thus, the signals induced by almost all of the bone-resorbing factors are transduced into osteoblasts/stromal cells to recruit osteoclasts in the coculture. Therefore, we proposed that osteoblasts/stromal cells express ODF, which was hypothesized to be a membrane-bound factor in promoting the differentiation of osteoclast progenitors into osteoclasts through a mechanism involving cell-to-cell contact (Suda *et al.*, 1992) (Fig. 1). Chambers *et al.* (1993) also proposed a similar hypothesis, in which stromal osteoclast-forming activity (SOFA) is expressed by osteoblasts/stromal cells to support osteoclast formation.

Discovery of M-CSF as an Essential Factor for Osteoclastogenesis

Experiments with the *op/op* mouse model established the role of M-CSF for osteoclast formation. Yoshida *et al.*

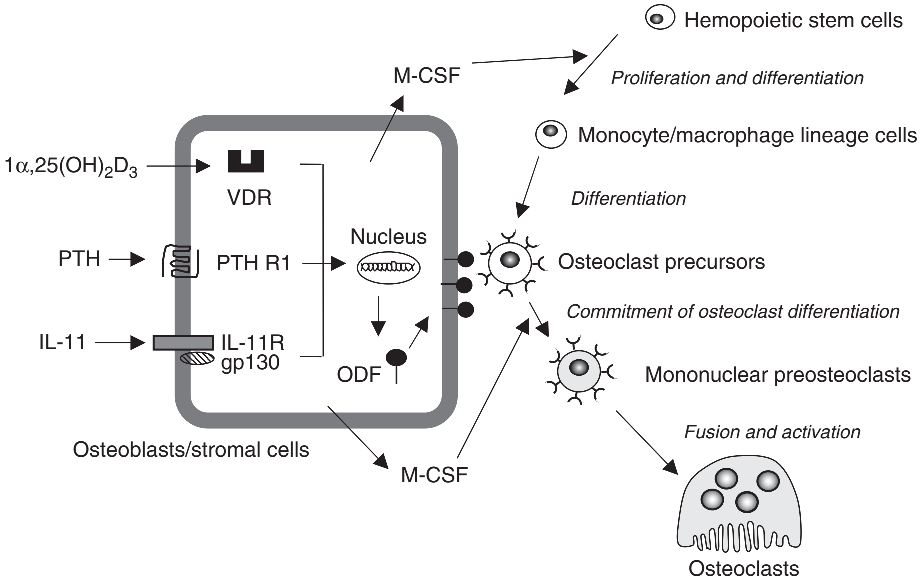

FIGURE 1 A hypothetical concept of osteoclast differentiation and a proposal for osteoclast differentiation factor (ODF). Osteotropic factors such as $1\alpha,25(OH)_2D_3$, PTH, and IL-11 stimulate osteoclast formation in mouse cocultures of osteoblasts/stromal cells and hematopoietic cells. The target cells of these osteotropic factors are osteoblasts/stromal cells. Three independent signaling pathways mediated by $1\alpha,25(OH)_2D_3$-VDR, PTH-PTHR1, and IL-11-IL-11R/gp130 interactions induce ODF as a membrane-associated factor in osteoblasts/stromal cells in similar manners. Osteoclast progenitors of the monocyte-macrophage lineage recognize ODF through cell–cell interaction with osteoblasts/stromal cells and differentiate into osteoclasts. M-CSF produced by osteoblasts/stromal cells is a prerequisite for both proliferation and differentiation of osteoclast progenitors. This hypothetical concept has been proved molecularly by the discovery of the RANKL–RANK interaction.

(1990) demonstrated that there is an extra thymidine insertion at base pair 262 in the coding region of the M-CSF gene in *op/op* mice. This insertion generated a stop codon (TGA) 21 bp downstream, suggesting that the M-CSF gene of *op/op* mice cannot code for the functionally active M-CSF protein. In fact, administration of recombinant human M-CSF restored the impaired bone resorption of *op/op* mice *in vivo* (Felix *et al.*, 1990; Kodama *et al.*, 1991). Calvarial osteoblasts obtained from *op/op* mice could not support osteoclast formation in cocultures with normal spleen cells, even in the presence of 1α,25$(OH)_2D_3$ (Takahashi *et al.*, 1991). The addition of M-CSF to the coculture with *op/op* osteoblastic cells induced osteoclast formation from normal spleen cells in response to 1α,25$(OH)_2D_3$. In contrast, spleen cells obtained from *op/op* mice were able to differentiate into osteoclasts when cocultured with normal osteoblasts. It was shown that M-CSF is involved in both proliferation of osteoclast progenitors and their differentiation into osteoclasts (Tanaka *et al.*, 1993; Felix *et al.*, 1994).

Begg *et al.* (1993) investigated age-related changes in osteoclast activity in *op/op* mice. Femurs of newborn *op/op* mice were infiltrated heavily with bone, and the marrow hemopoiesis was reduced significantly. However, the femoral marrow cavity of *op/op* mice enlarged progressively with the concomitant appearance of tartrate-resistant acid phosphatase (TRAP, a marker enzyme of osteoclasts)-positive osteoclasts, and by 22 weeks of age the marrow hemopoiesis was comparable to that of controls. Niida *et al.* (1999) reported that a single injection in *op/op* mice with recombinant human vascular endothelial growth factor (VEGF) induced osteoclast recruitment. VEGF receptor 1 (VEGFR-1/Flt-1) is a high-affinity tyrosine kinase (TK) receptor for VEGF and regulates angiogenesis as well as monocyte/macrophage functions. It was also reported that when a VEGFR-1 signaling deficiency [Flt1(TK)(−/−)] was introduced into *op/op* mice, the double mutant [*op/op* Flt1(TK)(−/−)] mice exhibited severer osteoclast deficiency (Niida *et al.*, 2005). These results suggest that factors other than M-CSF, including VEGF, can be substituted for M-CSF to induce osteoclast formation under special occasions.

Role of Osteoblasts/Stromal Cells in Osteoclast Function

One of the major technical difficulties associated with the analysis of mature osteoclasts is their strong adherence to plastic dishes. A collagen gel-coated culture using mouse bone marrow cells and osteoblasts/stromal cells was developed to obtain a cell preparation containing functionally active osteoclasts (Suda *et al.*, 1997a). The purity of osteoclasts in this preparation was only 2–3%, contaminated with numerous osteoblasts. However, this crude osteoclast preparation proved to be useful for establishing a reliable resorption pit assay on dentine slices. The crude osteoclast preparation was further purified using a 30% Percoll solution (Jimi *et al.*, 1996). Interestingly, these highly purified osteoclasts (purity: 50–70%) cultured for 24 hours on dentine slices failed to form resorption pits. Resorptive capability of the purified osteoclasts was restored when osteoblasts/stromal cells were added to the purified osteoclast preparation. Similarly, Wesolowski *et al.* (1995) obtained highly purified mononuclear and binuclear prefusion osteoclasts using echistatin-treated cocultures of mouse bone marrow cells and osteoblastic MB 1.8 cells. These enriched prefusion osteoclasts failed to form resorption pits on bone slices, but their bone-resorbing activity was induced when both MB 1.8 cells and 1α,25$(OH)_2D_3$ were added to the prefusion osteoclast cultures. These results suggested that osteoblasts/stromal cells play an essential role not only in the stimulation of osteoclast differentiation, but also in the activation of mature osteoclasts to resorb bone, which also depends on a cell-to-cell contact-dependent process (Suda *et al.*, 1997b).

RANKL–RANK INTERACTION FOR OSTEOCLASTOGENESIS

Discovery of OPG

OPG was cloned as a member of the TNF receptor superfamily in an expressed sequence tag cDNA project (Simonet *et al.*, 1997). Interestingly, OPG lacked a transmembrane domain and presented as a secreted form (Fig. 2). Hepatic expression of OPG in transgenic mice resulted in severe osteopetrosis. Osteoclastogenesis inhibitory factor (OCIF), which inhibited osteoclast formation in the coculture of osteoblasts and spleen cells, was isolated as a heparin-binding protein from the conditioned medium of human fibroblast cultures (Tsuda *et al.*, 1997). The cDNA sequence of OCIF was identical to that of OPG (Yasuda *et al.*, 1998a). OPG/OCIF contains four cysteine-rich domains and two death domain homologous regions (see Fig. 2). The death domain homologous regions share structural features with “death domains” of TNF type I receptor (p55) and Fas, both of which mediate apoptotic signals. Analysis of the domain deletion mutants of OPG revealed that the cysteine-rich domains are essential for inducing biological activity *in vitro*. Although the biological significance of the death domain homologous regions in the OPG molecule has to be elucidated in the future, the cysteine residue at position 400 (Cys-400) is necessary for homodimerization (Yamaguchi *et al.*, 1998).

OPG strongly inhibited osteoclast formation induced by 1α,25$(OH)_2D_3$, PTH, PGE_2, or IL-11 in cocultures. Analyses of transgenic mice overexpressing OPG and animals injected with OPG have demonstrated that this factor increases bone mass by suppressing bone resorption

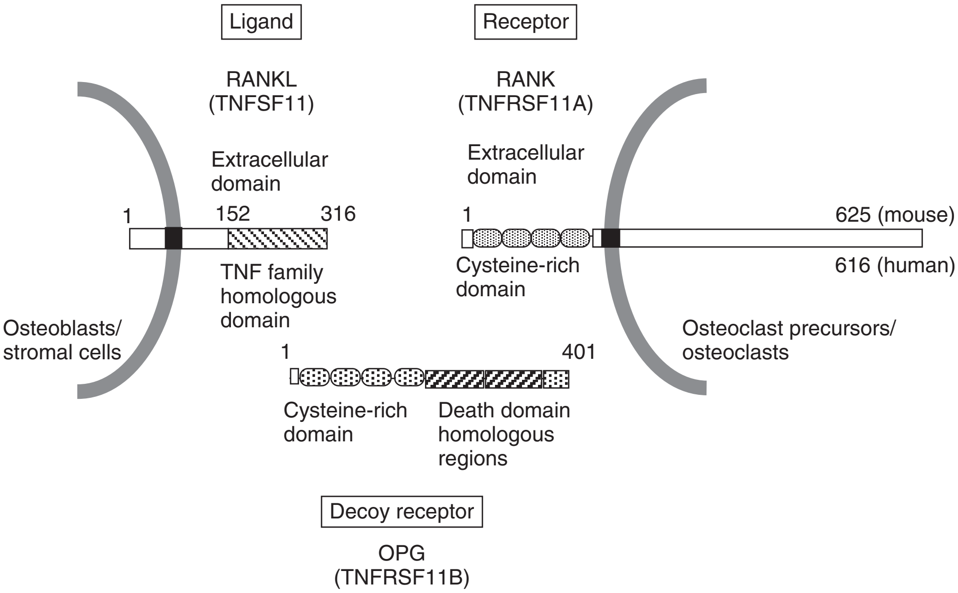

FIGURE 2 A diagrammatic representation of the ligand, receptor, and decoy receptor of the newly discovered TNF receptor-ligand family members essentially involved in osteoclastogenesis. RANKL is a type II transmembrane protein composed of 316 amino acid residues. The TNF homologous domain exists in Asp^{152}-Asp^{316}. Mouse RANK is a type I transmembrane protein of 625 amino acid residues. Human RANK composed of 616 amino acid residues. Four cysteine-rich domains exist in the extracellular region of the RANK protein. OPG, a soluble decoy receptor for RANKL, is composed of 401 amino acid residues without a transmembrane domain. OPG also contains four cysteine-rich domains and two death domain homologous regions. The cysteine-rich domains but not the death domain homologous regions of OPG are essential for inhibiting osteoclast differentiation and function. RANKL, RANK, and OPG are also named TNFSF11, TNFRSF11A, and TNFRSF11B, respectively.

(Simonet *et al.*, 1997; Yasuda *et al.*, 1998a). The physiological role of OPG was investigated further in OPG-deficient mice (Bucay *et al.*, 1998; Mizuno *et al.*, 1998). These mutant mice were viable and fertile, but adolescent and adult OPG(−/−) mice exhibited a decrease in bone mineral density (BMD) characterized by severe trabecular and cortical bone porosity, marked thinning of parietal bones of the skull, and a high incidence of fractures. Interestingly, osteoblasts derived from OPG(−/−) mice strongly supported osteoclast formation in coculture without adding any bone-resorbing agents (Udagawa *et al.*, 2000). These results indicate that OPG produced by osteoblasts/stromal cells functions as an important negative modulator in osteoclast differentiation and activation *in vivo* and *in vitro*.

RANKL–RANK Interaction in Osteoclast Differentiation and Function

Using OPG as a probe, Yasuda *et al.* (1998b) cloned a cDNA with an open reading frame encoding 316 amino acid residues from an expression library of the mouse bone marrow-derived stromal cell line ST2. The OPG-binding molecule was a type II transmembrane protein of the TNF ligand family. Because the OPG-binding molecule satisfied major criteria of ODF, this molecule was renamed ODF. Lacey *et al.* (1998) also succeeded in the molecular cloning of the ligand for OPG (OPGL) from an expression library of the murine myelomonocytic cell line 32D. Molecular cloning of ODF/OPGL demonstrated that it was identical to TRANCE (Wong *et al.*, 1997b) and RANKL (Anderson *et al.*, 1997), which had been identified independently by other research groups. A new member of the TNF receptor family, termed “RANK,” was cloned from a cDNA library of human dendritic cells (Anderson *et al.*, 1997). The mouse RANK cDNA encodes a type I transmembrane protein of 625 amino acid residues with four cysteine-rich domains in the extracellular region. The N-terminal region of RANK has a similar structure to that of OPG, a decoy receptor for RANKL (see Fig. 2).

A genetically engineered soluble form of RANKL, together with M-CSF, induced osteoclast formation from spleen cells in the absence of osteoblasts/stromal cells. Treatment of calvarial osteoblasts with $1\alpha,25(OH)_2D_3$, PTH, PGE_2, or IL-11 upregulated the expression of RANKL mRNA. Survival, fusion, and pit-forming activity of mouse osteoclasts formed *in vitro* are also induced by RANKL (Jimi *et al.*, 1999a). RANKL also increased bone resorption by isolated rat authentic osteoclasts (Burgess *et al.*, 1999; Fuller *et al.*, 1998). These results suggested that osteoblasts/stromal cells are essentially involved in both differentiation and activation of osteoclasts through the expression of RANKL as a membrane-associated factor (Fig. 3).

Polyclonal antibodies against the extracellular domains of RANK (anti-RANK Ab) induced osteoclast formation in

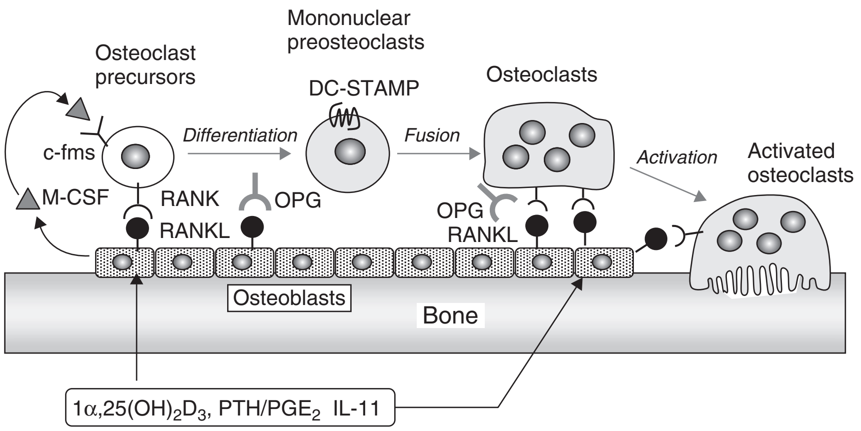

FIGURE 3 A schematic representation of osteoclast differentiation and function regulated by RANKL and M-CSF. Osteotropic factors such as 1α,25(OH)$_2$D$_3$, PTH, and IL-11 stimulate expression of RANKL in osteoblasts/stromal cells. Osteoblasts/stromal cells constitutively express M-CSF. The membrane-associated form of RANKL expressed by osteoblasts/stromal cells is responsible for the induction of osteoclast differentiation in the coculture. Osteoclast precursors express c-Fms and RANK, and recognize RANKL through cell–cell interaction with osteoblasts/stromal cells and differentiate into mononuclear preosteoclasts in the presence of M-CSF. Mononuclear preosteoclasts express DC-STAMP. RANKL also directly stimulates fusion and activation of osteoclasts. DC-STAMP plays a role in fusion of mononuclear preosteoclasts to form multinucleated osteoclasts. Osteoclasts express RANKL, which stimulates bone-resorbing activity of osteoclasts. Mainly osteoblasts/stromal cells produce OPG, a soluble decoy receptor of RANKL. OPG strongly inhibits the entire differentiation, fusion, and activation processes of osteoclasts induced by RANKL.

spleen cell cultures in the presence of M-CSF (Hsu *et al.*, 1999; Nakagawa *et al.*, 1998). In contrast, the anti-RANK antibody, which lacks the Fc fragment (the Fab fragment), completely blocked the RANKL-mediated osteoclastogenesis (Nakagawa *et al.*, 1998). Transgenic mice expressing a soluble RANK-Fc fusion protein showed osteopetrosis, similar to OPG transgenic mice (Hsu *et al.*, 1999). Taken together, these results suggest that RANK acts as the sole signaling receptor for RANKL in inducing differentiation and subsequent activation of osteoclasts (see Fig. 3). Thus, ODF, OPGL, TRANCE, and RANKL are different names for the same protein, which is important for the development and function of T cells, dendritic cells and osteoclasts. The terms RANKL, RANK, and OPG are used in this chapter in accordance with the guidelines of the American Society for Bone and Mineral Research President's Committee on Nomenclature (2000). According to the nomenclature of TNF and TNF receptor (TNFR) super family (SF) members, RANKL, RANK, and OPG are also named TNFSF11, TNFRSF11A, and TNFRSF11B, respectively (see Fig. 2).

Phenotypes of RANKL- and RANK-Deficient Mice

The physiological role of RANKL was investigated by generating RANKL-deficient mice (Kong *et al.*, 1999b). RANKL(−/−) mice lacked osteoclasts but had normal osteoclast progenitors that can differentiate into functionally active osteoclasts when cocultured with normal osteoblasts/stromal cells. Osteoblasts obtained from RANKL(−/−) mice failed to support osteoclast formation in the coculture with wild-type bone marrow cells even in the presence of 1α,25(OH)$_2$D$_3$ and PGE$_2$. RANKL(−/−) mice exhibited defects in early differentiation of T and B lymphocytes. In addition, RANKL(−/−) mice showed normal splenic structure and Peyer's patches, but lacked all lymph nodes.

The physiological role of RANK was also investigated by generating RANK-deficient mice (Dougall *et al.*, 1999). The phenotypes of RANK(−/−) mice were essentially the same as those of RANKL(−/−) mice. Like RANKL-deficient mice, RANK(−/−) mice were characterized by severe osteopetrosis resulting from an apparent block in osteoclast differentiation. RANK(−/−) mice also exhibited a marked deficiency of B cells in the spleen. RANK(−/−) mice retained mucosal-associated lymphoid tissues, including Peyer's patches, but completely lacked all the other peripheral lymph nodes. Li *et al.* (2000a) further showed that RANK(−/−) mice had a profound defect in bone resorption and remodeling and in the development of the cartilaginous growth plates of endochondral bone. Osteopetrosis observed in RANK(−/−) mice was rescued by the transplantation of bone marrow from rag1 (recombinase-activating gene 1)(−/−) mice, indicating that RANK(−/−) mice have an intrinsic defect in osteoclast lineage cells. Osteoclastogenesis in RANK(−/−) mice was also rescued by the transferring the RANK cDNA back into hematopoietic precursors. These data indicate that RANKL–RANK interaction is an absolute requirement not only for osteoclast development, but also in lymphocyte development and lymph node organogenesis.

Discovery of DC-STAMP as a Cell Fusion Factor in Osteoclast Generation

Multinucleated osteoclasts are formed by cell–cell fusion of mononuclear preosteoclasts. Recent studies have shown that the dendritic cell-specific transmembrane protein (DC-STAMP), a seven-transmembrane protein, is responsible for the cell–cell fusion of osteoclasts (Kukita *et al.*, 2004; Yagi M *et al.*, 2005). DC-STAMP expression in osteoclast precursors was upregulated during differentiation into osteoclasts. No multinucleated osteoclasts were observed in DC-STAMP knockout mice, but many mononuclear preosteoclasts expressing osteoclast-specific markers were detected in those bone tissues. Bone-resorbing activity was considerably lower in DC-STAMP(−/−) osteoclasts than in the wild-type osteoclasts (Yagi M *et al.*, 2005). DC-STAMP(−/−) mice developed mild osteopetrosis. These results suggest that DC-STAMP is an essential molecule for osteoclast fusion, and multinucleated osteoclasts have higher bone-resorbing activity than mononuclear preosteoclasts. Thus, osteoclasts are the specifically differentiated multinucleated cells specialized for bone resorption.

Modulators of RANK Signals

Molecules That Stimulate ITAM Signals

Immunoreceptor tyrosine-based activation motif (ITAM) is an important signaling component for a number of immunoglobulin-like receptors including T-cell receptors, B-cell receptors, NK-cell receptors, and Fc receptors. Kim *et al.* (2002) succeeded in the molecular cloning of a new member of such receptors expressed by osteoclasts and named it "the osteoclast-associated receptor (OSCAR)." Subsequently, Koga *et al.* (2004) reported that molecules containing ITAM such as DNAX-activating protein 12 (DAP12) and Fc receptor common γ chain (FcRγ) play important roles in osteoclastogenesis. Deficiency in both FcRγ and DAP12 caused osteopetrotic phenotypes in mice. FcRγ and DAP12 are adaptor molecules that associate with immunoglobulin-like receptors such as OSCAR, triggering receptor expressed on myeloid cells 2 (TREM2), signal-regulatory protein β1 (SIRPβ1), and paired immunoglobulin-like receptor A (PIR-A). ITAM-mediated signals cooperate with RANK to stimulate calcium oscillation. This signal is crucial for the robust induction of nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), that leads to osteoclastogenesis (Takayanagi, 2007). NFATc1 is a transcription factor essential for osteoclastogenesis, and the role of NFATc1 in osteoclastogenesis is described in detail in Chapter 9. The ligands for these receptors are not yet known, but will have to be identified in the future.

TGF-β

Bone is a major storage site for transforming growth factor β (TGF-β). Osteoclastic bone resorption releases TGF-β from bone matrix. Receptors for TGF-β superfamily members are a family of transmembrane serine/threonine kinases and are classified as type I and type II receptors according to their structural and functional characteristics. Formation of a type I–type II receptor complex is required for the ligand-induced signals. Sells Galvin *et al.* (1999) reported that TGF-β enhanced osteoclast differentiation in cultures of mouse bone marrow cells stimulated by RANKL and M-CSF. Fuller *et al.* (2000) also showed that osteoclast formation induced by RANKL was abolished completely by adding soluble TGF-β receptor type II, suggesting that TGF-β is an essential cofactor for osteoclastogenesis. These results support the previous findings (1) that transgenic mice expressing TGF-β_2 developed osteoporosis owing to enhanced osteoclast formation (Erlebacher and Derynck, 1996) and (2) that osteoclast formation was reduced in transgenic mice expressing a truncated TGF-β type II receptor in the cytoplasmic domain (Filvaroff *et al.*, 1999). Fox *et al.* (2000) and Quinn *et al.* (2001) independently reported that TGF-β directly promoted TNFα-induced osteoclast formation in bone marrow macrophage cultures, suggesting that TGF-β released from bone matrix acts as a costimulatory factor in osteoclast formation. (For more detail on the role of TNFα in osteoclast differentiation, see "RANKL-Independent Osteoclast Differentiation and Activation.") However, TGF-β has been shown to strongly inhibit osteoclast formation in the coculture of osteoblasts and bone marrow cells (Quinn *et al.*, 2001; Thirunavukkarasu *et al.*, 2001). These results suggest that TGF-β is a critical modulator for the control of physiological and pathophysiological osteoclastogenesis.

PGE$_2$

PGE$_2$ synergistically enhanced RANKL-induced osteoclastic differentiation of precursor cells through EP2 and EP4 (Wani *et al.*, 1999; Kobayashi *et al.*, 2005). RANKL-induced degradation of IκBα and phosphorylation of MAPKs in RAW264.7 cells were upregulated by PGE$_2$ in a cAMP/protein kinase A (PKA)-dependent manner, suggesting that PGE$_2$ signals crosstalk with RANK signals (Kobayashi *et al.*, 2005). TGF-β-activated kinase 1 (TAK1) possesses a PKA recognition site at amino acids 409–412. PKA directly phosphorylated TAK1 in RAW264.7 cells transfected with wild-type TAK1 but not with Ser^{412}Ala mutant TAK1. Ser^{412}Ala TAK1 served as a dominant-negative mutant in PKA-enhanced degradation of IκBα, phosphorylation of p38 MAPK, and PGE$_2$-enhanced osteoclast differentiation in RAW264.7 cells. Furthermore, forskolin enhanced TNFα-induced osteoclastic differentiation and IκBα degradation in RAW264.7 cells. Indeed, Ser^{412}Ala TAK1 abolished the stimulatory effects of forskolin on osteoclast differentiation induced by TNFα. These results suggest that the phosphorylation of the Ser^{412} residue in TAK1 is essential for cAMP/PKA-induced upregulation of osteoclast differentiation induced by RANKL and TNFα (Kobayashi *et al.*, 2005). PGE_2 produced at the inflammatory environment may directly enhance osteoclast formation.

REGULATION OF RANKL AND OPG EXPRESSION

Osteoblasts/Stromal Cells

Treatment of calvarial osteoblasts with osteotropic factors such as $1\alpha,25(OH)_2D_3$, PTH, PGE_2, or IL-11 upregulated the expression of RANKL mRNA. O'Brien *et al.* (1999) reported that the expression of dominant-negative signal transducer and activator of transcription (STAT) 3 or dominant-negative gp130 suppressed RANKL expression in a stromal/osteoblastic cell line (UAMS-32) and osteoclast formation-supporting activity stimulated by IL-6 together with soluble IL-6 receptor, oncostatin M, or IL-11 but not by PTH or $1\alpha,25(OH)_2D_3$. The gp130-dependent signals use at least two intracellular pathways: STAT 1/3 pathway and Src homology 2 domain-containing protein tyrosine phosphatase 2 (SHP2)/ras/mitogen-activated protein kinase(MAPK) pathway. To define the role of gp130 signals *in vivo*, Sims *et al.* (2004) analyzed mice in which gp130 signaling via either STAT1/3 or SHP2/ras/MAPK pathway was attenuated. They found that the STAT1/3 pathway was involved in the differentiation and function of osteoblasts and chondrocytes, whereas the SHP2/ras/MAPK pathway directly inhibited osteoclastogenesis in a cell lineage-autonomous manner. These results suggest that the gp130/STAT1/3 signaling pathway induces RANKL expression in osteoblasts.

The involvement of PGE receptor subtypes in PGE_2-induced RANKL expression in osteoblasts/stromal cells was examined using specific agonists for the EPs. Both the EP2 agonist and the EP4 agonist induced cAMP production and expression of RANKL mRNA in osteoblastic cells (Suzawa *et al.*, 2000). Sympathetic signaling via β2-adrenergic receptors (β2AR) expressed in osteoblasts has been shown to control bone formation downstream of leptin (Ducy *et al.*, 2000; Takeda *et al.*, 2002). Using β2AR-deficient mice, Elefteriou *et al.* (2005) reported that the sympathetic nervous system favors bone resorption by increasing expression in osteoblasts/stromal cells of RANKL. This sympathetic function requires cAMP/PKA signaling, followed by phosphorylation of activating transcription factor 4 (ATF4), a cell-specific cAMP response element binding protein (CREB)-related transcription factor, that enhances osteoblast differentiation and function. These results further confirm that cAMP-PKA signaling induces RANKL expression in osteoblasts/stromal cells.

Compounds that have a capacity of elevating intracellular calcium, such as ionomycin, cyclopiazonic acid, and thapsigargin, also induced osteoclast formation in mouse cocultures of bone marrow cells and primary osteoblasts (Takami *et al.*, 1997). Similarly, high calcium concentrations of the culture medium induced osteoclast formation in the cocultures. Treatment of primary osteoblasts with these compounds or high medium calcium stimulated the expression of RANKL mRNA (Takami *et al.*, 2000). Phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C (PKC), also stimulated the expression of RANKL mRNA in primary osteoblasts. PKC inhibitors, such as calphostin and staurosporin, suppressed ionomycin- and PMA-induced expression of RANKL mRNA in primary osteoblasts. Thus, calcium/PKC signals induce RANKL expression in osteoblasts/stromal cells. The calcium/PKC signal is proposed to be the fourth signal pathway involved in the induction of RANKL mRNA expression, which in turn stimulates osteoclast formation. It was also shown that lipopolysaccharide (LPS) and IL-1 stimulate RANKL expression in osteoblasts through calcium/PKC signals (Kikuchi *et al.*, 2001; Suda *et al.*, 2004).

These results suggest that at least four signals are independently involved in RANKL expression by osteoblasts/stromal cells: VDR-mediated signals, cAMP/PKA-mediated signals, gp130/STAT3-mediated signals, and calcium/PKC-mediated signals (Fig. 4). Treatment of primary osteoblasts with $1\alpha,25(OH)_2D_3$, PTH, PGE_2, and IL-11 suppressed OPG mRNA expression, suggesting that the balance between RANKL and OPG expression regulates osteoclast formation.

T Lymphocytes

RANKL expression in T cells is induced by antigen receptor engagement. Kong *et al.* (1999a) reported that activated T cells directly triggered osteoclastogenesis through RANKL expression. RANKL was detected on the surface of activated T cells. Activated T cells also secreted soluble RANKL into culture medium (Gillespie, 2007). Systemic activation of T cells *in vivo* also induced a RANKL-mediated increase in osteoclastogenesis and severe bone loss. In a T-cell-dependent model of rat adjuvant arthritis characterized by severe joint inflammation, treatment with OPG at the onset of the disease prevented bone and cartilage destruction but not inflammation. These results suggest that both systemic and local T-cell activation can lead to RANKL production and subsequent bone loss. Horwood *et al.* (1999) also reported that human peripheral blood-derived T cells, prepared with anti-CD3 antibody-coated magnetic beads, supported osteoclast differentiation from mouse spleen cells in the presence of concanavalin A together with IL-1 or TGF-β in the coculture. The expression of RANKL mRNA was stimulated in peripheral blood-derived T cells treated with the same factors. In synovial tissue sections with lymphoid infiltrates from patients with rheumatoid

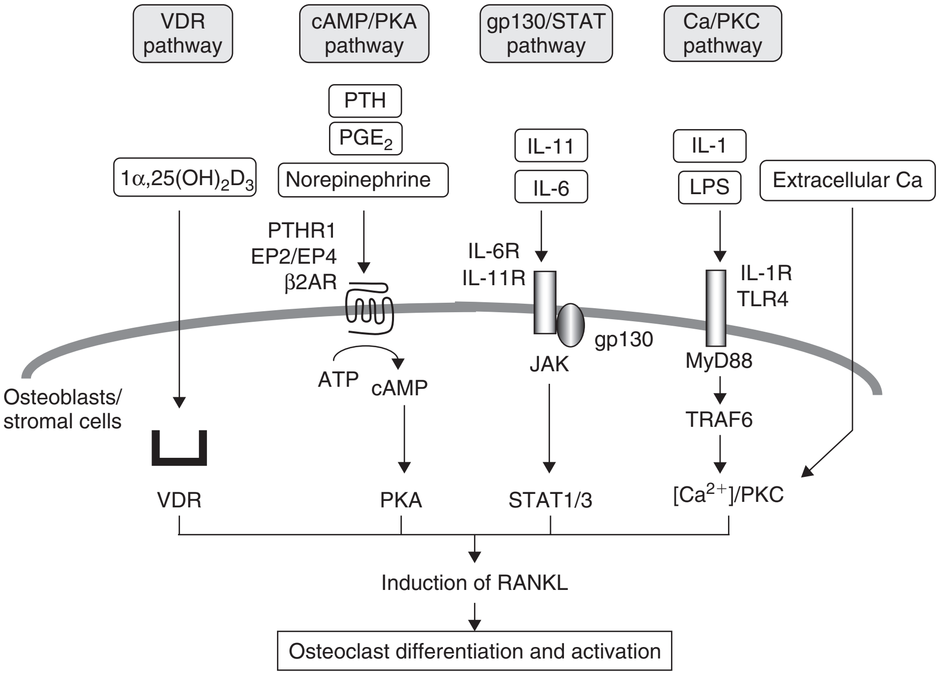

FIGURE 4 Signaling pathways for the induction of RANKL in osteoblasts/stromal cells. Three independent signals have been proposed to induce RANKL expression in osteoblasts/stromal cells: VDR-mediated signals by 1α,25$(OH)_2D_3$, cAMP/PKA-mediated signals by PTH, PGE-2 and β2AR agonists such as norepinephrine, gp130-mediated signals by IL-11 and IL-6. The calcium/PKC signal in osteoblasts/stromal cells, which is induced by ionomycin or high calcium concentrations of the culture medium, is now proposed to be the fourth signaling pathway involved in the induction of RANKL mRNA expression. LPS and IL-1 stimulate RANKL expression in osteoblasts through the calcium/PKC signal. RANKL expression induced by these four signals in osteoblasts/stromal cells in turn stimulates osteoclast differentiation and function.

arthritis, the expression of RANKL was demonstrated in CD3-positive T cells. The ability of activated T cells to support osteoclast formation may provide a mechanism for the potentiation of osteoclast formation and bone destruction in diseases such as rheumatoid arthritis and periodontitis. Using specific inhibitors, Kong *et al.* (1999a) showed that the induction of RANKL by T cells depends on PKC, phosphoinositide-3 kinase, and calcineurin-mediated signaling pathways. These results suggest that calcium/PKC signals are involved in RANKL expression by activated T cells.

The inflammation recruits hematopoietic and immune cells such as activated T cells at the site. Teng *et al.* (2000) transplanted human peripheral blood lymphocytes from periodontitis patients into NOD/SCID mice. Human CD4(+) T cells, but not CD8(+) T cells or B cells, were identified as essential mediators of alveolar bone destruction in the transplanted mice. Stimulation of CD4(+) T cells by *Actinobacillus actinomycetemcomitans*, a well-known gram-negative anaerobic microorganism that causes human periodontitis, induced production of RANKL. *In vivo* inhibition of RANKL function with OPG diminished alveolar bone destruction and reduced the number of periodontal osteoclasts after microbial challenge. In addition to the ability of T cells to directly support osteoclastogenesis, T cells also secrete inflammatory cytokines such as IL-1, IL-6, and IL-17, each of which can stimulate RANKL expression by osteoblasts/stromal cells. Recent studies have revealed that the IL-17-producing helper T-cell subset (Th17) is responsible for bone destruction in autoimmune arthritis (Sato *et al.*, 2006). Th17 cells are responsive to IL-23, which regulates their expansion and survival. The importance of IL-17 and IL-23 in rheumatoid arthritis and other inflammatory diseases was highlighted in studies using IL-17- and IL-23-deficient mice (Nakae *et al.*, 2003; Murphy *et al.*, 2003). Both IL-17-deficient mice and IL-23-deficient mice showed marked resistance to autoimmune responses. These results suggest that Th17 cells function as important immunomodulators of osteoclastic bone resorption in autoimmune arthritis.

Wnt Signals

Wnt proteins (Wnts) are palmitoylated and glycosylated ligands that play a central role in early development of organs and tissues. The discovery that loss-of-function mutations in low-density lipoprotein receptor-related protein 5 (LRP5), a Wnt coreceptor, brought about a low bone mass in humans and revealed the possible role of Wnt signaling in the regulation of bone remodeling (Gong *et al.*, 2001). There are two pathways of Wnt signaling: β-catenin-dependent canonical and -independent noncanonical pathways. Wnts act on osteoblast precursor cells and promote their differentiation into mature osteoblasts through the β-catenin-dependent canonical pathway (Kato *et al.*, 2002). In addition, Wnts suppress bone resorption by regulating RANKL/OPG ratio through the same pathway in mature osteoblasts. Wnt3a strongly inhibits $1\alpha,25(OH)_2D_3$-induced osteoclast formation in cocultures of stromal ST2 cells and bone marrow cells (Yamane *et al.*, 2001). However, Wnt3a fails to inhibit RANKL-induced osteoclast formation in bone marrow macrophage cultures. These results suggest that the inhibitory effect of Wnt3a on osteoclast formation is mediated by osteoblasts/stromal cells. Glass *et al.* (2005) developed mice expressing a stabilized form of β-catenin in osteoblasts (β-catenin mutant mice), and reported that the β-catenin mutant mice developed osteopetrosis with the defect of tooth eruption and the decreased number of osteoclasts. Microarray analysis to compare gene expression in LRP5(−/−) mice and β-catenin mutant mice showed that OPG mRNA is upregulated in osteoblasts in those mice. When β-catenin was inactivated selectively in mature osteoblasts using α1(I) collagen Cre mice, the bone mass was decreased owing to the enhancement of bone resorption (Glass *et al.*, 2005). Activation of the canonical Wnt pathway was shown to stimulate OPG expression in osteoblasts. In addition, the canonical Wnt pathway suppresses the expression of RANKL in MC3T3E1 cells and MG-63 cells (Spencer *et al.*, 2006). These results suggest that the activation of the canonical Wnt pathway in osteoblasts suppresses bone resorption through upregulation of OPG expression and downregulation of RANKL expression.

Regulation of Shedding of RANKL

OPG(−/−) mice exhibit severe osteoporosis because of enhanced osteoclast differentiation and function. The serum concentration of RANKL was elevated in OPG(−/−) mice (Yamamoto *et al.*, 2006). A homozygous deletion of the OPG gene was found in patients with juvenile Paget's disease, and the serum level of RANKL was markedly elevated in one such a patient as well (Whyte *et al.*, 2002). Osteoblasts obtained from OPG(−/−) mice released a large amount of RANKL. When OPG was added to the culture of OPG(−/−) osteoblasts, the release of RANKL into the culture medium was strikingly inhibited. Activated T cells from WT mice also released a large amount of RANKL. Similarly, the release of RANKL from activated T cells was inhibited by adding OPG. These results suggest that activated T cells release RANKL because of the lack of OPG production, and that the binding of OPG to membrane-associated RANKL expressed by either osteoblasts or activated T cells suppresses the shedding of RANKL. OPG(−/+) and T-cell-double-deficient mice were created by cross-breeding of OPG(−/−) mice and athymic nude (Fox n1nu/nu) mice (Nakamichi *et al.*, 2007). Serum levels of RANKL in OPG(−/−) and T cell-double-deficient mice were as high as those in OPG(−/−) mice. These results suggest that the high levels of serum RANKL detected in OPG(−/−) mice are not derived from T cells but from bone tissues. Hikita *et al.* (2006) also reported that matrix metalloproteinase (MMP) 14 and a disintegrin and metalloproteinase (ADAM) 10 have strong RANKL-shedding activity. Suppression of MMP14 in primary osteoblasts increased membrane-bound RANKL and promoted osteoclastogenesis in cocultures with macrophages. These results suggest that some proteases such as MMP 14 and ADAM 10 are involved in the RANKL shedding and the binding of OPG to the membrane-associated RANKL protects RANKL against attack by those proteases.

MUTATIONS OF RANK, OPG, AND RANKL FOUND IN HUMANS

RANK Mutation

Discovery of the RANKL/OPG/RANK signaling pathway has led the identification of some bone diseases in humans (Fig. 5). In 2000, Hughes *et al.* reported for the first time that the gene responsible for familial expansile osteolysis and familial Paget's disease of bone was mapped to the gene encoding RANK (TNFRSF11A). So far, four different insertion mutations in the gene encoding RANK (TNFRSF11A) have been described. Familial expansile osteolysis is a rare autosomal dominant disorder of bone characterized by focal areas of increased bone remodeling. The osteolytic lesions, which usually develop in the long bones during early adulthood, show increased activities for both osteoblasts and osteoclasts. Two mutations of the heterozygous insertion were detected in the first exon of the RANK gene in affected members of four families. One mutation was a duplication of 18 bp (84dup18) and the other a duplication of 27 bp (75dup27), both of which affected the signal peptide (SP) region of the RANK molecule (Hughes *et al.*, 2000; Palenzuela *et al.*, 2002). Nakatsuka *et al.* (2003) reported the clinical and radiographic features of individuals with the 75dup27 mutation. Whyte and Hughes (2002) found a 15-bp duplication in the RANK gene (84dup15) in a mother and a daughter affected by expansile skeletal hyperphosphatasia. Johnson-Pais *et al.* (2003) also identified an 18-bp tandem duplication (83dup18) in the RANK gene in two patients with familial expansile osteolysis.

Expression of recombinant forms of the mutant RANK proteins revealed perturbations in the expression levels and lack of normal cleavage of the signal peptide (Hughes *et al.*, 2000). Both mutations caused an increase in RANK-mediated NF-κB signaling *in vitro*, consistent with the presence of an activating mutation. Thus, activating mutations in RANK cause familial expansile osteolysis and

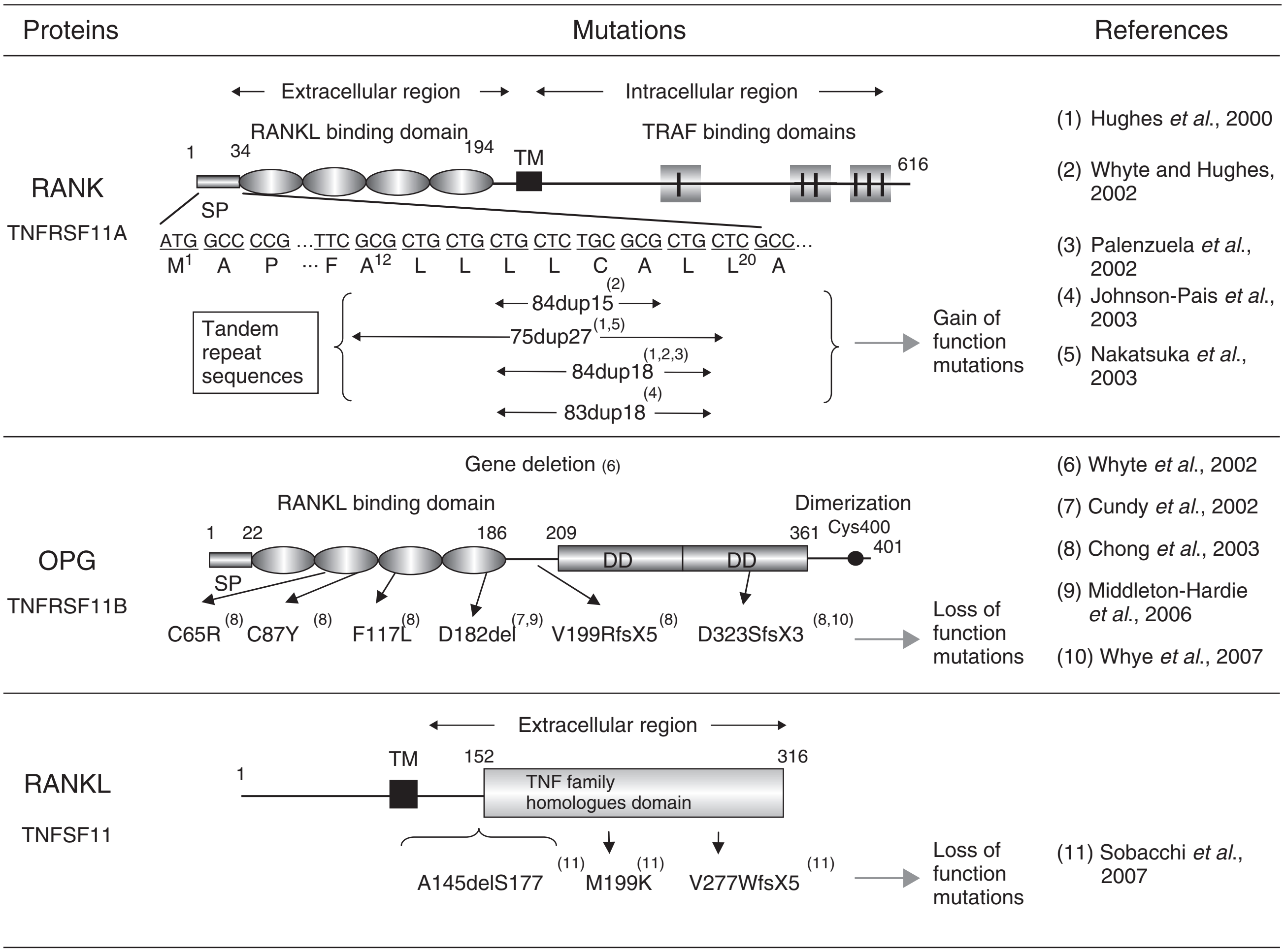
Proteins
Mutations
References
Extracellular region
Intracellular region
RANKL binding domain
TRAF binding domains
1
34
194
TM
616
RANK
TNFRSF11A
SP
ATG GCC CCG …TTC GCG CTG CTG CTG CTC TGC GCG CTG CTC GCC…
M1 A P … F A12 L L L L C A L L20 A
Tandem repeat sequences
84dup15 (2)
75dup27 (1,5)
84dup18 (1,2,3)
83dup18 (4)
Gain of function mutations
(1) Hughes et al., 2000
(2) Whyte and Hughes, 2002
(3) Palenzuela et al., 2002
(4) Johnson-Pais et al., 2003
(5) Nakatsuka et al., 2003
Gene deletion (6)
RANKL binding domain
Dimerization
1
22
186
209
361
Cys400
401
OPG
TNFRSF11B
SP
DD
DD
C65R (8)
C87Y (8)
F117L (8)
D182del (7,9)
V199RfsX5 (8)
D323SfsX3 (8,10)
Loss of function mutations
(6) Whyte et al., 2002
(7) Cundy et al., 2002
(8) Chong et al., 2003
(9) Middleton-Hardie et al., 2006
(10) Whye et al., 2007
Extracellular region
RANKL
TNFSF11
1
TM
152
316
TNF family homologues domain
A145delS177 (11)
M199K (11)
V277WfsX5 (11)
Loss of function mutations
(11) Sobacchi et al., 2007

FIGURE 5 Mutations of RANK, OPG, and RANKL found in humans. Three different insertion mutations have been identified in the signal peptide (SP) region of the RANK (TNFRSF11A) gene. These three mutations lead to the gain-of-function mutations. The total deletion and six different mutations have been identified in the OPG (TNFRSF11B) gene. The six mutations found in the OPG gene result in the loss-of-function mutations. In the RANKL (TNFSF11) gene, three mutations have been identified in the extracellular region. These three mutations lead to the loss of function of RANKL. Figures in the shoulder parentheses indicate references. The expression, like that of "fsX5," means the frame shift and appearance of a new stop codon after four new amino acids.

expansile skeletal hyperphosphatasia, both of which show hyperphosphatasia with increased bone remodeling.

OPG Mutation

Juvenile Paget's disease, an autosomal recessive osteopathy, is characterized by rapidly remodeling woven bone, osteopenia, fractures, and progressive skeletal deformity. Whyte *et al.* (2002) found defects in the gene encoding OPG defects in the two Navajo patients with juvenile Paget's disease. Both patients had a homozygous deletion of the OPG gene. Serum levels of OPG were undetectable, but those of soluble RANKL were markedly increased. Cundy *et al.* (2002) subsequently reported that mutations of the OPG gene cause idiopathic hyperphosphatasia (also known as Juvenile Paget's disease) (see Fig. 5). Three affected siblings showed the same homozygous mutation of a 3-bp inframe deletion in exon 3 of the OPG gene, resulting in the loss of an aspartate residue (D182del). Recombinant mutant OPG failed to inhibit bone resorption *in vitro*. Middleton-Hardie *et al.* (2006) also studied the characteristics of OPG (D182del) in more detail. OPG (D182del) inhibited osteoclast formation less effectively than wild-type OPG and had a reduced ability to bind to RANKL. In addition, the mutant OPG was retained within the cells, probably because of the change in the folding structure. These results suggest that deletion of aspartate 182 in OPG is the loss-of-function mutation.

Chong *et al.* (2003) evaluated the relationships between phenotypes and genotypes of the OPG gene in patients of nine families with idiopathic hyperphosphatasia. They found homozygous mutations in the OPG gene in affected members from six of nine families with idiopathic hyperphosphatasia. Four of the six mutations occurred in the cysteine-rich ligand-binding domain (C65R, C87Y, F117L, and D182del). One mutation was found to be a deletion mutation/insertion at the junction of exon 3 and intron 3. This mutation is predicted to produce a truncated protein consisting of amino acids 1–198 of the wild type with six additional nonnatural amino acids at the C terminus (V199RfsX5). The other mutation is the insertion/deletion mutation at the C-terminal end of the protein (D323SfsX3). The severity of the phenotype was related to the predicted effects of the mutations on OPG function. Three mutations occurred in and near the cysteine-rich ligand-binding domain (C65R, C87Y, and V199RfsX5) and were predicted to disrupt the binding of OPG to RANKL. These mutations result in severe phenotypes. Noncysteine missense mutations in the ligand-binding domain (F117L and D182del) were associated with an intermediate phenotype. A mutation at the C-terminal end of the protein (D323SfsX3) was associated with the mildest phenotype (Chong *et al.*, 2003; Whyte *et al.*, 2007). Thus, mutations in the OPG gene account for the majority of, but not all, cases of idiopathic hyperphosphatasia, and there are distinct genotype–phenotype relationships.

The effects of recombinant OPG were investigated in two adult siblings with juvenile Paget's disease (D182del) (Cundy *et al.*, 2005). Bone resorption (assessed by N-telopeptide excretion) was markedly suppressed by once-a-week subcutaneous doses of 0.3 to 0.4 mg per kg body weight. Plasma alkaline phosphatase activity and osteocalcin levels were similarly suppressed into a normal range. After 15 months of treatment, radial bone mass was increased in one patient by 9% and in the other, by 30%. Apart from mild hypocalcemia and hypophosphatemia, no apparent adverse events occurred. These results provide corroborative evidence that juvenile Paget's disease is indeed the result of OPG deficiency and confirms the critical role of OPG in regulating bone turnover in humans.

RANKL Mutation

Autosomal recessive osteopetrosis is usually associated with normal or elevated numbers of nonfunctional osteoclasts. Sobacchi *et al.* (2007) reported mutations in the gene encoding RANKL in six individuals with autosomal recessive osteopetrosis whose bone biopsy specimens lacked osteoclasts (see Fig. 5). These individuals did not show any obvious defects in immunological parameters. The mutation of the RANKL gene was found in four families of the six families examined. Family 1 showed the deletion of amino acids 145–177 in RANKL. Families 2 and 4 had the substitution of Met to Lys at 199 of RANKL (M199K). Family 3 had the deletion of the 828–829 nucleotides, which results in the appearance of a stop codon at 281 (V277WsfX5). Osteoclast progenitors appear to be normal, because RANKL induced formation of functional osteoclasts from monocytes of the patients. Hematopoietic stem cells were transplanted into three patients. All three patients showed good levels of hematological engraftment, but no improvement in bone remodeling was observed in those patients after hematopoietic stem cell transplantation. This suggests that the defect is not intrinsic to osteoclasts. Hematopoietic stem cell transplantation apparently did not provide sufficient donor stromal precursors or mesenchymal stem cells to correct the bone defect. It is also suggested that RANKL expressed by hematopoietic cells including T cells cannot functionally cure osteopetrosis in normal bone metabolism. Taken together, these results demonstrate that mutations in the gene encoding RANKL lead to an osteoclast-poor form of osteopetrosis. These findings confirm the notion that RANKL expressed by osteoblasts/stromal cells also plays an essential role in osteoclast differentiation and function in humans.

RANKL-INDEPENDENT OSTEOCLAST DIFFERENTIATION AND ACTIVATION

Accumulating evidence has shown that RANKL is important not only for physiological bone development, but also for pathological bone destruction in postmenopausal osteoporosis, rheumatoid arthritis, periodontal diseases, and tumor-induced osteolysis. In addition, recent studies have shown that RANKL is not the sole factor responsible for osteoclast differentiation and function. TNFα stimulates osteoclast differentiation and IL-1 stimulates osteoclast function in the absence of the RANKL–RANK interaction.

RANKL-Independent Regulation of Osteoclast Differentiation

The cytoplasmic tail of RANK interacts with TNF receptor-associated factor 1 (TRAF1), TRAF2, TRAF3, TRAF5, and TRAF6 (Darnay *et al.*, 1998; Galibert *et al.*, 1998; Schneeweis *et al.*, 2005). TRAF6-mediated signals appear to be important for osteoclast differentiation and function, because TRAF6(−/−) mice develop severe osteopetrosis (Lomaga *et al.*, 1999; Naito *et al.*, 1999). Azuma *et al.* (2000) and Kobayashi *et al.* (2000) independently found that TNFα stimulates osteoclast differentiation in the absence of the RANKL–RANK interaction. Osteoclast formation induced by TNFα was inhibited by the addition of antibodies against TNF receptor type I (TNFRI, p55) and TNF receptor type II (TNFRII, p75), respectively, but not by OPG. Osteoclasts induced by TNFα formed resorption pits on dentine slices only in the presence of IL-1. Kim *et al.* (2005) reported that hematopoietic precursors from RANKL(−/−), RANK(−/−), or TRAF6(−/−) mice differentiated into osteoclasts *in vitro* when they were stimulated with TNFα in the presence of cofactors such as TGF-β. These results suggest that RANK/TRAF6-mediated signals are not essentially required for osteoclast differentiation induced by TNFα.

TNFRI and TNFRII use TRAF2 as a common signal transducer in the target cells, suggesting that TRAF2-mediated signals play a role in osteoclast differentiation.

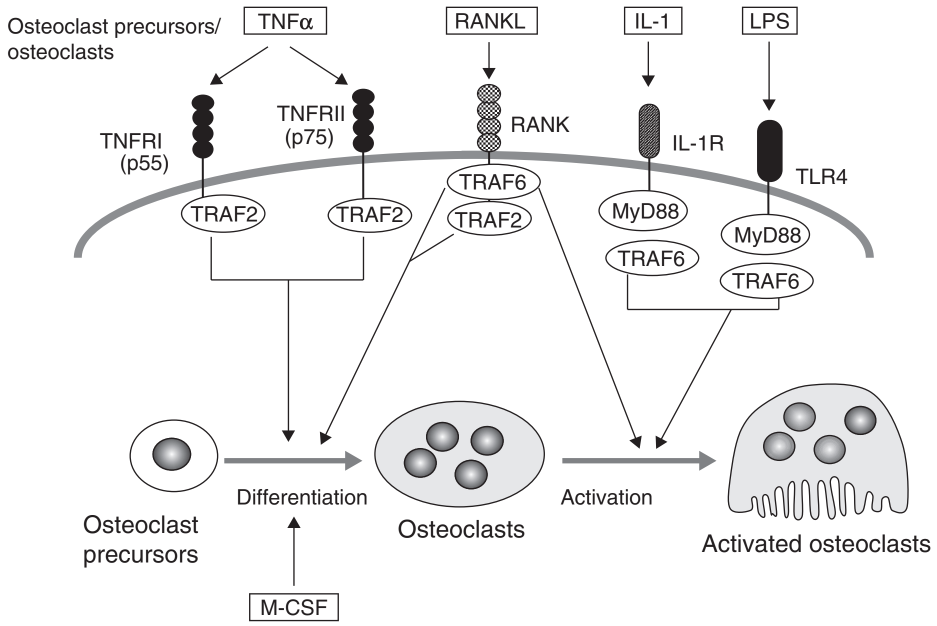

FIGURE 6 A schematic representation of RANKL-dependent and -independent regulation of osteoclast differentiation and function. The binding of RANKL to RANK induces association of RANK and TRAF family members including TRAF2 and TRAF6. TNFα and RANKL stimulate osteoclast differentiation independently. Osteoclast differentiation induced by TNFα occurs via TNFRI (p55) and TNFRII (p75) expressed by osteoclast precursors. RANKL induces osteoclast differentiation through RANK-mediated signals. M-CSF is a common factor required for both TNFα- and RANKL-induced osteoclast differentiation. TNFRI and TNFRII use TRAF2 as a common signal transducer in the target cells, suggesting that TRAF2-mediated signals play important roles in osteoclast differentiation. Activation of osteoclasts is induced by RANKL, IL-1, and LPS through RANK, IL-1 receptor (IL-1R), and TLR4, respectively. Both TLR4 and IL-1R use common signaling molecules such as MyD88 and TRAF6. Osteoclast function appears to require preferentially TRAF6-mediated signals.

Kanazawa and Kudo (2005) examined the involvement of TRAF2 in osteoclast differentiation by using TRAF2-deficient mice. RANKL-induced osteoclastogenesis gave a reduction of 20% in the progenitors from TRAF2-deficient mice compared with that of the cells from littermate wild-type mice, whereas TNFα-induced osteoclastogenesis was severely impaired in the cells from the TRAF2-deficient mice. TRAF2 overexpression induced differentiation of osteoclast progenitors from wild-type mice into osteoclasts. It has been reported that when osteotropic factors such as 1α,25(OH)$_2$D$_3$, PTH, and IL-1 were administered into RANK(−/−) mice, neither TRAP-positive cell formation nor hypercalcemia was induced (Li *et al.*, 2000a). In contrast, administration of TNFα into RANK(−/−) mice induced TRAP-positive cells near the site of injection even though the number of TRAP-positive cells induced by TNFα was not large. These results suggest that TRAF2 plays an important role in TNFα-induced osteoclastogenesis (Fig. 6).

Lam *et al.* (2000) reported that differentiation of macrophages into osteoclasts was dramatically enhanced by TNFα, when macrophages were pretreated with a small amount of RANKL, suggesting that osteoblasts/stromal cells are somehow involved in osteoclastogenesis in TNFα-induced osteoclastogenesis. To evaluate the role of osteoblasts/stromal cells in TNFα-driven osteoclast formation *in vivo*, Kitaura *et al.* (2005) generated chimeric mice in which wild-type (WT) bone marrow-derived osteoclast precursors are transplanted into lethally irradiated mice deleted of both TNFRI and TNFRII [TNFRI/II(−/−)], and in which bone marrow cells derived from TNFRI/II(−/−) mice are transplanted into lethally irradiated WT mice. WT to WT and TNFRI/II(−/−) to TNFRI/II(−/−) irradiated and transplanted mice were used as positive and negative controls, respectively. Those chimeras were administered daily with TNFα and sacrificed on day 5. As expected, TNFRI/II(−/−) to TNFRI/II(−/−) mice failed to respond to TNFα, whereas WT to WT mice generated osteoclasts in a dose-dependent manner. Although the increase in the number of osteoclasts induced by TNFα was observed in both chimeric mice, the responsiveness to TNFα was higher in TNFRI/II(−/−) to WT mice than WT to TNFRI/II(−/−) mice. These results indicate that TNF receptors expressed by osteoblasts/stromal cells contribute more than those by osteoclast progenitors to TNF-induced osteoclastogenesis *in vivo*. Nevertheless, these results also suggest that TNFα directly induces differentiation of osteoclast precursors into osteoclasts. TNFα appears to be a more convenient target in arresting inflammatory osteolysis.

RANKL-Independent Regulation of Osteoclast Function

IL-1 stimulates osteoclast function through IL-1 receptor type I (Jimi *et al.*, 1999b). As described earlier, purified osteoclasts placed on dentine slices failed to form resorption pits. When IL-1 or RANKL was added to the purified osteoclast cultures, resorption pits were formed on dentine slices within 24 hours (Jimi *et al.*, 1999b). Osteoclasts express IL-1 type 1 receptors, and IL-1 activated NF-κB rapidly in purified osteoclasts. The pit-forming activity of osteoclasts induced by IL-1 was inhibited completely by adding IL-1 receptor antagonist (IL-1ra) but not by OPG (Jimi *et al.*, 1999a). Fuller *et al.* (2006) carefully examined the role of humoral agents, including RANKL, TGF-β, IL-1α, and TNFα in murine osteoclast formation *in vitro*. They reported that IL-1α and TNFα substituted for RANKL for stimulation of bone-resorbing activity of osteoclasts, but the potency of TNFα to induce osteoclast function was much weaker than that of IL-1. These results suggest that IL-1 directly stimulates osteoclast function through IL-1 type 1 receptors in mature osteoclasts.

LPS, a major constituent of gram-negative bacteria, has been proposed to be a potent stimulator of bone resorption in inflammatory bone diseases including periodontal diseases. Toll-like receptor (TLR) family members are recognition receptors for a diverse group of microbial ligands, including components of bacteria, fungi, and viruses. TLR4 is a critical receptor for LPS. The cytoplasmic signaling cascade of TLR4 is quite similar to that of the IL-1 receptor, because both receptors possess cytoplasmic Toll/IL-1 receptor (TIR) domains. Both TLR4 and IL-1 receptors use common signaling molecules such as myeloid differentiation factor 88 (MyD88) and TRAF6 (Akira and Takeda., 2004). LPS and IL-1 enhanced the survival of osteoclasts and pit-forming activity of osteoclasts even in the presence of OPG (Kikuchi *et al.*, 2001; Suda *et al.*, 2004). The stimulatory effects of LPS and IL-1 on the pit-forming activity were not observed when MyD88-deficient osteoclasts were cultured on dentine slices (Sato *et al.*, 2004). In contrast, RANKL stimulated pit-forming activity of MyD88-deficient osteoclasts. These results suggest that LPS and IL-1 commonly use MyD88 and TRAF6 to induce osteoclast function, whereas RANKL uses TRAF6 to induce osteoclast function (see Fig. 6). TNFα failed to induce pit-forming activity of osteoclasts. These results suggest that TRAF6 signals are preferentially used for activation of osteoclasts. Thus, IL-1 and some bacterial components directly stimulate osteoclast function independent of the RANK-mediated pathway (see Fig. 6).

CONCLUSION

The discovery of the RANKL–RANK interaction now opens a wide new area in bone biology focused on the investigation of the molecular mechanism of osteoclast development and function. Osteoblasts/stromal cells, through the expression of RANKL and M-CSF, are involved throughout the osteoclast lifetime of their differentiation, survival, fusion, and activation. OPG produced by osteoblasts/stromal cells is an important negative regulator of osteoclast differentiation and function. Both RANKL(−/−) mice and RANK(−/−) mice show similar features of osteopetrosis with a complete absence of osteoclasts in bone. Gain-of-function mutations of RANK have been found in patients suffering from familial expansile osteolysis and familial Paget's disease of bone. Loss-of-function mutation of OPG also results in juvenile Paget's disease in bone. Loss-of-function mutations in the gene encoding RANKL were found in patients with autosomal recessive osteopetrosis whose bone biopsy specimens lacked osteoclasts. These findings confirm that the RANKL–RANK interaction is indispensable for osteoclastogenesis, not only in mice, but also in humans. Studies have also shown that inflammatory cytokines, TNFα and IL-1, can substitute for RANKL in inducing osteoclast differentiation and function. These results suggest that in addition to RANKL, inflammatory cytokines play important roles in osteoclastic bone resorption under pathological conditions.

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Chapter 10

Osteoclast Function: Biology and Mechanisms

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INTRODUCTION

There is a unanimous consensus among biologists that the main function of osteoclasts is to resorb mineralized bone, dentine, and calcified cartilage. Actually, according to our current knowledge, this seems to be the only function for those large and multinucleated cells that reveal several unique features. However, their close relationship with immune cells as well as mesenchymal stem cells in a bone marrow environment may indicate also new, previously unrecognized functions (Kollet *et al.*, 2006). Resorption of mineralized tissues is obligatory for normal skeletal maturation, including bone growth and remodeling, as well as tooth eruption.

In evolution the appearance of osteoclasts opened a totally new strategy for skeletal development. However, it is very difficult to know exactly why natural selection during evolution has favored the development of osteoclasts in the first place. Was it because of the obvious advantages they offered for the flexible use of the skeleton? Perhaps the development of osteo- (chondro)clast-like cells was favored by natural selection because of the advantages of the effective regulation of calcium homeostasis. The third possibility could be the need and pressure for the development of a safe environment for the hematopoietic tissue. No firm conclusions can be drawn from the current evidence and knowledge. Given the importance of calcium homeostasis and hematopoiesis, one might speculate that resorptive cells were originally developed not at all for skeletal purposes, but to support those vital functions.

It is not known either for sure whether functional osteoclasts were originally developed for the resorption of bone or calcified cartilage. It also remains to be clarified at what stage of the evolution resorbing osteoclasts appeared. Most probably this took place more than 300 million years ago.

BONE RESORPTION IS THE ONLY RELIABLE CHARACTERIZATION FOR OSTEOCLASTS

During skeletal growth, osteoclasts are needed for the resorption of calcified cartilage and modeling of growing bone. In adult bone, resorptive cells are responsible for remodeling. If necessary, they fulfill the requirements of calcium homeostasis via excessive resorption beyond normal remodeling. In addition to osteoclasts, tumor cells, monocytes, and macrophages have been suggested to have bone-resorbing capacity. However, later studies have not been able to confirm that tumor cells can resorb bone directly. Instead they can induce recruitment, as well as activity of osteoclasts, by secreting a large number of osteoclast regulating factors (Ralston, 1990). Bone resorption by macrophages has been demonstrated only in vitro and is probably due to the phagocytosis of bone particles rather than the more specialized mechanisms used by the osteoclasts. It is also possible that mineralized bone per se can induce monocytes and tissue macrophages to differentiate into osteoclasts under culture conditions. A recent study by Kim *et al.* (2006b) indicated that in addition to receptor activator of NFκB ligand (RANKL), monocyte chemotactic peptide-1 together with macrophage colony-stimulating factor (M-CSF) was also able to induce multinuclear tartrate-resistant acid phosphatase (TRACP)-positive cells from circulating monocytes. Although these cells were positive for calcitonin receptor and revealed several other osteoclastic markers they were not able to resorb bone.

At present it is thus generally accepted that the osteoclast is the only cell that is able to resorb mineralized

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bone. Both mononuclear and multinuclear osteoclasts can resorb bone, but larger cells seem to be more effective than smaller ones, although there is no direct relationship between the resorption capacity and the number of nuclei (Piper *et al.*, 1992). Large osteoclasts predominate at sites of aggressive, pathological bone resorption and they may even be more sensitive to extracellular stimulating factors (Trebec *et al.*, 2007). The number of nuclei in osteoclasts also varies among species, being higher in birds than in mammals.

Osteoclasts in different types of bone have been thought to be similar. Some differences have been observed especially in their content of proteolytic enzymes between different bone locations. Lee *et al.* (1995) described mononuclear cells with ruffled borders on uncalcified septa of the growth plate cartilage and proposed that these septoclasts probably resorb transversal septa of the growth plate just before chondroclasts start resorption of longitudinal calcified septa. According to present knowledge, chondroclasts that resorb calcified cartilage are similar to osteoclasts, but there are perhaps also other types of resorbing cells present in specific areas of the growing skeleton.

LIFE SPAN OF THE OSTEOCLAST AND THE RESORPTION CYCLE

In the adult, stem cells for osteoclasts originate from the hematopoietic tissue (for review, see Chapter 7). They share a common differentiation pathway with macrophages and dendritic cells until the final differentiation steps. Differentiation is characterized by the sequential expression of different sets of genes. Several cytokines and growth factors are known to affect the differentiation pathway of the osteoclasts, and studies have confirmed the central role of the receptor activator of NFκB (RANK)-RANKL-osteoprotegerin (OPG) pathway in this process.

After proliferating in bone marrow, mononuclear preosteoclasts are guided to bone surfaces by mechanisms, which are so far unknown. It is not known in detail where and when fusion of mononuclear precursors to multinuclear osteoclasts actually takes place and what are the molecular mechanisms regulating fusion. There are certainly several types of molecular interactions between the membranes of two cells undergoing fusion. Some of these have recently been revealed. Yagi *et al.* (2005) demonstrated a critical role of dendritic cell–specific transmembrane protein (DC-STAMP) in the fusion process. They showed that osteoclast cell fusion was abrogated in DC-STAMP –/– mice and could be restored by retroviral induction of the gene. These results are in accordance with a conclusion that DC-STAMP is one of the key molecules in the osteoclast fusion, although it might not be the actual fusion protein. Earlier in vitro studies have suggested that proteolipid part of V-ATPase may have a role in membrane fusion (Peters *et al.*, 2001). It is thus of special interest that impaired fusion ability of preosteoclasts was observed in mice that were deficient in V-ATPase V0 subunit d2 (Lee *et al.*, 2006). Because there are only very limited numbers of multinucleated cells in the adult body, the cell fusion process may offer interesting new molecular targets to regulate bone resorption. However, cell type specificity of the fusion process may still appear to be a difficult challenge.

The fusion of mononuclear precursors into multinucleated osteoclasts in the bone marrow takes place mainly in the vicinity of bone surfaces, because multinuclear osteoclasts are seldom observed far away from the bone surface. Somehow, precursors are guided near to those sites that are determined to be resorbed. How this happens, how these sites are determined, and which cells actually make the decision where and when, for instance, a new remodeling unit is initiated are not known. Strongest candidates for this role are osteocytes and bone lining cells.

Both negative and positive regulation between osteocytes and osteoclasts could exist. In vitro osteoclasts resorb devitalized bone with a much higher efficiency than bone where osteocytes are alive (Gu *et al.*, 2005b). In addition, it has been shown that healthy osteocytes secrete a partially estrogen dependent biological activity that inhibits osteoclasts differentiation. At least part of this biological activity is transforming growth factor (TGF) β3 (Heino *et al.*, 2002). Thus, it is possible that healthy osteocytes secrete a factor or factors that prevent osteoclast differentiation and activation, whereas dying osteocytes promote osteoclast activity. There is indirect evidence, indeed, that such mechanisms are operating in vivo. Kurata *et al.* (2006) showed, using a 3-D coculture model, that local damage of osteocytes induces differentiation of bone marrow precursors that are near to the damaged area. In bone, osteocytes form an internal network that could sense the whole bone as a single unit (Aarden *et al.*, 1994) and are the most obvious cells to act as gatekeepers for the local remodeling processes. Learning the molecular details of this complicated biological cross-talk between osteocytes and osteoclasts remains a major challenge for bone biologists. It may well be a key to understand regulation of the bone remodeling.

It is also possible that bone matrix may undergo time- or stress-related changes that can regulate osteoclast activity. However, at present somewhat controversial data exist about these changes. During ageing, matrix molecules, especially collagen, undergo nonenzymatic glycation, which leads to the accumulation of advanced glycation end (AGE) products into the bone matrix. A receptor for AGE products, RAGE, a member of the immunoglobulin superfamily, has several ligands and has been suggested to have a role in several diseases. Osteoclasts can detect AGE products because they express RAGE (Zhou *et al.*, 2006). RAGE –/– mice have increased bone mass and decreased resorption capacity of their osteoclasts, suggesting that AGE products can, via RAGE, activate signaling pathways

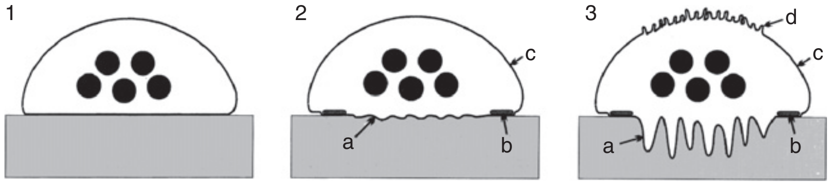

FIGURE 1 The nonresorbing osteoclast is polarized (1), but immediately after attachment for resorption it shows three different membrane domains (2): ruffled border (a), sealing zone (b), and basal membrane (c). Once matrix degradation has started (3), the fourth membrane domain, a functional secretory domain (FSD), appears in the basal membrane (d).

leading to osteoclast activation. This is in agreement with a study of Henriksen *et al.* (2007) showing that osteoclasts prefer aged bone. In contrast, Valcourt *et al.* (2007) have shown that AGE modifications inhibited osteoclasts, both their differentiation and activity. In conclusion, there is evidence, although indirect at this time, that osteocytes and bone matrix may both have a role in targeting osteoclasts to a correct resorption site.

Although it is not known exactly how resorption sites are determined, it is known that the first sign of a forthcoming resorption place on the endosteal surface is the retraction of bone-lining cells (Jones and Boyde, 1976). This retraction uncovers osteoid and after its removal by osteoblasts the osteoclasts can attach to the mineralized surface. The sequence of cellular events needed for bone resorption is called the resorption cycle (see Figs. 1 and 4). One resorption cycle of any individual osteoclast thus involves complicated multistep processes, which include osteoclast attachment, formation of the sealing zone, plasma membrane polarization, and resorption itself with final detachment and cell death (Lakkakorpi and Väänänen, 1995; Väänänen *et al.*, 2000). On the basis of in vitro studies, one osteoclast can undergo several consecutive resorption cycles before entering the apoptosis pathway (Kanehisa and Heerche, 1988). Following is a short description of each particular phase of the resorption cycle.

FORMATION OF THE SEALING ZONE AND CYTOSKELETAL CHANGES DURING THE RESORPTION CYCLE

The initial attachment of osteoclasts to bone matrix is mediated by integrins of heterodimeric adhesion receptors. So far five integrins have been identified in osteoclasts. $\alpha_v\beta_3$ (a classical vitronectin receptor), $\alpha_v\beta_5$, $\alpha_2\beta_1$ (collagen receptor), and $\alpha v\beta_1$, which binds to a variety of extracellular matrix proteins, including vitronectin, collagen, osteopontin, and bone sialoprotein (Nesbitt *et al.*, 1993). The latest one is $\alpha_9\beta_1$, which binds to ADAM8, a disintegrin and metalloproteinase (Rao *et al.*, 2006). The interactions between these integrins and bone matrix are functional important for osteoclast activation. Antibodies against the vitronectin receptor, Arg-Gly-Asp (RGD) mimetics, and RGD peptides, which block the attachment of the vitronectin receptor to RGD-containing bone matrix proteins, inhibit bone resorption in vitro (Sato *et al.*, 1990; Horton *et al.*, 1991; Lakkakorpi *et al.*, 1991) and in vivo (Fisher et al., 1993; Engelman et al., 1997). β_3- and α_9-integrin null mice have dysfunctional osteoclasts and impaired bone resorption (McHugh *et al.*, 2000; Rao *et al.*, 2006).

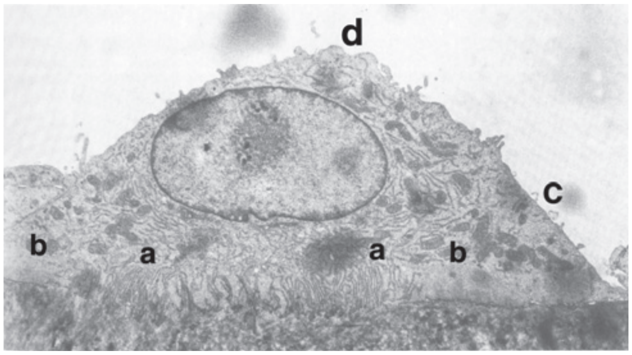

FIGURE 2 A transmission electron microscopic image of a bone-resorbing osteoclast. a, ruffled border; b, sealing zone; c, basal membrane; d, functional secretory domain. Original magnification: ×2500.

During osteoclast activation, the matrix-derived signals, transduced mainly through integrins, induce dramatic cytoskeleton and plasma membrane reorganization resulting in the formation of a ringlike structure of microfilaments known as the *actin ring* at the sealing zone (Lakkakorpi *et al.*, 1989). This structure surrounds a specialized plasma membrane domain, the ruffled border, thus forming an isolated resorptive microenvironment between the osteoclast and the underlying bone matrix (Väänänen *et al.*, 2000). The ultrastructure of a resorbing osteoclast (Fig. 2) clearly shows that the plasma membrane of resorbing osteoclast at the sealing zone is tightly bound to the matrix, offering a good diffusion barrier between the resorption lacuna and extracellular fluid (Väänänen and Horton, 1995). It has been demonstrated that a pH gradient really exists (Baron *et al.*, 1985), indicating that the sealing around the resorption lacuna is tight enough to maintain the low pH that is essential for bone mineral dissolution and the optimized activities of bone degradating enzymes (see Fig. 3). The permeability of the sealing zone is dynamically regulated during the resorption cycle (Stenbeck and Horton, 2000). This is the case not only in vitro; osteoclasts in bone also have a similar type of actin ring around the resorption lacuna (Sugiyama and Kusuhara, 1994).

The pioneer in vitro studies of osteoclasts on bone or dentine slices in late 1980s and early 1990s revealed that the microfilament pattern in osteoclasts undergoes rapid changes when preparing for resorption (Fig. 4) (Kanehisa *et al.*, 1990; Lakkakorpi *et al.*, 1989; Zambonin-Zallone *et al.*, 1988). In osteoclasts that are not resorbing, polymerized actin is accumulated in podosome-type structures throughout the whole bone-facing surface of the osteoclast. Gradually, an intense accumulation of podosomes to the local areas of the bone-facing membrane takes place (Lakkakorpi and Väänänen, 1991). Next, podosomes are collected into a large circular structure(s) and simultaneously the density of podosomes increases. At the last step, actin forms a dense beltlike structure where individual podosomes cannot be recognized under a light microscope. This dynamic process of podosome organization has been monitored in more detail recently by live confocal imaging and fluorescence recovery after photobleaching (FRAP) analysis in a monocytic cell line expressing an actin-green fluorescent protein (Destaing *et al.*, 2003). In individual podosomes, the polymerized actin is organized into a 300-nm dense actin core and a 500-nm "actin cloud," which correspond to the thick core bundle of actin fibers and a domelike radial meshwork of less densely packed actin fibers under the electron microscope, respectively (Luxenburg *et al.*, 2007). During osteoclast differentiation, these individual podosomes evolve from clusters in early stages, to dynamic rings at intermediate stages, to belts organized at the cell periphery in mature osteo-

matrix, isolated podosomes fuse into a continuous sealing zone. Because podosomes and the sealing zone have been exclusively detected in osteoclasts on collagen or plastic and those on mineralized matrix, respectively, Saltel *et al.* (2006) argue that the sealing zone is not derived from podosomes. However, by careful examination of cultured osteoclasts with high-resolution scanning electron microscopy, Luxenburg *et al.* (2007) found that the organization of individual podosomes in osteoclast on glass and bone as well as macrophages is similar, indicating a generalized basic structure of podosomes, independent of substrate or cell type. In all states of podosome organization, including the sealing zone, individual podosomes could still be detected. The sealing zone differs from individual or clustered podosomes mainly in their densities and degree of interconnectivity of podosomes. Although there is no direct evidence, the higher order of podosomes may physically segregate the sealing zone into two distinct regions governing barrier and adhesion functions, respectively.

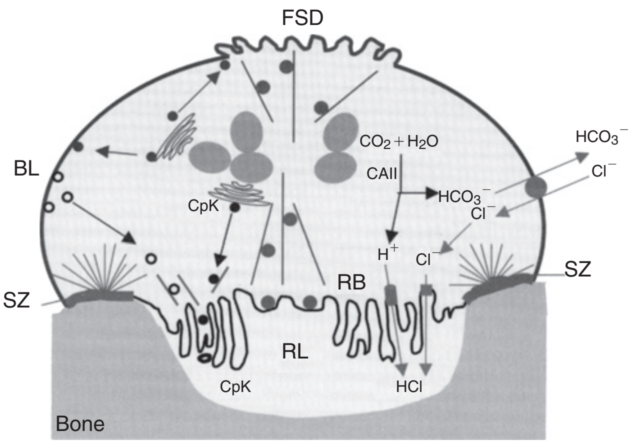

FIGURE 3 Schematic illustration of a bone-resorbing osteoclast. Intensive intracellular membrane trafficking is involved in the establishment of specific plasma membrane domains and resorption processes. Cathepsin K and protons are secreted vectorially to the resorption lacunae. Transcytotic vesicles are presented as brown full circles. BL, basolateral domain; CAII, carbonic anhydrase II; CpK, cathepsin K; FSD, functional secretory domain; RB, ruffled border; RL, resorption lacunae; SZ, sealing zone.

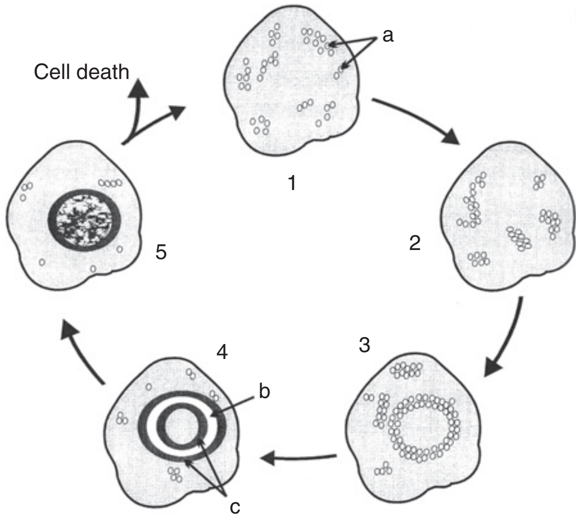

FIGURE 4 Organization of microfilaments in osteoclasts can be used to recognize different phases of the resorption cycle. When a nonresorbing cell (1) is induced to resorb, podosome-type structures (a) gather to certain areas of bone-facing surface (2) and finally form a large circular collection of podosomes (3). In the following step, the individual podosome-type structure disappears (4) and a distinct dense actin ring (b) appears between two broad vinculin rings (c). The vitronectin receptor is tightly colocalized with vinculin in those rings as well as in podosomes. After resorption, cytoskeletal rings disappear in a certain order (5) and the cell can either undergo apoptosis or return to the resting phase.

At the fully developed sealing zone, $\alpha_v\beta_3$ integrin and its associated proteins, such as talin, vinculin, and paxillin, form broad bands around the actin core (Lakkakorpi *et al.*, 1991). This distribution pattern suggests that molecules other than integrins are directly linking the actin core to the plasma membrane, although the signals derived from $\alpha_v\beta_3$ integrin are required for inducing actin reorganization and the sealing zone formation (McHugh *et al.*, 2000). This notion is also supported by observations from echistatin-treated mice (Masarachia *et al.*, 1998; Yamamoto *et al.*, 1998), which did not reveal changes in the sealing zone or ruffled borders.

A single podosome represents a unit of multiprotein complexes with F-actin and its regulators such as Arp2/3, WASP, cortactin, and gelsolin at the core, which is surrounded by integrins and their associated proteins (paxillin, talin, vinculin), adaptor proteins (Cbl, SH3BP2, and others), kinases (c-Src, Pyk2 and PI-3Ks), Rho GTPases (cdc42, Rho and Rac), enzymes (MT1-MMP, MMP-9), and endocytic regulators (e.g., dynamin) (Linder and Aepfelbacher, 2003; Linder and Kopp, 2005). The important role of many of these components in podosome and sealing zone formation in osteoclasts has been characterized during the past few years (see Table 1 and references therein). In addition to the regulating mechanisms at the podosome level, upstream signaling pathways induced by osteoclast activating cytokines are also critical for promoting cytoskeleton reorganization and the sealing zone formation. However, some of these cytokines, such as M-CSF and RANKL, are also required for osteoclast differentiation, making it very difficult to dissect their direct role in osteoclast cytoskeleton organization. To overcome this difficulty, Kim *et al.* (2006a) and Fuller *et al.*(2002) have developed an in vitro washaway-recovery system, in which actin rings in mature osteoclasts are disrupted quickly by cytokine starvation and allowed to re-form under stimulation by individual cytokines. This transient manipulation does not change the total number of osteoclasts on bone slices and does not induce apoptosis. Using this system we have revealed that M-CSF, RANKL, TNF and interleukin (IL)-1, but not culture medium alone, can directly stimulate actin-ring formation (Fig. 5). Because these cytokines do not induce sealing zone formation in osteoclasts cultured on plastic or glass, the data support the concept that signaling pathways downstream of both integrin and the cytokine receptors coordinately activate osteoclasts (Nakamura *et al.*, 2001; Faccio *et al.*, 2003).

Baron and Duong and their colleagues have identified a signaling complex, consisting of Pyk2/Src/Cbl/PI-3K, which links $\alpha_v\beta_3$-integrin signaling to actin-ring formation in osteoclasts (Sanjay *et al.*, 2001; Duong *et al.*, 1998). More recently, a novel protein complex mediating $\alpha_v\beta_3$-integrin's function in osteoclasts has been identified (Zou *et al.*, 2007). In this model, protein tyrosine kinases, c-Src and Syk, bind directly to the cytoplasmic tail of β_3-integrin and form a complex with immune receptors with ITAMs (immunoreceptor tyrosine-based activation motifs), FcRγ and Dap12. The complex then activates Vav3, which is a GEF (guanine exchange factor) of small GTPase Rac and promotes actin cytoskeleton organization and function of osteoclasts (Faccio *et al.*, 2005). In both models, c-Src plays a central role in $\alpha_v\beta_3$-integrin mediated osteoclast activation. c-Src-deficient mice, which suffer from osteoporosis, have a normal number of osteoclasts, which attach to bone but do not form a normal sealing zone and the ruffled border (Boyce *et al.*, 1992; Soriano *et al.*, 1991). Although the osteoclast phenotypes can be genetically rescued by kinase-deficient mutants of c-Src, the c-Src kinase activity, at least in vitro, is required for podosome and the sealing zone formation (Miyazaki *et al.*, 2004). Calpain, an intracellular cysteine protease, has recently been identified as regulating osteoclast cytoskeleton organization by selectively cleaving talin, which links integrin to the actin cytoskeleton (Hayashi *et al.*, 2005). Both IL-1 and RANKL can directly stimulate cytoskeleton organization and the sealing zone formation in osteoclasts (see Fig. 5 and Nakamura *et al.*, 2002; Burgess *et al.*, 1999). The cytoskeletal effects of both cytokines are likely mediated by a common downstream effector, TRAF6. IL-1 induces a TRAF6/c-Src complex formation in osteoclasts (Nakamura *et al.*, 2002), whereas gene rescuing experiments in RANK- and TRAF6-deficient osteoclasts have demonstrated that TRAF6, especially its RING finger domain, is essential for osteoclast cytoskeletal organization and resorptive function (Armstrong *et al.*, 2002; Kobayashi *et al.*, 2001).

Compared to what we know for actin, much less is known about the changes in the organization of microtubules and intermediate filaments during the resorption cycle. In osteoclasts cultured on glass, each nucleus preserves its own microtubule-organizing center during fusion (Moudjou *et al.*, 1989). However, in resorbing mammalian osteoclast, individual centrioles are eliminated and microtubules are radiating from perinuclear area (Mulari *et al.*, 2003b). In resorbing osteoclasts, microtubules form thick bundles in the middle of the cell, originating from the top of the cell and converging toward the ruffled border (Lakkakorpi and Väänänen, 1995). Data suggest that these microtubules actually extend from the ruffled border to the functional secretory domain and are most probably mediating transcytotic trafficking. Even less is known about the organization of intermediate filaments and their possible changes during the polarization of osteoclasts.

RESORBING OSTEOCLASTS ARE HIGHLY POLARIZED AND SHOW FOUR DIFFERENT MEMBRANE DOMAINS

Osteoclasts cycle between resorbing and nonresorbing phases, which are accompanied by drastic changes in their polarization (see Figs. 1 to 4). Resorbing osteoclasts are highly polarized cells containing several different plasma membrane domains, whereas those osteoclasts that are not resorbing do not reveal clear morphological features of polarity. In resorbing cells the sealing zone itself forms one distinctive membrane domain and simultaneously separates two

TABLE 1 The Components of Podosomes and Their Function in Osteoclasts

Molecules	Localization		Function	Refs.
	Podosomes	Osteoclasts on bone		
F-actin	Core	Sealing zone	Structure element sealing zone formation/polarization	Zambone-Zallone *et al.*, 1988
Actin regulators				
Arp2/3	Core	Sealing zone	Actin polymerization knockdown by RNAi disrupts sealing zone	Hurst *et al.*, 2004
WASP	Core	Sealing zone	Null osteoclasts are markedly depleted of podosomes and fail to form sealing zone	Calle *et al.*, 2004
Actin-binding proteins				
Cortactin	Core + ring	Sealing zone	Src substrate, Arp2/3 activator Knockdown by RNAi inhibits sealing zone formation	Tehrani *et al.*, 2006
Gelsolin	Core	Sealing zone	Uncapping and severing of F-actin podosome assembly Sealing zone formation	Wang *et al.*, 2003
α-Actinin	ND	ND	ND	
Rho GTPases				
Rho A	ND	ND	Podosome organization and sealing zone formation	Chellaiah *et al.*, 2000
Cdc42	Core	ND	WASP activation Sealing zone formation	Chellaiah *et al.*, 2005
Rac	ND	ND	Sealing formation	Razzouk *et al.*, 1999
Integrins				
β3	Ring	Basal membrane, surround sealing zone	Cell–matrix contact signal transduction, null osteoclasts have abnormal sealing zone	McHugh *et al.*, 2000
β1	Core + ring	Sealing zone	Sealing zone formation	Helfrich *et al.*, 1996
Adaptor proteins				
Talin	Ring	Surround sealing zone	Adaptor between integrins and actin-binding proteins Function in OC, ND	Lakkakorpi *et al.*, 1991
Vinculin	Ring	Sealing zone	Function in OCs, ND	Lakkakorpi *et al.*, 1991
Paxillin	Ring	Surround sealing zone	ND	Pfaff and Jurdic, 2001
Kinases				
c-Src	Ring	Sealing zone and ruffled border	Non-receptor tyrosine kinase, null OCs have no podosomes and sealing zone	Boyce *et al.*, 1992
Pyk2	Ring	Sealing	Dominated negative disrupts sealing zone formation produce IP3	Duong *et al.*, 1998
PI-3K	Ring	ND	PI-3K inhibitors destroy sealing zone formation	Nakamura *et al.*, 1995
Metalloproteases				
MT1-MMP	Core	Sealing zone	ECM degradation podosome dynamic, osteoclast migration	Sato *et al.*, 1997
MMP-9	Core	Sealing zone	ECM degradation podosome dynamic, osteoclast migration	Spessoto *et al.*, 2002
Motor proteins				
Dynamin	Core	Sealing zone	GTPases essential for endocytosis podosome invagination, podosome dynamic and sealing zone formation	Bruzzaniti *et al.*, 2005

ECM, *extracellular matrix*; ND, *not determined*; OC, *osteoclast*.

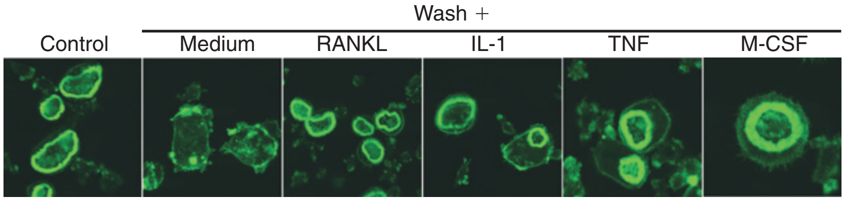

FIGURE 5 Mature osteoclasts were generated from bone marrow macrophages with M-CSF and RANKL for 5 days. The cells were either untreated (control) or washed with cold medium followed by culturing with medium alone (medium) or medium plus 100 ng/mL RANKL, 10 ng/mL IL-1, 10 ng/mL TNF, and 50 ng/mL M-CSF, respectively, for 60 minutes. The cells were then fixed and F-actin was stained with Alexa-488–phalloidin. (See plate section)

other membrane domains, the ruffled border (RB) and the basolateral domain (see Figs. 1 and 2). In addition to these three membrane domains, morphological and functional studies have revealed a distinct membrane domain in the basal membrane, namely a functional secretory domain (FSD; Salo *et al.*, 1996). This membrane domain has some characteristic features of the apical membrane domain in epithelial cells. Viral proteins that are usually targeted to the apical domain are, in osteoclasts, targeted to this domain. In addition, FSD is morphologically different from the rest of the membrane, and it has been also shown to be a target for transcytotic vesicles carrying bone degradation products (Salo *et al.*, 1997; Nesbitt and Horton, 1997). In resorbing osteoclasts, thick bundles of microtubules connect the RB and the FSD (Mulari *et al.*, 2003a), and a specific type of exocytotic vesicles, clastosomes, have been described in close association to the FSD (Salo, 2002). At present it is not known how formation of FSD is regulated.

The ruffled border membrane forms the actual "resorbing organ." The characteristics of this unique membrane domain do not perfectly fit any other known plasma membrane domain described so far. The ruffled border membrane is formed by the rapid fusion of acidic intracellular vesicles (Palokangas *et al.*, 1997). Many of the proteins reported to be present at the ruffled border are also found in endosomal and/or lysosomal membranes, including the vacuolar proton pump, mannose-6-phosphatase receptor, rab7, and 1 gp 110 (Baron *et al.*, 1988; Väänänen *et al.*, 1990; Palokangas *et al.*, 1997). Functional experiments, for instance, with labeled transferring and dextran, have further supported the conclusion that the ruffled border represents a plasma membrane domain with typical features of the late endosomal compartment (Palokangas *et al.*, 1997).

A small GTPase, rab7, regulates targeting and docking of acidic vesicles to the ruffled border (Zhao *et al.*, 2002). On their way to the ruffled border acidic vesicles are translocated from the microtubule-mediated trafficking to actin filaments (Mulari *et al.*, 2003a), and this transition could happen via specific protein–protein interaction between rab 7 and another small GTPase, Rac1 (Sun *et al.*, 2005).

Several membrane proteins reveal a nonhomogeneous distribution at the ruffled border. For instance, the vacuolar proton pump (Mattsson *et al.*, 1997), as well as rab7, is concentrated at the lateral edges of the ruffled border (Palokangas *et al.*, 1997). Our functional experiments have also shown that exocytotic vesicles in resorbing osteoclasts are found at the lateral areas of the ruffled border, and endocytotic vesicles mainly bud from the central area of the ruffled border (Zhao *et al.*, 2001). The ruffled border is thus composed of two different domains—lateral and central—where exocytosis and endocytosis occur, respectively. This allows simultaneous proton and enzyme secretion and the endocytosis of degradation products.

In order to create and maintain specialized membrane domains, as well as to transport products of biosynthetic or secretory machinery, the cell has distinct intracellular trafficking routes, originating from one compartment and reaching another. Intracellular vesicular routes shown to be operating in the resorbing osteoclasts were presented schematically in Fig. 3. It is obvious that these events could be highly specific and may offer new potential targets to inhibit or stimulate bone resorption. In addition to its role in the organization of membrane domains, the cytoskeleton also undergoes drastic reorganization during cell polarization. By following transferrin traffic in osteoclasts during different stages of the resorption cycle, one can see how dynamic membrane trafficking in osteoclasts actually is. In bone-resorbing cells, the bulk of the endocytosed transferrin is rapidly targeted to the ruffled border (Mulari *et al.*, 2003a), whereas in osteoclasts that are moving on the bone surface transferrin is mainly targeted via perinuclear recycling compartment to the leading edge (Sun *et al.*, unpublished).

HOW OSTEOCLASTS DISSOLVE BONE MINERAL

Bone mineral is mainly crystalline hydroxyapatite, and there are not many biological processes that could be responsible for the solubilization of crystals. In fact, the only process that has been suggested to be able to solubilize hydroxyapatite crystals in the biological environment is low pH. Thus the idea that bone resorption is facilitated by local acidification has been discussed among bone biologists for a long time, and Fallon *et al.* (1984) demonstrated that the resorption lacuna is really acidic. This observation was also confirmed later by other investigators who used the accumulation of acridine orange in acidic compartments as an indicator of low pH (Baron *et al.*, 1985).

The first experiments to explain the molecular mechanism of lacunar acidification suggested the presence of gastric-type proton pumps in osteoclasts (Baron *et al.*, 1985; Tuukkanen and Väänänen, 1986). However, it soon became clear that the main type of proton pump in the ruffled border of osteoclasts is a V-type ATPase (Bekker and Gay, 1990a; Blair *et al.*, 1989; Väänänen *et al.*, 1990).

V-type ATPases are electrogenic proton pumps that contain several different subunits forming two independently assembled complexes: cytoplasmic and membrane bound. (For a review, see Drory and Nelson, 2006.) V-type ATPases are found in all mammalian cells, and they are responsible for the acidification of various intracellular compartments, including endosomes, lysosomes, secretory vesicles, and synaptic vesicles. The membrane-bound complex is composed of at least five different subunits, and the soluble catalytic complex contains at least eight different subunits. Each pump contains several copies of each subunit, and two or even more isoforms for several subunits have been described. In addition to the marked differences in expression level in different cells and tissues, different isoforms are specifically expressed in certain cells, giving, at least in theory, a good possibility for a remarkable cell and tissue specificity.

Heterogeneity of the 116-kDa a3 subunit has turned out to be of special importance for bone biology. Four different isoforms of this subunit that stabilize the soluble complex to the membrane complex have been characterized. It has been shown that a marked number of patients suffering from malignant osteopetrosis have a mutation in the a3 subunit of vacuolar type ATPase (Kornak *et al.*, 2000; Frattini *et al.*, 2000). Further evidence for the importance of this particular subunit is provided by the severe osteopetrotic phenotype in knockout mice of this specific subunit (Li *et al.*, 2000) and also by the fact that oc/oc osteopetrotic mice have a deletion in this subunit (Scimeca *et al.*, 2000). Antisense oligonucleotides and siRNAs against different subunits of the proton pump complex have also been used to confirm the role of the proton pump in osteoclast function (Laitala and Väänänen, 1994; Hu *et al.*, 2005).

The low pH in the resorption lacuna is achieved by the action of the proton pump both at the ruffled border and in intracellular vacuoles. Cytoplasmic acidic vacuoles disappear at the time when the ruffled border appears, during the polarization of the cell. Thus the initial acidification of the subcellular space is achieved by the direct exocytosis of acid during the fusion of intracellular vesicles to form the ruffled border. This ensures rapid initiation of mineral dissolution, and further acidification may be obtained by the direct pumping of protons from the cytoplasm to the resorption lacuna. As mentioned above small GTPases Rab7 and Rac1 regulates targeting of acidic vesicles to the ruffled border. This targeting is obviously also partially mediated via subunit B of V-ATPase since direct interaction between subunit B and actin filament has also been described in osteoclasts (Zuo *et al.*, 2006).

In vitro studies with isolated osteoclasts and bafilomycin A1 were first used to show the functional importance of the proton pump for mineral dissolution (Sundquist *et al.*, 1990). An elegant in vivo study using local administration of bafilomycin A1 confirmed that inhibition of the vacuolar proton pump can potentially be used to inhibit bone resorption (Sundquist and Marks, 1995). The development of new types of vacuolar proton pump inhibitors with improved specificity and selectivity has opened a whole new opportunity for treating osteoporosis and other bone metabolic bone diseases (for review, see Niikura, 2006). However, it is still not known whether any present inhibitor is suitable for the clinical use.

In addition to the proton pump, several other molecules have critical functions in the acidification of the resorption lacuna. Minkin and Jennings (1972) showed that bone resorption in mouse calvarial cultures could be inhibited by carbonic anhydrase (CA) inhibitors and decrease of CA II expression by using antisense technology leads to the inhibition of bone resorption in cultured rat osteoclasts (Laitala and Väänänen, 1994). In humans, Ca II deficiency causes nonfunctional osteoclasts and osteopetrosis (Sly and Hu, 1995), and aging CA-II deficient mice also show mild osteopetrosis (Peng *et al.*, 2000). In addition to soluble CA II at least two membrane-bound carbonic anhydrases, namely CA IV and CA XIV, are expressed in osteoclasts (Riihonen *et al.*, 2006).

Pumping of protons through the ruffled border is balanced by the secretion of anions (see Fig. 3). The presence and functional importance of a high number of chloride channels in the ruffled border membrane have been shown by demonstrating that loss of the chloride channel isoform CIC-7 leads to osteopetrosis both in patients and mice (Kornak *et al.*, 2001). CIC-7 is associated with another membrane protein, Ostm1, a protein of unknown function (Lange *et al.*, 2006), and it is of interest that mutations in this gene lead to osteopetrosis both in mice (grey-lethal mice) and in humans (Ramirez *et al.*, 2004). Polymorphisms of the same gene are associated with bone mineral density (Pettersson *et al.*, 2005). It is not thus surprising that CIC-7 is considered as one of the most interesting drug targets for osteoporosis (Schaller *et al.*, 2005).

In addition to the proton pump and chloride channel, the ruffled border also seems to have K+/Cl-cotransporter KCC1, which participates somehow in proton extrusion (Kajiya *et al.*, 2006). The outflow of chloride anions through the ruffled border is most likely compensated by the action of a HCO_3/Cl exchanger in the basal membrane. The basolateral membrane of resorbing osteoclasts also contains a high concentration of Na/K-ATPase (Baron *et al.*, 1986) and Ca-ATPase (Bekker and Gay, 1990b).

HOW OSTEOCLASTS DEGRADE ORGANIC MATRIX

After solubilization of the mineral phase, the organic matrix is degraded and removed from the resorption lacuna. The roles of two major classes of proteolytic enzymes—lysosomal cysteine proteinases and matrix metalloproteinases (MMPs)—have been studied most extensively. The question of the role of proteolytic enzymes in bone resorption can be divided into at least four subquestions. First, what are the proteolytic enzymes, which are needed to remove unmineralized osteoid from the site of future resorption? Second, what are the proteolytic enzymes, which take part in the degradation of organic matrix in the resorption lacuna? Third, is there intracellular matrix degradation in osteoclasts, and what proteolytic enzymes, if any, are responsible for the intracellular degradation process? Fourth, where and how are different proteolytic enzymes targeted during the resorption process?

Sakamoto and Sakamoto (1982) and Chambers *et al.* (1985) suggested that osteoblast-derived collagenase (MMP-1) plays a major role in the degradation of bone covering osteoid. Removal of the osteoid layer seems to be a necessary or even obligatory step for the future action of osteoclasts. Although the role of osteoblasts seems to be essential in this early phase of bone resorption, it has not been shown definitively that osteoblasts in situ are responsible for the production of all proteolytic enzymes necessary for osteoid degradation. The possibility cannot be ruled out that some proteinases necessary for matrix degradation have already been produced and stored in the matrix during bone formation.

In addition to MMP-1, a membrane-bound matrix metalloproteinase (MT1-MMP) clearly has a role in bone turnover. MT1-MMP-deficient mice develop dwarfism and osteopenia among other connective-tissue disorders (Holmbeck *et al.*, 1999). The analysis of deficient mice, however, suggests that the primary defects are in bone-forming cells rather than in osteoclasts, although some studies have also indicated a high expression on MT1-MMP in osteoclasts (Sato *et al.*, 1997; Pap *et al.*, 1999). It appears to be localized in specific areas of cell attachment, but at the moment there are no direct data pointing to the specific function of MT1-MMP in the resorption process. In conclusion, it is evident that several MMPs that are in osteoblastic cells play an important role in the regulation of bone turnover, but specific mechanisms remain to be elucidated.

It is obvious that a substantial degradation of collagen and other bone matrix proteins during bone resorption takes place in the extracellular resorption lacuna. Two enzymes, cathepsin K and MMP-9, have been suggested to be important in this process. Data that cathepsin K is a major proteinase in the degradation of bone matrix in the resorption lacuna are now very convincing. First of all, it is highly expressed in osteoclasts and is also secreted into the resorption lacuna (Everts *et al.*, 2006; Troen, 2006). Second, it has been shown that it can degrade insoluble type I collagen, and inhibition of its enzymatic activity in in vitro and in vivo models prevents matrix degradation (Bossard *et al.*, 1996; Votta *et al.*, 1997). Third, deletion of the cathepsin K gene in mice leads to osteopetrosis (Saftig *et al.*, 1998; Gowen *et al.*, 1999). Finally, human gene mutations of cathepsin K lead to pyknodysostosis (Gelb *et al.*, 1996; Johnson *et al.*, 1996).

These data have encouraged several researchers and pharmaceutical companies to try to develop specific inhibitors of cathepsin K to be used in the treatment of osteoporosis (Kumar *et al.*, 2007; Palmer *et al.*, 2005). Because mineral dissolution is not prevented by such inhibitors, it is possible that inhibition of this enzyme could lead to the accumulation of an unmineralized matrix. This is supported by a recent finding of Li *et al.* (2006) showing increased bone fragility despite high bone mass in mice lacking cathepsin K. This may be an important contraindication to the long-term use of cathepsin K inhibitors in the treatment of bone diseases.

Osteoclasts are also rich in cystatin B, a natural inhibitor of cysteine proteinases. Addition of cystatin B to the culture medium of bone marrow cells cultured on bone slices protected osteoclasts from experimentally induced apoptosis (Laitala-Leinonen *et al.*, 2006), suggesting that as in neuronal cells, a balance of cysteine proteinases and their natural inhibitors regulates cell survival. This hypothesis is further supported by Chen *et al.* (2007), who demonstrated that cathepsin K –/– mice showed premature cell senescence. A number of other lysosomal cysteine proteinases—cathepsins B, D, L, and S—have been suggested to play a role in osteoclasts, and data exist showing that at least B and L are also secreted into the resorption lacuna (Goto *et al.*, 1994). An interesting proteomic analysis of lysosomal acid hydrolases secreted by osteoclasts indicated that almost all known cathepsins were produced by Raw 264.7 cell derived osteoclasts (Czupalla *et al.*, 2006). These results also suggest that osteoclasts target several lysosomal hydrolases for secretion and do not release them only by fusion of endosomes/lysosomes to the ruffled border.

MMP-9 is highly expressed in osteoclasts and secreted into the resorption lacuna. MMP-9 knockout mice show an interesting phenotype of transient osteopetrosis (Vu *et al.*, 1998) suggesting that it is mainly needed in the degradation of calcified cartilage during animal growth. Fuller *et al.* (2007) recently demonstrated that inhibitors of MMPs had no effect on CTX-I release in in vitro resorption assay with human osteoclasts. These studies together with some previous in vitro studies (Everts *et al.*, 1998) support the conclusion that MMP-9 is not important in the resorption process of bone matrix as such but plays a role, for example, during migration and attachment of osteoclasts, and perhaps in the resorption of calcified cartilage. Some other studies suggest that the role of MMP-9 in different populations of osteoclasts could be different, and furthermore that osteoclasts may be a more heterogeneous cell population than previously thought (Everts *et al.*, 2006).

In conclusion, evidence indicates that several MMPs and lysosomal proteinases, especially cathepsin K and some other cysteine proteinases, play a major role in the matrix degradation (Everts *et al.*, 2006; Fuller *et al.*, 2007). The coordinated action of several proteinases may be needed to solubilize completely fibrillar type I collagen and other bone matrix proteins. Some of these proteinases obviously act mainly extracellularly and some intracellularly or both. Extracellular matrix degradation may be further enhanced by production of free oxygen radicals and this may continue also during the transcytosis through the cell. In addition, osteoclasts may produce and target several lysosomal enzymes not only to the resorption lacuna and intracellular compartments but also to other membrane domains than the ruffled border for secretion (Czupalla *et al.*, 2006). Specific function(s) of these secreted enzymes remain to be found.

HOW BONE DEGRADATION PRODUCTS ARE REMOVED FROM THE RESORPTION LACUNA

During bone resorption the ruffled border membrane is continuously in very close contact with the bone matrix. Thus, it is somewhat misleading to speak about a resorption lacuna, which is only a very narrow space between the cell membrane and the matrix components. This tentatively suggests that degradation products must be somehow removed continuously from the resorption space in order to allow the resorption process to continue. Theoretically, there are two different routes for resorption products. They can either be released continuously from the resorption lacuna beneath the sealing zone or be transcytosed through the resorbing cell.

Salo *et al.* (1994) provided the first evidence for the transcytosis of bone degradation products in vesicles through the resorbing osteoclasts from the ruffled border to the functional secretory domain of the basal membrane. This finding was later confirmed by demonstrating fragments of collagen and other matrix proteins in the transcytotic vesicles (Salo *et al.*, 1997; Nesbit *et al.*, 1997). When matrix degradation products are endocytosed, it is possible that further degradation of matrix molecules takes place during the transcytosis. Experiments where bone degradation products and cathepsin K were localized simultaneously in resorbing osteoclasts revealed that they exist in a same pool of transcytotic vesicles (Vääräniemi *et al.*, 2004). Results further showed that TRACP-containing vesicles were fused into matrix and cathepsin K–containing vesicles on their way to the functional secretory domain. On the other hand it has been shown that TRACP can generate highly destructive reactive oxygen species (ROS) that are able to destroy collagen and other proteins (Halleen *et al.*, 1999). In addition, cleavage of TRACP with cathepsin K increases its activity for ROS formation (Fagerlund *et al.*, 2006). All these results together suggest a new function for TRACP in the final destruction of matrix components during the transcytosis. The observed phenotype of mild osteopetrosis in TRACP knockout mice (Hayman *et al.*, 1996) is in good agreement with this.

In addition to collagen and other protein fragments, calcium and phosphate also have to be removed from the resorption lacuna. Our initial observations with tetracycline-labeled bone matrix suggested that calcium is also transcytosed from the resorption lacuna (Salo *et al.*, 1997). A recent study by Yamaki *et al.* (2006) supports this suggestion, demonstrating the presence and transport of calcium in vesicles towards the functional secretory domain. However, until now it has not been possible to exclude a leakage of calcium and phosphate directly from the resorption lacuna to the extracellular medium. This mechanism would at least consume less energy than vesicular transcytosis. In addition to these two routes, there is also some evidence to suggest that a nonvesicular transcytosis could be a possibility, although the evidence is still rather vague. This possibility is supported by the finding that a high amount of Ca-channel TRPV5 is located at the ruffled border, and when the gene is knocked out, osteoclasts lose the capacity to resorb bone (van der Eerden, 2005). This option would also involve the active participation of cytoplasmic calcium binding proteins such as calmodulin. Only a very limited number of indirect data are available about phosphate transport from the resorption lacuna during bone resorption. Ito *et al.* (2005) found, using osteoclast-like cells, that these cells had Na-dependent phosphate transport that was induced by acidic pH.

Stenbeck and Horton (2000) suggested that the sealing zone is a more dynamic structure than previously thought and could be loose enough to allow the diffusion of molecules from the extracellular fluid into the resorption lacuna. On the basis of their findings, the authors concluded that the sealing zone may also allow diffusion of the resorption products out from the lacuna. It remains to be seen whether all these three mechanisms are operational and if one of them is more important than others.

WHAT HAPPENS TO OSTEOCLASTS AFTER RESORPTION

In vitro studies have shown that an osteoclast can go through more than one resorption cycle (Kanehisa and Heersche, 1988; Lakkakorpi and Väänänen, 1991). Unfortunately, we do not yet know if this also happens in vivo or if the osteoclast continues its original resorption cycle as long as it is functional. Regardless, there must be a mechanism that destroys multinucleated osteoclasts in situ. There are at least two different routes that the multinucleated osteoclast can take after it has fulfilled its resorption task. It can undergo fission into mononuclear cells or it can die.

Very little evidence exists to support the idea that multinucleated cells are able to undergo fission back to mononuclear cells. It is thus very likely that all osteoclasts die via apoptosis. It is becoming more evident that changing the osteoclast life span is a major mechanism to regulate bone resorption activity. Many physiological as well as pharmacological agents seem to use this particular mechanism of action.

At present many physiological as well as pharmacological agents are known to induce apoptosis in osteoclasts, for instance, bisphosphonates, which are widely used to inhibit bone resorption and prevent bone loss. Clodronate and aminobisphosphonates both induce apoptosis, but their mechanisms of action are different. Aminobisphosphonates inhibit protein prenylation by blocking an enzyme, farnesyl pyrophosphate synthetase (Russell, 2006), and this inhibition leads to disturbances of intracellular vesicular trafficking and finally to cell death (Luckman *et al.*, 1998). In clodronate-treated cells apoptosis is due to the inhibition of mitochondrial function, and the target molecule is mitochondrial ADP/ATP translocase (Lehenkari *et al.*, 2002). Another example of drug-induced apoptosis is reveromycin A, which is an acid-activated isoleucyl-tRNA synthetase inhibitor with a remarkable specificity to osteoclasts in experimental animals (Woo *et al.*, 2006).

In addition to bisphosphonates, estrogen and calcitonin have been suggested to regulate osteoclast apoptosis (Hughes *et al.*, 1996; Selander *et al.*, 1996a). It is quite evident that cell–matrix interactions are also important signals for osteoclast survival. The importance of the right extracellular milieu for the osteoclast phenotype and for survival is seen clearly when one compares their sensitivity to extracellular calcium. A moderate concentration of extracellular calcium has been shown to promote apoptosis in osteoclasts cultured on an artificial substrate (Lorget *et al.*, 2000), whereas if cultured on bone, the cells can tolerate high concentrations of extracellular calcium (Lakkakorpi *et al.*, 1996).

A good example of a prolonged life span of osteoclasts on the bone metabolism is SHIP–/– mice (Takeshita *et al.*, 2002) which shows a marked osteoporosis due to increased number of hyperactive osteoclasts. A study by Kim *et al.* (2006a), with targeted knockout of glucocorticoid receptor in osteoclasts, suggests that osteoclasts are a primary target of glucocorticoids and may even regulate bone formation via an osteoclast-mediated pathway. Another animal model, targeted overexpression of 11-β-hydroxysteroid dehydrogenase 2, an enzyme that inactivates glucocorticoids, seems to support also a direct effect on osteoclasts (Jia *et al.*, 2006).

It is most likely that the molecular pathways leading to apoptosis in osteoclasts are similar to those described in other cells. It is possible, however, that the highly specific phenotype of the osteoclast also includes cell-specific features of cell survival and death (Tanaka *et al.*, 2006). If this turns out to be a case, it will open a number of new strategies to regulate osteoclast activity in various bone diseases.

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Chapter 11

Receptor Activator of NF-κB (RANK) Signaling

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INTRODUCTION

Osteoclasts are multinucleated cells of monocyte/macrophage origin that degrade bone matrix. Congenital lack or dysfunction of osteoclasts causes osteopetrosis, the investigation of which has provided insights into the essential molecules for osteoclast differentiation and function (Asagiri and Takayanagi, 2007; Teitelbaum and Ross, 2003). Naturally occurring or genetically modified osteopetrotic mice have provided information on the molecules essential for osteoclastogenesis, including receptor activator of nuclear factor (NF)-κB (RANK), RANK ligand (RANKL), and macrophage colony-stimulating factor (M-CSF). Osteoclasts are generated *in vitro* from bone marrow cells stimulated with recombinant RANKL and M-CSF. This osteoclast differentiation system allows a rough but extremely useful visualization of the differentiation process, and the cells undergoing differentiation can then be subjected to extensive molecular analyses. Here we focus on studies of the intracellular signal transduction of RANK, and summarize recent progress in the understanding of the mechanisms of osteoclastogenesis.

LESSONS FROM OSTEOPETROTIC MICE

Osteopetrotic mice revealed a number of genes essential for osteoclast differentiation, which are categorized into two types (Fig. 1): genes involved in the generation of osteoclast precursor cells and those involved in the promotion of the differentiation process. The first group of genes includes *M-CSF* (Yoshida *et al.*, 1990), *Csf1r/c-Fms* (encoding M-CSF receptor) (Dai *et al.*, 2002), and the transcription factor *PU.1* (Tondravi *et al.*, 1997), which are involved in the generation of common progenitors for macrophages and osteoclasts. The deletion of these genes results in the lack of both macrophages and osteoclasts. M-CSF induces the proliferation of osteoclast precursor cells, supports their survival, and upregulates RANK expression, which is a prerequisite for their further differentiation (Ross and Teitelbaum, 2005; Arai *et al.*, 1999). The transcription factor PU.1 binds to the promoter region of *Csf1r* and positively regulates transcription (Zhang *et al.*, 1994), and mice deficient in *PU.1* display an osteopetrotic phenotype similar to *op/op* mice (Tondravi *et al.*, 1997). MITF is activated by M-CSF signaling (Weilbaecher *et al.*, 2001) and binds to the *Bcl-2* promoter (McGill *et al.*, 2002). MITF regulates the expression of the anti-apoptotic protein Bcl-2 in the osteoclast lineage (McGill *et al.*, 2002), and both *mi/mi* mice (carrying a mutation at the *MITF* locus) and *Bcl-2*-deficient mice exhibit osteopetrosis (McGill *et al.*, 2002; Hodgkinson *et al.*, 1993).

The second group includes *RANKL* (Kong *et al.*, 1999), *RANK* (Dougall *et al.*, 1999; Li *et al.*, 2000), *TRAF6* (Kobayashi *et al.*, 2003; Lomaga *et al.*, 1999; Naito *et al.*, 1999), *c-Fos* (Johnson *et al.*, 1992; Wang *et al.*, 1992), *NF-κB (p50/p52)* (Franzoso *et al.*, 1997; Iotsova *et al.*, 1997), *NFATc1* (Asagiri *et al.*, 2005), and *Fc receptor common* γ *subunit (FcR*γ*)/DNAX-activating protein 12* (*DAP12*) (Koga *et al.*, 2004; Mocsai *et al.*, 2004), the deficiency of which leads to the loss of multinucleated osteoclasts despite a normal (or increased) number of macrophages. *DC-STAMP* (Yagi *et al.*, 2005), *Gab2* (Wada *et al.*, 2005), and *IKK*β (Ruocco *et al.*, 2005) are also included in this group, but the osteopetrotic phenotype that develops with a deficiency of these genes is less severe. In a culture system, *Syk* (Koga *et al.*, 2004; Mocsai *et al.*, 2004), *NIK* (Novack *et al.*, 2003), and *IKK*α (Ruocco *et al.*, 2005) have also been shown to be included in this group. Despite the long list of molecules possibly involved in RANK signaling, we cannot fully understand the differentiation process until sequential molecular events activated by RANK are revealed.

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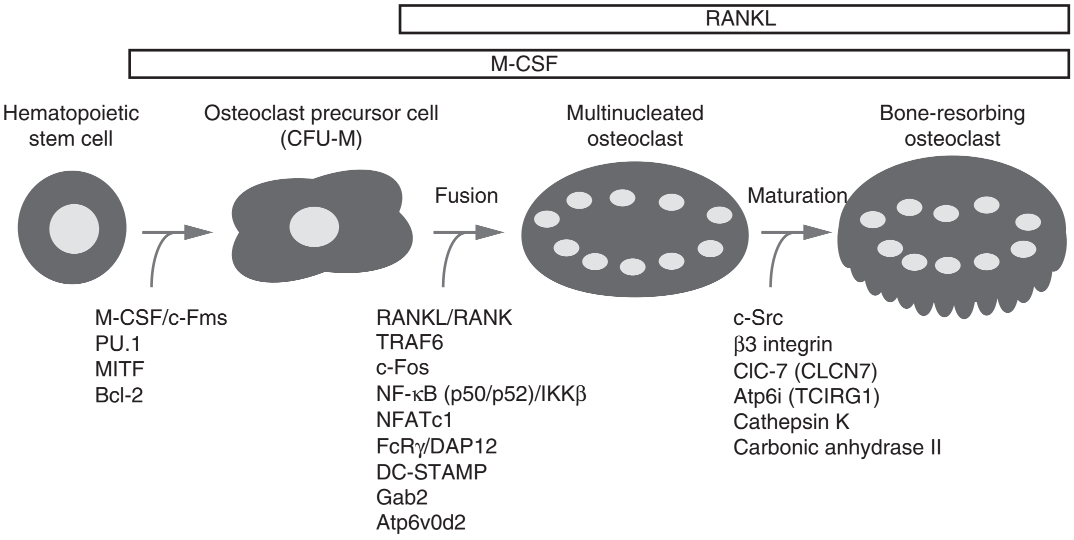

FIGURE 1 Key molecules for osteoclast differentiation. Osteoclast differentiation requires two major cytokines, M-CSF and RANKL, at certain indicated periods. Hematopoietic stem cells undergo differentiation into macrophage colony-forming units (CFU-M), which are the common precursor cells of macrophages and osteoclasts. The differentiation step from CFU-M to multinucleated osteoclasts is characterized by cell–cell fusion, which is mainly induced by RANKL. Mature osteoclasts acquire bone-resorbing activity, which is also dependent on M-CSF and RANKL.

Another set of genes is comprised of *c-Src* (Lowe *et al.*, 1993; Soriano *et al.*, 1991), *ClC-7 (CLCN7)* (Kornak *et al.*, 2001), *Atp6i (TCIRG1)* (Frattini *et al.*, 2000; Li *et al.*, 1999), and *cathepsin K* (Saftig *et al.*, 1998). Mice deficient in these molecules have osteoclasts with no or very little bone-resorbing activity, indicating these molecules are crucial for osteoclast function.

TRAF6

RANK lacks intrinsic enzymatic activity in its intracellular domain, and the analyses of molecules associating with the cytoplasmic domain of RANK revealed that it transduces signaling by recruiting adaptor molecules such as the TRAF family of proteins (Darnay *et al.*, 1998; Galibert *et al.*, 1998; Wong *et al.*, 1999; Wong *et al.*, 1998). The TRAF family contains seven members (TRAFs 1, 2, 3, 4, 5, 6, and 7) and mainly mediates signals induced by the TNF family of cytokines and pathogen-associated molecular patterns (PAMPs) (Bouwmeester *et al.*, 2004; Inoue *et al.*, 2000). The cytoplasmic tail of RANK contains three TRAF6-binding sites, and the phenotype of knockout mice revealed TRAF6 to be the major adaptor molecule linking RANK to osteoclastogenesis (Lomaga *et al.*, 1999; Naito *et al.*, 1999; Gohda *et al.*, 2005). In contrast to the essential role of TRAF6 in osteoclastogenesis, the contributions of other TRAF members seem to be relatively limited.

The binding of TRAF6 to RANK induces the trimerization of TRAF6, leading to the activation of NF-κB and mitogen-activated kinases (MAPKs), including Jun N-terminal kinase (JNK) and p38 (Wong *et al.*, 1998; Kobayashi *et al.*, 2001). TRAF6 contains an N-terminal RING finger domain and a stretch of predicted zinc finger domains (Kobayashi *et al.*, 2001). The ubiquitin ligase activity mediated by the RING finger motif of TRAF6 has been shown to be important for NF-κB activation in immune cells (Deng *et al.*, 2000) as well as osteoclasts (Lamothe *et al.*, 2007). However, deletion analysis indicated that the RING finger domain of TRAF6 is dispensable for the formation of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (Kobayashi *et al.*, 2001). Further studies are needed to resolve this discrepancy. TRAF6 forms a signaling complex containing RANK and TAK-1-binding protein (TAB) 2, which results in TGFβ-activated kinase (TAK)1 activation (Mizukami *et al.*, 2002). Dominant negative forms of TAK1 and TAB2 inhibit the NF-κB activation induced by RANKL, and endogenous TAK1 has been shown to be activated in response to RANKL stimulation in RAW 264.7 cells (Mizukami *et al.*, 2002). Thus, *in vitro* experiments suggest that TAK1 is involved in the TRAF6-mediated activation of NF-κB and MAPKs during osteoclastogenesis.

TRAF6 was originally identified as an adaptor molecule that binds CD40, but CD40 has only one TRAF6-binding site. However, the osteoclastogenic activity of RANK, which contains three binding sites is 100 times higher than CD40 (Gohda *et al.*, 2005). Therefore, there appear to exist qualitative differences between RANK and other receptor signaling events. It is still not clear the mechanisms by which RANK alone among the TRAF6-binding receptors is able to stimulate osteoclastogenesis so powerfully. One possible explanation is that RANK has specific adaptor protein(s) not associated with other TRAF6-binding receptors. For example, Gab2 has been shown to be associated with RANK and to play an important role in its signal transduction (Wada *et al.*, 2005). Atypical PKC (aPKC)-interacting protein p62 is one of the candidate molecules putatively involved in the regulation of TRAF6 (Duran *et al.*, 2004).

NF-κB

NF-κB activation is among the very early molecular events induced by RANK (Anderson *et al.*, 1997) and is mediated by TRAF6. NF-κB is a family of dimeric transcription factors that include Rel (cRel), RelA (p65), RelB, NF-κB 1 (p50), and NF-κB 2 (p52, which is processed from its precursor, p100). Whereas the Rel proteins contain transcriptional activation domains, such domains are absent in p50 or p52, the activation function of which depends on heterodimerization with any of the three Rel proteins (Ghosh and Karin, 2002; Hayden and Ghosh, 2004). Mice doubly deficient in *p50* and *p52* develop osteopetrosis because of a defect in osteoclast differentiation, indicating that NF-κB (p50 and p52) plays an indispensable role in osteoclastogenesis (Franzoso *et al.*, 1997; Iotsova *et al.*, 1997).

NF-κB activation depends on classical and noncanonical pathways. The classical NF-κB signaling pathway involves activation of the inhibitor of the κB (IκB) kinase (IKK) complex that phosphorylates and degrades the IκB, which retains most of the NF-κB dimer, including p50:RelA, in the cytoplasm (Ghosh and Karin, 2002; Hayden and Ghosh, 2004). The alternative pathway induces the p52:RelB dimer, which are generated through the processing of the p100:RelB complex by NF κB-inducing kinase (NIK) (Ruocco *et al.*, 2005; Novack *et al.*, 2003; Ghosh and Karin, 2002; Hayden and Ghosh, 2004). If p50 and p52 are exclusively involved in the classical and alternative pathways, respectively, the phenotype of *p50/p52*-deficient mice suggests that both pathways are important for osteoclastogenesis. However, osteoclast formation is not completely abrogated in mice lacking NIK (Novack *et al.*, 2003). Targeted disruption of *IKK*α, which is important in the alternative pathway, results in an impairment of osteoclastogenesis only *in vitro*, not *in vivo*, while targeted disruption of *IKK*β, which is an essential catalytic IKK in the classical pathway, results in the impairment of osteoclastogenesis both *in vitro* and *in vivo* (Ruocco *et al.*, 2005). These results suggest that the classical, not the alternative pathway, is crucial for osteoclastogenesis; however, this is not completely consistent with the finding that *p50*-deficient mice exhibit no bone abnormalities. Thus, the precise roles of the two NF-κB activation pathways and the function of each NF-κB component in osteoclastogenesis are at present not entirely clear.

NFATc1

The NFAT transcription factor family was originally identified in T cells, and is comprised of five members. Among all the known transcription factors, the mRNA of NFATc1 (NFAT2) is the most strongly induced by RANKL (Takayanagi *et al.*, 2002). The necessary and sufficient role of *NFATc1* in osteoclastogenesis was suggested by the *in vitro* observation that *NFATc1*$^{-/-}$ embryonic stem cells do not differentiate into osteoclasts, and that ectopic expression of NFATc1 causes bone marrow-derived precursor cells to undergo osteoclast differentiation in the absence of RANKL (Takayanagi *et al.*, 2002). The activation of NFATc1 is mediated by a specific phosphatase, calcineurin, which is activated by calcium/calmodulin signaling. Consistent with this, calcineurin inhibitors such as FK506 and cyclosporin A potently inhibit osteoclastogenesis (Takayanagi *et al.*, 2002; Ishida *et al.*, 2002). Although *in vivo* analysis of *NFATc1*-deficient mice has been hampered by embryonic lethality (de la Pompa *et al.*, 1998; Ranger *et al.*, 1998), the adoptive transfer of hematopoietic stem cells to osteoclast-deficient mice and blastocyst complementation experiments have made it possible to fully demonstrate the critical function of NFATc1 in osteoclastogenesis *in vivo* (Asagiri *et al.*, 2005).

c-Fos and AP-1

The essential role of c-Fos in osteoclastogenesis has long been established (Grigoriadis *et al.*, 1994). The AP-1 transcription factor is a dimeric complex composed of the Fos (c-Fos, FosB, Fra-1, Fra-2), Jun (c-Jun, JunB, JunD), and ATF (ATFa, ATF2, ATF3, ATF4, B-ATF) proteins (Eferl and Wagner, 2003; Wagner and Eferl, 2005). RANK activates the transcription factor complex AP-1 partly through an induction of its critical component, c-Fos (Takayanagi *et al.*, 2002; Wagner and Eferl, 2005), but it is unclear whether RANK signaling is involved in the functional modification of c-Fos. The expression of c-Fos is actually increased after RANKL stimulation, but the molecular mechanism underlying its induction has yet to be fully understood.

NFATc1 induction by RANKL is completely abrogated in *c-Fos*-deficient cells (Takayanagi *et al.*, 2002; Matsuo *et al.*, 2004) and overexpression of NFATc1 rescues osteoclastogenesis in *c-Fos*-deficient cells (Matsuo *et al.*, 2004). ChIP experiments have shown c-Fos is recruited to the *NFATc1* promoter after RANKL stimulation (Asagiri *et al.*, 2005). Thus, c-Fos plays an essential role in osteoclastogenesis by functioning as a direct transcriptional regulator of NFATc1. Another member of the Fos family, Fra-1, which is a transcriptional target of c-Fos during osteoclast differentiation, compensates for the loss of *c-Fos* both *in vivo* and *in vitro*, but *Fra-1*-deficient mice do not exhibit osteopetrosis (Eferl *et al.*, 2004). This suggests that Fra-1 has an ability to compensate for the loss of *c-Fos* but is not the exclusive downstream molecule. It is reported that transgenic mice expressing dominant negative c-Jun (which inhibits all AP-1 dimers) under the control of the

TRAP promoter exhibit osteopetrosis, clearly indicating that AP-1 activity is critical for osteoclastogenesis (Ikeda *et al.*, 2004).

In contrast to the critical role of c-Fos, the Jun family of proteins, which are partners of the Fos family of proteins in the context of AP-1, play a redundant role. Mice lacking Jun family proteins such as c-Jun and JunB are embryonically lethal, but conditional knockout mice revealed that a deficiency in JunB or c-Jun leads to a considerable decrease in osteoclast formation, but not to the complete blockade of this process, suggesting that Jun members can substitute for each other during osteoclastogenesis (Wagner and Eferl, 2005; David *et al.*, 2002; Kenner *et al.*, 2004).

Role of NF-κB in the NFATc1 induction

As mentioned earlier, NFATc1 is the most important transcriptional target of c-Fos. What is the role of NF-κB in the regulation of c-Fos and NFATc1? Since NFATc1 induction was shown to be impaired in *TRAF6*$^{-/-}$ cells (Takayanagi *et al.*, 2002), it has been suggested that the induction of NFATc1 by RANKL requires NFκB activation. This idea was also supported by the observation that an NF-κB inhibitor was shown to suppress RANKL-stimulated induction of NFATc1 (Takatsuna *et al.*, 2005) and that NFATc1 induction is also impaired in *p50/p52*-deficient cells (Yamashita *et al.*, 2007). These results collectively indicate that the RANKL-mediated induction of NFATc1 is dependent on NF-κB.

Is NFATc1 the direct transcriptional target of NF-κB? Chromatin immunoprecipitation (ChIP) experiments revealed that NF-κB is recruited to the *NFATc1* promoter immediately after RANKL stimulation (Asagiri *et al.*, 2005). In fact, the *NFATc1* promoter contains κB sites and NF-κB overexpression activated the *NFATc1* promoter in a luciferase reporter gene assay (Asagiri *et al.*, 2005). However, osteoclastogenesis in *p50/p52*-deficient cells was shown to be rescued by the overexpression of c-Fos as well as NFATc1 (Yamashita *et al.*, 2007). In addition, the induction of c-Fos was impaired in *p50/p52*-deficient cells (Yamashita *et al.*, 2007). Although it is not clear whether NF-κB is directly recruited to the *c-fos* promoter, these results do suggest that NF-κB contributes to the induction of NFATc1 through the induction of c-Fos, at least in part. It is likely that NF-κB contributes to the induction of NFATc1 by both directly acting on the *NFATc1* promoter, and indirectly, through c-Fos induction.

Another molecule that is recruited to the *NFATc1* promoter is NFATc2, a member of the NFAT family of transcription factors. NFATc2 is present prior to RANKL stimulation and is recruited to the *NFATc1* promoter at the same time as NF-κB. NFATc2 and NF-κB cooperatively activate the *NFATc1* promoter within minutes of RANKL stimulation (Asagiri *et al.*, 2005). Physiologically, this (initial induction of NFATc1) is an important step toward the robust induction of NFATc1 at the next stage of differentiation. However, because NFATc2 is dispensable for osteoclastogenesis *in vivo* (Asagiri *et al.*, 2005), the initial induction of NFATc1, which is dependent on NF-κB and NFATc2, is not absolutely essential for osteoclastogenesis.

Autoamplification of NFATc1 and Epigenetic Regulation

Because NFATc1 and NFATc2 play a redundant role in the immune system (Peng *et al.*, 2001), the question arises as to how NFATc1 plays such an exclusive function in osteoclastogenesis. An interesting observation was obtained from rescue experiments using *NFATc1*$^{-/-}$ osteoclast precursor cells: osteoclast formation in *NFATc1*$^{-/-}$ cells was recovered not only by forced expression of NFATc1 but also by that of NFATc2 (Asagiri *et al.*, 2005). How are we to reconcile the indispensable *in vivo* role of NFATc1 in osteoclastogenesis with the observation that *NFATc1* deficiency is compensated for by forced expression of NFATc2? We considered the following explanation: the NFATc1 and NFATc2 proteins have a similar function of inducing osteoclastogenesis if and when they are ectopically expressed at a similarly high level. Therefore, the essential role of NFATc1 is not achieved by the unique function of the protein, but by an NFATc1-specific gene regulatory mechanism.

Accordingly, we analyzed the mRNA expression of *NFATc1* and *NFATc2* genes during osteoclastogenesis. The mRNA of *NFATc1* is induced selectively and potently by RANKL, while *NFATc2* mRNA is expressed constitutively in precursor cells at a low level (Asagiri *et al.*, 2005; Takayanagi *et al.*, 2002). Importantly, FK506, which suppresses the activity of NFAT through an inactivation of calcineurin, downregulates the induction of *NFATc1,* but not *NFATc2*. This suggests that *NFATc1* is selectively autoregulated by NFAT during osteoclastogenesis. As expected, ChIP experiments revealed that NFATc1 is recruited to the *NFATc1* but not the *NFATc2* promoter 24 h after RANKL stimulation, and the occupancy persists during the terminal differentiation of osteoclasts, indicating the autoamplification mechanism by NFATc1 is specifically operative in the *NFATc1* promoter (Asagiri *et al.*, 2005).

Why does this autoamplification occur only in *NFATc1* gene regulation? If NFAT-binding sites are only found in the *NFATc1* promoter, the selective recruitment would be easily explained. However, NFAT-binding sites are in fact found in both the *NFATc1* and the *NFATc2* promoters; thus, the promoter sequence cannot be the answer. Histone acetylation is a marker of the transcriptionally active chromatin structure, and transcriptional coactivators such as CBP and PCAF have histone acetylase activity (de la Serna *et al.*, 2006; Ansel *et al.*, 2006). Investigation of the recruitment of CBP and PCAF to the *NFATc1* promoter yielded positive results. The rate of histone acetylation in the *NFATc1* promoter increased gradually after RANKL stimulation, and methylation of histone H3 lysine 4, which is characteristic of a transcriptionally active locus, is also upregulated exclusively in the *NFATc1* promoter; however, this was not observed in the *NFATc2* promoter (Asagiri *et al.*, 2005). Conversely, the *NFATc2* promoter is constantly associated with methylated DNA-binding proteins, such as methyl-CpG-binding protein 2 (MeCP2), suggesting epigenetic modification of the *NFATc2* promoter is responsible for the muted pattern of gene expression (Asagiri *et al.*, 2005; Robertson, 2002). Thus, contrasting epigenetic modification of the *NFATc1* and the *NFATc2* promoters might account for their unique spatiotemporal induction pattern during osteoclastogenesis. In conclusion, the essential role of the *NFATc1* gene is determined not only by the function of the encoded protein, but also by an NFATc1-specific gene regulatory mechanism. It remains an issue to be pursued in future work to determine the reason such a specific epigenetic regulation is evidently at work only in osteoclasts.

Transcriptional Targets and Partners of NFATc1

Accumulating evidence indicates that a number of osteoclast-specific genes are directly regulated by NFATc1. Based on promoter and/or ChIP analyses, the *TRAP* (Takayanagi *et al.*, 2002; Matsuo *et al.*, 2004; Kim *et al.*, 2005), *calcitonin receptor* (Takayanagi *et al.*, 2002; Matsuo *et al.*, 2004; Kim *et al.*, 2005; Anusaksathien *et al.*, 2001), *cathepsin K* (Kim *et al.*, 2005; Matsumoto *et al.*, 2004), *β3 integrin* (Crotti *et al.*, 2006), and osteoclast-associated receptor (OSCAR) have all been shown to be regulated by NFATc1 (Kim *et al.*, 2005; Kim *et al.*, 2005). It is not understood how the target genes of NFATc1 promote the differentiation process, but it is notable that the genes involved in osteoclast fusion are also regulated by NFATc1. DC-STAMP, a putative seven-transmembrane spanning protein, is essential for the cell–cell fusion of osteoclasts (Yagi *et al.*, 2005; Kukita *et al.*, 2004). Its expression is rapidly induced in osteoclast precursor cells by RANKL and forced expression of DC-STAMP induces the formation of TRAP-positive multinucleated cells. Because the RANKL-mediated induction of *NFATc1* in *DC-STAMP*$^{-/-}$ cells is normal (Yagi *et al.*, 2005), DC-STAMP may function downstream of NFATc1 to promote multinucleation. In fact, a recent report showed that DC-STAMP is regulated by NFATc1(Yagi *et al.*, 2007). The D2 isoform of the vacuolar (H(+)) ATPase (v-ATPase) V(0) domain (Atp6v0d2), which was also found to be crucial for osteoclast fusion, was revealed to be a transcriptional target of NFATc1(Kim *et al.*, 2007).

The AP-1 complex is known to be a transcriptional partner of NFAT in lymphocytes, and crystal structure analysis revealed the formation of the NFAT:AP-1 complex to be crucial for DNA binding (Chen *et al.*, 1998). Likewise, an NFAT:AP-1 complex is important for the induction of the *TRAP* and *calcitonin receptor* genes as well as the robust autoamplification of NFATc1 (Takayanagi *et al.*, 2002). It has also been shown that NFATc1 cooperates with PU.1 and MITF on the *cathepsin K* and the *OSCAR* promoters (Kim *et al.*, 2005; Matsumoto *et al.*, 2004). It is noteworthy that both PU.1 and MITF, which are thought to be important for the survival of osteoclast precursor cells, also participate in osteoclast-specific gene induction at the terminal stage of differentiation. Thus, NFATc1 forms an osteoclast-specific transcriptional complex containing AP-1 (Fos/Jun), PU.1, and MITF for the efficient induction of osteoclast-specific genes (Asagiri *et al.*, 2005). It should also be noted that the components of the NFATc1 complex are not always the same: the cooperation between NFATc1 and PU.1/MITF was not observed on the *calcitonin receptor* promoter, suggesting that the differential composition of the transcriptional complex contributes to the spatiotemporal expression of each gene during osteoclastogenesis (Kim *et al.*, 2005).

Sequential Molecular Events During Osteoclastogenesis

The process of osteoclast differentiation can be effectively divided into three stages in the context of transcriptional control by NFATc1, as shown in Fig. 2: (i) The binding of RANKL to RANK results in the recruitment of TRAF6, leading to the activation of downstream molecules such as NF-κB. NFATc2 is recruited to the *NFATc1* promoter at a very early phase. Cooperation of NFATc2 and NF-κB initiates the induction of *NFATc1*. (ii) Stimulated by calcium signaling, NFATc1 is activated and binds its own promoter. This leads to the robust induction of *NFATc1* (i.e., autoamplification). AP-1 (containing c-Fos) is critical for this autoamplification. RANKL induces selective recruitment of NFATc1 to the promoter of *NFATc1*, but not to that of *NFATc2*, which is explained by epigenetic regulation. (iii) A number of osteoclast-specific genes such as *cathepsin K*, *TRAP*, *calcitonin receptor,* and *OSCAR* are activated by a transcriptional complex containing NFATc1 and cooperators such as AP-1, PU.1, and MITF.

MAPKs

In vitro experiments have suggested that MAPKs play an important role in osteoclastogenesis, but corroborating *in vivo* evidence has yet to be obtained. MAPKs are involved in the activation of AP-1 components (Chang and Karin, 2001) and therefore may have a role in osteoclastogenesis by modulating AP-1 activity, but the detailed molecular

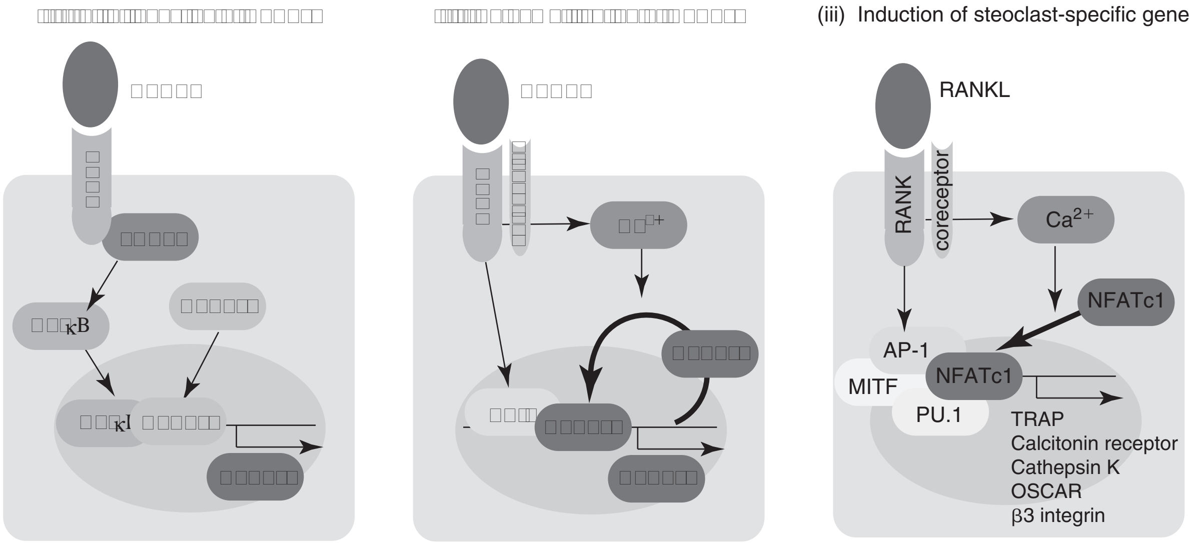

FIGURE 2 RANKL-mediated osteoclast differentiation and the induction of NFATc1. (i) The binding of RANKL to its receptor RANK results in the recruitment of TRAF6, which activates NF-κB. NF-κB and NFATc2 are involved in the initial induction of NFATc1. (ii) NFATc1 expression is autoamplified by its binding to the own promoter. The AP-1 component c-Fos is essential for the robust induction of NFATc1. Costimulatory receptors for RANK mediate the activation of the calcium signaling required for the nuclear localization and activation of NFATc1. (iii) NFATc1 in cooperation with partner transcription factors activates the induction of osteoclast-specific genes.

mechanisms of this action are not well elucidated. Mammals express at least four distinctly regulated groups of MAPKs, p38-MAPKs (p38α/β/γ/δ), JNK1/2/3, ERK1/2, and ERK5 (Chang and Karin, 2001). Many of the MAPKs have been shown to be activated downstream of RANK. Based on the effect of a specific inhibitor of p38α and β (SB203580) in RAW 264.7 cells, it was suggested that p38α and/or β are involved in osteoclast formation (Matsumoto *et al.*, 2000). It has also been shown that p38 is important for the induction of the *cathepsin K* gene (Matsumoto *et al.*, 2004). The functions of the other p38-MAPK isoforms, p38γ and δ, remain to be determined. MEKs (ERK1/2 kinases) are also activated by RANKL; however, inhibition of ERK activity by a MEK inhibitor did not suppress osteoclastogenesis (Matsumoto *et al.*, 2000) but rather potentiated it (Hotokezaka *et al.*, 2002), suggesting that the ERK pathway negatively regulates osteoclastogenesis. Although mice with *JNK1/2/3* genes individually inactivated are viable and do not display obvious bone abnormalities, an *in vitro* study has indicated that at least JNK1 is involved, albeit partially (David *et al.*, 2002). More detailed *in vivo* analyses are needed to obtain conclusive evidence on the role of the MAPKs in osteoclastogenesis.

Costimulatory Signals for RANK

It has been suggested that the RANK and M-CSF receptor together transmit signals that are sufficient for osteoclastogenesis (Boyle *et al.*, 2003). In addition, a novel type of receptor was identified in osteoclasts. OSCAR is an immunoglobulin-like receptor involved in the cell–cell interaction between osteoblasts and osteoclasts (Kim *et al.*, 2002). OSCAR associates with an adaptor molecule, FcRγ (Koga *et al.*, 2004; Merck *et al.*, 2004), which harbors an immunoreceptor tyrosine-based activation motif (ITAM) critical for the activation of calcium signaling in immune cells (Reth, 1989). Another ITAM-harboring adaptor, DAP12, has been reported to be involved in the formation and function of osteoclasts (Kaifu *et al.*, 2003). Notably, mice doubly deficient in FcRγ and DAP12 exhibit severe osteopetrosis owing to the differentiation blockade of osteoclasts, demonstrating that the immunoglobulin-like receptors associated with FcRγ and DAP12 are essential for osteoclastogenesis (Koga *et al.*, 2004; Mocsai *et al.*, 2004). These receptors include OSCAR, triggering receptor expressed in myeloid cells (TREM)-2, signal-regulatory protein (SIRP) β1, and paired immunoglobulin-like receptor (PIR)-A, although the ligands and exact function of each of these receptors remain to be determined. The importance of the ITAM-harboring adaptors and the receptors associated with them in bone metabolism is also underscored by reports that mutations in the *DAP12* and *TREM-2* genes cause severe skeletal and behavior abnormalities known as Nasu–Hakola disease (Paloneva *et al.*, 2000; Paloneva *et al.*, 2002).

ITAM-mediated signals cooperate with RANK to stimulate calcium signaling through ITAM phosphorylation and the resulting activation of Syk and PLCγ. Therefore, these signals should properly be called costimulatory signals for RANK (Fig. 3). Initially characterized in natural killer and myeloid cells, the immunoglobulin-like receptors

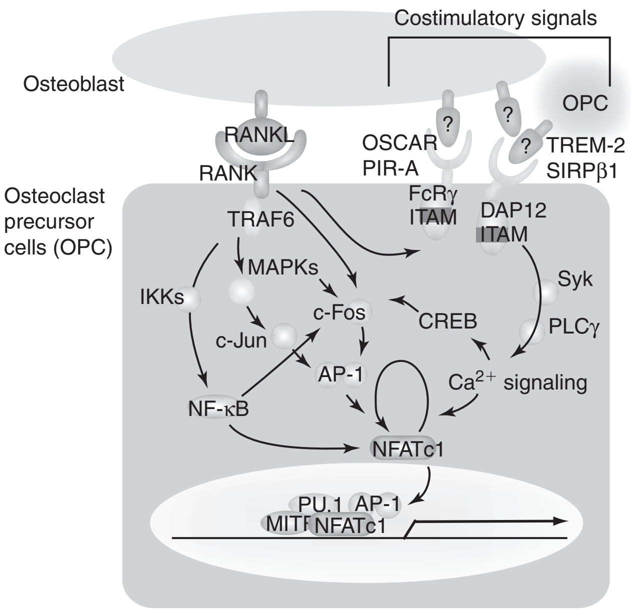

FIGURE 3 Cooperation of RANK and immunoreceptor tyrosine-based activation motif (ITAM) signals in osteoclastogenesis. RANK activates the NF-κB and c-Fos pathways that stimulate the induction of NFATc1. Immunoglobulin-like receptors associate with Fc receptor common γ subunit (FcRγ) and DNAX-activating protein 12 (DAP12), both of which contain the ITAM motif. RANK and ITAM signaling cooperate to phosphorylate phospholipase Cγ(PLCγ) and activate calcium signaling, which is critical for the autoamplification of NFATc1.

associated with FcRγ or DAP12 are thus identified as unexpected but nevertheless essential partners of RANK during osteoclastogenesis (Koga *et al.*, 2004; Baron, 2004; Takayanagi, 2005). It is not fully understood how RANK can specifically induce osteoclastogenesis in cooperation with ITAM signaling, but it is partly explained by the observation that phosphorylation of ITAM is upregulated by RANKL (Koga *et al.*, 2004). In addition, RANKL stimulation results in an increased expression of immunoreceptors such as OSCAR, thereby augmenting the ITAM signal (Kim *et al.*, 2005; Kim *et al.*, 2005). It is also conceivable that RANK activates an as yet unknown pathway that specifically synergizes with or upregulates ITAM signaling. Recent studies showed that RGS10 is activated by RANK and mediates calcium oscillation. We have recently unpublished results suggesting that Tec family tyrosine kinases such as Btk and Tec are activated by RANK and are involved in the phosphorylation of PLCγ (Shinohara *et al.*, 2008).

The role of inhibitory type receptors containing immunoreceptor tyrosine-based inhibitory motif (ITIM) in osteoclast differentiation remains elusive, but osteoclastogenesis is enhanced in mice lacking phosphatases such as Src homology (SH)-2-containing protein tyrosine phosphatase (SHP)-1 (Aoki *et al.*, 1999; Umeda *et al.*, 1999) or SH-2-containing inositol 5′,-phosphatase (SHIP)-1 (Takeshita *et al.*, 2002), which counterbalance the ITAM signal in the immune system.

Calcium/calmodulin signaling is related not only to phosphatases such as calcineurin, but also to kinases, including calcium/calmodulin-dependent kinases. These kinases are thus also activated during RANKL-induced osteoclastogenesis and participate in the activation of RANK signaling mainly by upregulating c-Fos expression through CREB (Sato *et al.*, 2006). The regulation of c-Fos has been enigmatic, but recent studies suggest that RANK induction of c-Fos is under the control of multiple transcriptional regulators including NF-κB (Yamashita *et al.*, 2007), CREB (Sato *et al.*, 2006), and PPARγ (Wan *et al.*, 2007) as well as MAPKs.

CONCLUSION

The discovery of RANKL and RANK has greatly enriched our understanding of the mechanisms of osteoclast differentiation, activation, and survival. Much attention and effort have been paid to RANK signaling from both the biological and therapeutic points of view. Despite this progress, it is still not clear why only RANK induces osteoclastogenesis. The signaling events activated by RANK are integrated by NFATc1, but nothing is known about the downstream molecules that orchestrate the late phase of the differentiation process. Therefore, much work remains to be done in this field, but the tremendous effort entailed will surely be rewarded because the rationality of targeting RANK signaling in therapy has already been demonstrated by the efficacy of anti-RANKL antibody in several human trials against bone diseases (McClung *et al.*, 2006).

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Chapter 12

Regulating Bone Resorption: Targeting Integrins, Calcitonin Receptor, and Cathepsin K

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INTRODUCTION

Skeletal growth and repair during adult life occurs via bone remodeling, a coordinated and tightly regulated process. The maintenance of normal bone mass depends on maintaining the appropriate balance between bone formation and bone resorption. The current view on normal bone remodeling is that the processes of bone resorption and formation are tightly coupled, so that a change in either usually leads to a subsequent compensating change in the other (Martin and Sims, 2005). However, normal age-related physiological changes and certain pathological conditions result in excessive bone resorption, leading to osteoporosis.

Bone resorption is carried out by osteoclasts, which are multinucleated, terminally differentiated cells derived from the monocyte/macrophage lineage (Roodman, 1999; Suda *et al.*, 1999; Teitelbaum, 2000). Each bone remodeling cycle begins with a resorption phase, in which osteoclasts are recruited to a remodeling site on the bone surface, where the cells excavate bone surface over a period of 2 to 4 weeks. This is followed by a reversal phase, when the osteoclasts move away from the resorption site and mononuclear precursors are recruited and differentiated into osteoblasts. During the subsequent 2- to 4-month-long formation phase, mature osteoblasts deposit an organic matrix that then becomes mineralized (Grey and Reid, 2005; Rodan, 1998).

The rate of bone resorption varies as a function of changes in both the number and activity of osteoclasts, the only cells capable of resorbing bone (Baron, 1989). Osteoclastogenesis is controlled by the proliferation and homing of the progenitors to bone and their differentiation and fusion to form multinucleated cells. A comprehensive review of the regulation of osteoclast generation has been presented in a previous chapter.

Osteoclast function starts with adhesion to the bone matrix, which initiates a polarization of the cell that involves cytoskeletal reorganization and the formation of a tight sealing zone that is enriched in filamentous actin and encloses the resorption lacuna (Duong *et al.*, 2000). The cytoskeletal organization associated with osteoclastic bone resorption is also discussed in another chapter. Within the area surrounded by the sealing zone, targeted insertion of intracellular transport vesicles into the plasma membrane forms a deeply folded membrane, the ruffled border, which secretes protons and hydrolytic enzymes into the resorption lacuna. The acidification of the lacuna by the secreted protons demineralizes the bone matrix, exposing the organic components, primarily collagen type I, which are subsequently degraded by the secreted lysosomal proteolytic enzymes (Baron *et al.*, 1988, 1985; Väänänen *et al.*, 2000; Fig. 1).

Current antiresorptive therapies, which predominantly target osteoclast development and survival, include bisphosphonates, selective estrogen receptor modulators (SERMs),

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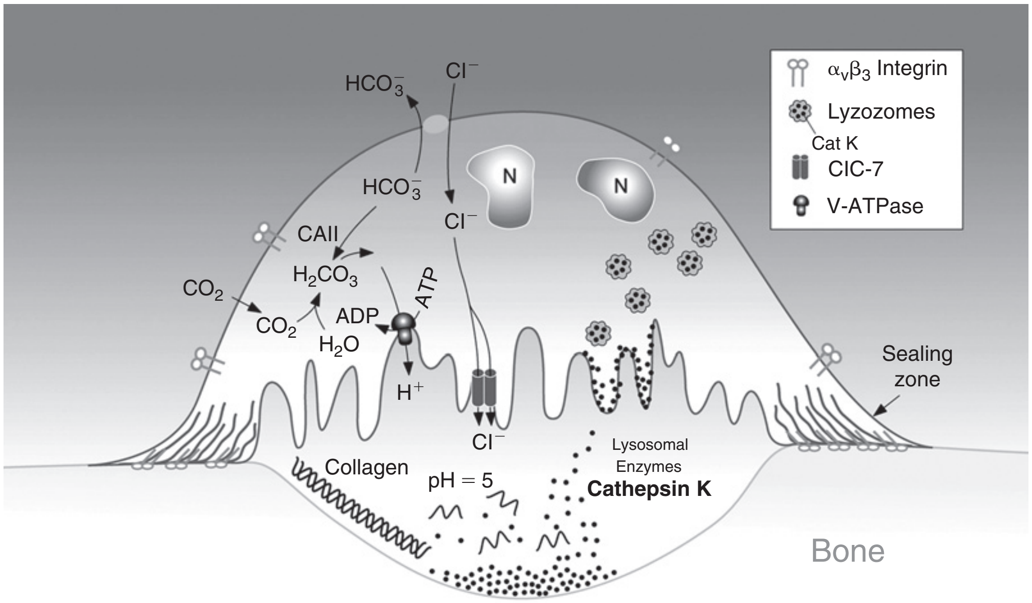

FIGURE 1 Schematic illustration of a bone-resorbing osteoclast. Several targets are genetically and pharmacologically validated as rate-limiting in regulating osteoclast bone resorption activity including the adhesion receptor $\alpha_v\beta_3$ integrin for osteoclast polarization and movement; carbonic anhydrase II (CAII), V-ATPase, and chloride channel ClC-7 for acidification; and cathepsin K for the removal of organic bone matrix in the resorption lacunae.

and anti–receptor activator of NFκB (RANK) ligand (Boyce *et al.*, 2006; Grey and Reid, 2005; Rodan and Martin, 2000). However, pharmacological or genetic methods used to disrupt osteoclast function (Rodan and Martin, 2000; Teitelbaum, 2000) identified many molecules that are involved either in the degradation of bone matrix proteins such as cathepsin K or metalloproteinases (MMPs), or in the regulation of acidification, including carbonic anhydrase type II, tartrate-resistant acid phosphatase (TRAP), the a3 subunit of the vacuolar H^+-ATPase, and the ClC-7 chloride channel, which can be targeted for therapeutic regulation of osteoclast activity (Frattini *et al.*, 2000; Kornak *et al.*, 2001; Li *et al.*, 1999; Scimeca *et al.*, 2000). A new generation of antiresorptive agents has been developed to specifically interfere with osteoclast bone-resorbing activity (Boyce *et al.*, 2006; Rodan and Martin, 2000; Vasiljeva *et al.*, 2007). These antiresorptives include agents that directly compromise osteoclast activation by modulating cellular polarization and secretion, including calcitonin, $\alpha_v\beta_3$ integrin antagonists, and Src kinase inhibitors, as well as agents that either directly inhibit acidification of the resorption lacunae by blocking the osteoclast-specific subtype of carbonic anhydrase type II or the V-ATPase or the ClC-7 chloride channel, or block degradation of bone matrix proteins, such as inhibitors of cathepsin K. This chapter discusses the signal transduction pathways mediated by integrin and calcitonin receptor in the context of their regulation of cytoskeletal reorganization during osteoclastic bone resorption, as well as the role of cathepsin K in bone resorption.

ADHESION AND CYTOSKELETAL ORGANIZATION IN OSTEOCLASTS—$\alpha_V\beta_3$ INTEGRINS

Osteoclastic bone resorption is a process requiring physical intimacy between resorptive cell and bone matrix. Thus, cell matrix attachment molecules, particularly integrins, play a central role in the capacity of osteoclasts to degrade bone (Carron *et al.*, 2000; Feng *et al.*, 2001). Integrins, a superfamily of heterodimeric transmembrane receptors, mediate cell–matrix and cell–cell interaction. Integrin-mediated adhesion and signaling regulate a variety of cell processes (Clark and Brugge, 1995; Giancotti and Ruoslahti, 1999; Schlaepfer *et al.*, 1999). In addition to cell adhesion, integrin assembly also organizes extracellular matrices, modulates cell shape changes, and participates in cell spreading and motility (Wennerberg *et al.*, 1996; Wu *et al.*, 1996).

Integrin signaling is bidirectional. Extracellular matrix molecules interact with the integrin external domain and stimulate outside-in signaling that induces changes in cytoskeletal organization and intracellular signaling, whereas inside-out signaling induced by the interaction with cytoskeletal proteins or actions of intracellular signaling effectors prompt conformational changes in the integrin's ligand binding site (Takagi *et al.*, 2002) that alter its affinity, thereby modulating its binding capabilities and, ultimately intracellular events (Butler *et al.*, 2003; Geiger *et al.*, 2001; Pelletier *et al.*, 1995).

$\alpha_v\beta_3$-Mediated Osteoclast Function

Adhesion of osteoclasts to the bone surface involves the interaction of integrins with the extracellular matrix proteins within bone. Bone consists mainly of type I collagen (>90%) and of non-collagenous proteins interacting with a mineral phase of hydroxyapatite. Osteoclasts express very high levels of the vitronectin receptor, $\alpha_v\beta_3$ (Duong *et al.*, 2000; Horton, 1997; Rodan and Martin, 2000). Mammalian osteoclasts also express lower levels of collagen/laminin receptor $\alpha_2\beta_1$ and the vitronectin/fibronectin receptor $\alpha_v\beta_1$, whereas osteoclast precursors express $\alpha_2\beta_2$ and $\alpha_v\beta_5$ (Athanasou and Quinn, 1990; Lane *et al.*, 2005). $\alpha_v\beta_{3,}$ like all members of the α_v integrin family, recognizes the amino acid Arg-Gly-Asp (RGD), which is present in a variety of bone matrix proteins, including osteopontin and bone sialoprotein. Osteoclasts attach to and spread on these substrates in an RGD-dependent manner and, most important, competitive ligands arrest bone resorption *in vivo* (Engleman *et al.*, 1997; Murphy *et al.*, 2005; Yamamoto *et al.*, 1998). Additionally, osteoclastic bone resorption *in vitro* can be inhibited by RGD-containing peptides and disintegrins or by antibodies that block $\alpha_v\beta_3$ binding.

Whereas $\alpha_v\beta_3$ is abundantly expressed on mature osteoclasts and is a marker of their phenotype (McHugh *et al.*, 2000), it is absent in marrow macrophages, which express $\alpha_v\beta_5$. With exposure to receptor activator of NFκB ligand (RANKL), the β_5 subunit disappears and is replaced by β_3 (Ross and Teitelbaum, 2005). Ovariectomy of $\beta_5^{-/-}$ mice induces increased osteoclastogenesis and more bone loss than in their wild-type counterparts (Lane *et al.*, 2005). Thus, although $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are compositionally similar, they are differentially expressed in response to RANKL and exert different effects on osteoclast function.

Attachment of $\alpha_v\beta_3$ to the matrix induces signaling events that include changes in intracellular calcium, protein tyrosine phosphorylation, and cytoskeletal reorganization (Duong *et al.*, 1998; Duong and Rodan, 2000; Eliceiri *et al.*, 1998; Faccio *et al.*, 2003; Sanjay *et al.*, 2001b). The importance of $\alpha_v\beta_3$ in osteoclast function is underscored by the fact that the targeted disruption of the β_3 integrin subunit results in a progressive increase in the bone mass due to osteoclast dysfunction. Osteoclasts isolated from these $\beta_3^{-/-}$ mice fail to spread, do not form actin rings, and have abnormal ruffled membranes and exhibit reduced bone resorption activity *in vivo* (McHugh *et al.*, 2000). In echistatin-treated mice, where bone resorption was inhibited, the number of osteoclasts on the bone surface was unchanged rather than decreased, suggesting that osteoclast inhibition by $\alpha_v\beta_3$ integrin antagonists is not due to the detachment of osteoclasts from the bone surface (Masarachia *et al.*, 1998; Yamamoto *et al.*, 1998). Consistent with this observation, the number of osteoclasts in the bones of $\beta_3^{-/-}$ mice was normal, but the formation of the ruffled border was impaired (McHugh *et al.*, 2000). These data suggest, as indicated by the analysis of the integrin repertoire of osteoclasts, that in the absence of functional $\alpha_v\beta_3$ receptors, adhesion per se is not compromised and that other integrins, or different attachment proteins, participate in this process. However, some unique and not completely characterized signaling properties of $\alpha_v\beta_3$ may well be critical for cell motility and for the formation of the ruffled border (McHugh *et al.*, 2000; Nakamura *et al.*, 2001; Sanjay *et al.*, 2001b). Thus, $\alpha_v\beta_3$ plays a role in the adhesion and spreading of osteoclasts on bone and the subsequent resorptive process by transmitting bone matrix–derived signals, ultimately activating intracellular events that regulate cytoskeletal reorganization that are essential for osteoclast migration and polarization.

Research has focused on characterizing the $\alpha_v\beta_3$-regulated signaling cascade and identifying structural proteins required for this cytoskeletal reorganization. Studies using both genetic and biochemical approaches, such as immunolocalization and co-precipitation, have partially elucidated the $\alpha_v\beta_3$-induced signaling in osteoclasts, identifying complexes that includes Pyk2, Src, Cbl, dynamin, and Syk as potentially important components.

Pyk2 Couples $\alpha_v\beta_3$ Integrins to Src in Osteoclasts

The engagement of the matrix by either $\alpha_v\beta_3$ or $\alpha_v\beta_2$ initiates a signaling mechanism in osteoclasts and osteoclast precursors that activates the nonreceptor tyrosine kinase, Pyk2 (Duong *et al.*, 1998; Faccio *et al.*, 2003; Lakkakorpi *et al.*, 2003; Miyazaki *et al.*, 2004; Nakamura *et al.*, 1998; Sanjay *et al.*, 2001b), a member of the focal adhesion kinase (FAK) family, by a mechanism that involves an increase in cytosolic free Ca^{2+} and the binding of Pyk2 to the cytoplasmic domain of the β integrin subunit (Duong and Rodan, 2000; Faccio *et al.*, 2003; Pfaff and Jurdic, 2001; Sanjay *et al.*, 2001b). Pyk2 is highly expressed in osteoclasts (Duong *et al.*, 1998), where it colocalizes with the podosomes in osteoclasts plated on glass and in sealing zones of resorbing osteoclasts on bone (Duong *et al.*, 1998; Gil-Henn *et al.*, 2007; Sanjay *et al.*, 2001b; Zhang *et al.*, 2002).

Pyk2 is required for normal osteoclast function. Pyk2$^{-/-}$ mice are osteopetrotic, and Pyk2$^{-/-}$ osteoclasts and osteoclasts infected with Pyk2 antisense-expressing adenovirus resorb bone less efficiently and exhibit defects in cell adhesion, spreading, and sealing zone organization (Duong *et al.*, 2001; Gil-Henn *et al.*, 2007; Sims *et al.*, 1999). Following integrin engagement, integrin-induced phosphorylation of Pyk2 Tyr^{402} creates a binding site for Src, another nonreceptor tyrosine kinase, and the Src-associated adaptor/ubiquitin ligase Cbl (Duong *et al.*, 1998; Lakkakorpi *et al.*, 2003; Miyazaki *et al.*, 2004).

Pyk2's kinase activity, Tyr^{402}, and focal adhesion targeting (FAT) domain, which targets Pyk2 to adhesion complexes

(Schaller and Sasaki, 1997), are all required to one degree or another for normal Pyk2 function in osteoclasts. Mutating Tyr^{402} or disabling the catalytic site reduces but does not eliminate the ability of recombinant Pyk2 to restore the bone-resorbing activity of $Pyk2^{-/-}$ osteoclasts, while deleting the FAT domain essentially eliminates the normalization of bone resorbing activity (Gil-Henn *et al.*, 2007). Interestingly, $Pyk2^{Y402F}$ has a powerful dominant-negative effect when overexpressed in wild-type osteoclasts, while kinase-dead Pyk2 has little negative effect (Lakkakorpi *et al.*, 2003; Miyazaki *et al.*, 2004), notwithstanding the similar efficacies of these two mutants in restoring $Pyk2^{-/-}$ osteoclast bone resorbing activity (Gil-Henn *et al.*, 2007).

Deleting the β_3 integrin gene or disabling the Pyk2-binding site on β_3 integrin results in a dramatic reduction in the attachment-induced interaction of Src with Pyk2 and Src and Cbl phosphorylation (Faccio *et al.*, 2003), indicating that Pyk2 couples $\alpha_v\beta_3$ to the Src-Cbl signaling cascade (discussed later). Interestingly, attachment-induced phosphorylation of Pyk2 Tyr^{402} in osteoclasts generated *in vitro* is affected more by eliminating the β_2 integrin than by eliminating the β_3 integrin (Faccio *et al.*, 2003), suggesting that Pyk2 performs both Src/Cbl-dependent and Src/Cbl-independent attachment-induced functions in osteoclasts downstream of different integrins, and that a yet-to-be-identified integrin-specific factor regulates the binding of Src to Pyk2 phosphotyrosine 402.

The binding of Src by Pyk2 is also regulated by the large GTPase dynamin, which reduces the phosphorylation level of Pyk2 Tyr^{402} by an as yet incompletely characterized mechanism, thereby eliminating the Src binding site (Bruzzaniti *et al.*, 2005a). The process involves the binding and/or hydrolysis of GTP by dynamin and appears to be induced by Src-catalyzed phosphorylation of dynamin, suggesting that the process acts as a feedback loop by which Src limits the duration of the Pyk2-Src association.

Pyk2 also associates with phospholipase C-γ (Nakamura *et al.*, 2001), leupaxin (Gupta *et al.*, 2003), $p130^{Cas}$ (Lakkakorpi *et al.*, 1999), and gelsolin (Wang *et al.*, 2003) in osteoclasts and promotes the formation of the peripheral podosome belt by a microtubule-dependent mechanism (Gil-Henn *et al.*, 2007). Both $Pyk2^{Y402F}$ and kinase-dead Pyk2 normalize podosome belt formation in $Pyk2^{-/-}$ osteoclasts but not bone resorption, indicating that Src-Pyk2 association is required for bone resorption even when podosome organization is normal. Thus, Pyk2's ability to recruit Src and its associated proteins to sites of $\alpha_v\beta_3$ integrin activation appears to be a required function of Pyk2 in coupling downstream of activated $\alpha_v\beta_3$.

c-Src Regulates $\alpha_v\beta_3$-Mediated Cytoskeletal Organization and Cell Motility

Src is a key signaling effector that couples $\alpha_v\beta_3$-initiated signals to multiple signaling pathways and cellular responses (Faccio *et al.*, 2003; Miyazaki *et al.*, 2004; Sanjay *et al.*, 2001b; Zou *et al.*, 2007). Src has been reported to play multiple roles in integrin signaling, in the regulation of the cell cytoskeleton and in cell migration (Alper and Bowden, 2005; Frame, 2004; Playford and Schaller, 2004). $Src^{-/-}$ mice are osteopetrotic (Soriano *et al.*, 1991) to a greater extent than the $Pyk2^{-/-}$ mice, and mice that lack both Src and Pyk2 are more osteopetrotic than either the $Src^{-/-}$ or the $Pyk2^{-/-}$ mice (Sims *et al.*, 1999). Src appears to play a unique role in osteoclasts, because the absence of other Src family kinases that are normally expressed in osteoclasts (c-Fyn, c-Yes, Hck, and Fgr; Horne *et al.*, 1992; Lowell *et al.*, 1996) fails to produce osteopetrosis (Lowell *et al.*, 1996; Stein *et al.*, 1994), although $Src^{-/-}\cdot Hck^{-/-}$ double mutant mice are significantly more osteopetrotic than the $Src^{-/-}$ animals (Lowell *et al.*, 1996), suggesting that Hck may partially compensate for the absence of Src. Re-expressing Src in $Src^{-/-}$ osteoclasts *in vitro* restores bone-resorbing activity (Miyazaki *et al.*, 2004), and targeting re-expression of Src to osteoclasts in the $Src^{-/-}$ mouse reverses the osteopetrotic phenotype (Schwartzberg *et al.*, 1997).

$Src^{-/-}$ osteoclasts fail to spread normally on extracellular matrix, exhibit abnormal organization of F-actin-containing adhesion complexes, and are significantly less mobile than wild-type osteoclasts (Lakkakorpi *et al.*, 2001; Sanjay *et al.*, 2001b). The notable absence of a ruffled border in $Src^{-/-}$ osteoclasts (Boyce *et al.*, 1992) suggests that Src also contributes to the regulation of exocytic and/or endocytic vesicle trafficking in osteoclasts, as it appears to do in other types of cells (Ahn *et al.*, 1999; Foster-Barber and Bishop, 1998). The absence of the ruffled border could also be a consequence of the defects in attachment and motility, because attachment to the bone surface and establishment of the sealing zone must precede the formation of the ruffled border and secretion into the resorption compartment. The formation of the ruffled border is therefore likely to be induced by signals emanating from attachment complexes in the sealing zone (outside-in signaling), which may require Src.

Src activation is associated with the formation of the podosome adhesion structures (Tarone *et al.*, 1985) that characterize osteoclasts and other highly motile cells (Linder and Aepfelbacher, 2003; Marchisio *et al.*, 1988, 1984). Podosomes contain a dense central column of F-actin, the actin core, surrounded by a more loosely organized F-actin meshwork called the podosome cloud (Collin *et al.*, 2006; Destaing *et al.*, 2003). Numerous adaptor and regulatory proteins, including Pyk2, Src, Cbl, paxillin, vinculin, integrins, talin, dynamin2, the Arp2/3 complex, Wasp, and cortactin, colocalize with the podosome core and/or cloud (Bruzzaniti *et al.*, 2005b; Destaing *et al.*, 2003; Luxenburg *et al.*, 2006b; Ochoa *et al.*, 2000; Pfaff and Jurdic, 2001; Tehrani *et al.*, 2006). Actin polymerizes continuously into the podosome core and cloud throughout the brief (2- to 4-minute) life span of the podosome (Destaing *et al.*, 2003), revealing the dynamic nature of these specialized attachment structures.

Deletion or inhibition of Src leads to reduced osteoclast motility and alterations of the osteoclast cytoskeleton (Sanjay *et al.*, 2001b), including fewer podosomes, a reduced rate of actin polymerization within the podosome (measured as the recovery from photobleaching), and an increased podosome life span (Destaing *et al.*, 2003, 2008; Luxenburg *et al.*, 2006b). Src's tyrosine kinase activity and adaptor function are both required for normal podosome organization and dynamics, because neither kinase-dead Src nor a Src mutant with both the SH2 and SH3 binding domains disabled rescue podosome function (Destaing *et al.*, 2008; Luxenburg *et al.*, 2006b). Thus, Src is involved in regulating multiple aspects of podosome structure, organization, and dynamics. Pyk2 also modulates the podosome organization. The rate of actin polymerization within podosomes of Pyk2$^{-/-}$ osteoclasts is reduced, with the time of recovery from photobleaching increasing by a factor of 2, although podosome life span is largely unaffected (Gil-Henn *et al.*, 2007), suggesting that Src may regulate actin polymerization in cooperation with Pyk2 but regulate podosome life span by a Pyk2-independent mechanism.

The key Src substrates in the regulation of podosome assembly and function remain to be identified. Src phosphorylates several actin regulatory proteins that contribute to podosome formation and functions, including N-Wasp (Park *et al.*, 2005; Torres and Rosen, 2003), dynamin2 (Ochoa *et al.*, 2000; Werbonat *et al.*, 2000), and cortactin (Lua and Low, 2005; Martinez-Quiles *et al.*, 2004). Silencing cortactin expression in osteoclasts inhibits podosome formation (Tehrani *et al.*, 2006), and mutating cortactin's Src-phosphorylated tyrosine residues causes defective podosome formation (Tehrani *et al.*, 2006) and affects podosome life span (Luxenburg *et al.*, 2006a). Src could therefore regulate actin polymerization and life span of podosomes at least in part by regulating cortactin activity. Src could also modulate podosome activity by promoting the dynamin-induced dephosphorylation of Pyk2 (discussed earlier) or by regulating the interaction of the PI(4,5)P2 modifying enzyme PIPKI-γ90 with talin (Lee *et al.*, 2005).

The Role(s) of Cbl Downstream of the Integrin-Pyk2-Src Complex

As noted previously, the Pyk2-Src signaling complex that forms in response to the engagement of $\alpha_v\beta_3$ to the matrix includes the adaptor protein Cbl, which is phosphorylated by Src (Sanjay *et al.*, 2001b; Tanaka *et al.*, 1996). Cbl and the highly homologous Cbl-b also function as ubiquitin ligases within multiprotein signaling complexes, targeting receptor tyrosine kinases and other signaling proteins for ubiquitylation (Sanjay *et al.*, 2001a; Swaminathan and Tsygankov, 2006; Thien and Langdon, 2001). The single deletions of either the Cbl or the Cbl-b gene cause relatively mild but distinctly different bone phenotypes (Chiusaroli *et al.*, 2002, 2003; Murphy *et al.*, 1998).

Deletion of Cbl induces a mild reduction in bone resorption that is transiently detected primarily during endochondral bone formation at the stage of formation of the marrow cavity and is thought to be the result of reduced osteoclast motility, observed both *in vitro* and *in vivo* (Chiusaroli *et al.*, 2003; Sanjay *et al.*, 2001b). The reduced osteoclast motility may be due in part to the loss of Cbl-mediated ubiquitylation, because expression on a Cbl$^{-/-}$ background of a Cbl with a disabling point mutation in the RING domain (c-Cbl$^{C379A/-}$; Thien *et al.*, 2005) results in osteoclasts with reduced motility, and the motility defect is rescued by re-expressing wild-type c-Cbl (Itzstein *et al.*, 2006). Thus, Cbl-dependent mechanisms promote the motility of osteoclasts *in vitro*, consistent with the role of Cbl in the regulation of cell adhesion, spreading, and migration via mechanisms that involve Src and phosphatidylinositol 3-kinase (PI3K; Feshchenko *et al.*, 1999; Meng and Lowell, 1998; Scaife and Langdon, 2000) and contribute to the osteoclast's ability to invade and resorb bone and mineralized cartilage *in vivo* (Chiusaroli *et al.*, 2003). The moderate and transient Cbl$^{-/-}$ bone phenotype suggests that Cbl-b might largely compensate for the absence of c-Cbl.

The bone phenotype of Cbl-b$^{-/-}$ mice is distinctly different from the Cbl$^{-/-}$ phenotype and more pronounced, with significantly reduced bone mass (Chiusaroli *et al.*, 2002). Bone formation is unaltered in these mice, but urine deoxypyridinoline crosslinks, a marker of bone resorption, are increased, as is *in vitro* bone resorbing activity (Sanjay *et al.*, 2004), suggesting that the osteopenia results from increased osteoclast activity. Expressing recombinant Cbl-b in the Cbl-b$^{-/-}$ osteoclasts restores *in vitro* resorbing activity to normal via a mechanism that requires both the phosphotyrosine-binding TKB domain and the ubiquitin conjugating enzyme-binding RING domain, whereas overexpressing c-Cbl has no effect (Nakajima *et al.*, 2006). These different phenotypes suggest that Cbl and Cbl-b each perform some unique functions in osteoclasts, presumably as a consequence of the differences in the specific regulatory proteins that bind to each Cbl protein.

In contrast to the distinct but relatively mild phenotypes of the single Cbl gene deletions, the simultaneous depletion of both Cbl and Cbl-b disrupts the podosome belt, reduces migration and bone resorption, and increases osteoclast apoptosis (Purev, unpublished observation), indicating that these homologous Cbl proteins also redundantly perform certain key functions in osteoclasts, as they do in other cells (Naramura *et al.*, 2002). These common functions of Cbl and Cbl-b may be required for the Src-dependent signaling mechanisms downstream of integrin activation that are responsible for the reduced bone resorbing activity of Src$^{-/-}$ osteoclasts. The fact that overexpressing Cbl proteins with reduced binding to Src or PI3K reduces the bone-resorbing activity of wild-type osteoclasts (Miyazaki *et al.*, 2004; Sanjay *et al.*, 2006), in contrast to the effects of deleting either Cbl (no change) or Cbl-b (increased resorption), supports this possibility.

$\alpha_v\beta_3$-Dependent Activation of Phosphatidylinositol 3-Kinase

Phosphatidylinositol 3-kinase (PI3K) is a key signaling effector that associates with activated $\alpha_v\beta_3$ and Src in osteoclasts (Hruska *et al.*, 1995). Activation of $\alpha_v\beta_3$ increases PI3K activity and its association with gelsolin-containing cytoskeleton complexes (presumably the podosomes; Chellaiah *et al.*, 1998; Hruska *et al.*, 1995). Inhibiting PI3K disrupts the actin ring and interferes with osteoclast attachment, spreading, and bone-resorbing activity (Lakkakorpi *et al.*, 1997; Nakamura *et al.*, 1995). In adhering and spreading osteoclasts and macrophages, PI3K is activated and translocates to the membrane and/or actin cytoskeleton (Lakkakorpi *et al.*, 1997; Meng and Lowell, 1998). Attachment of macrophages to the matrix induces Src family kinase-dependent phosphorylation of Cbl and Cbl's association with PI3K (Meng and Lowell, 1998). Src-catalyzed phosphorylation of Cbl Tyr^{731} creates a binding site for PI3K (Ueno *et al.*, 1998). Overexpressing Cbl^{Y731F}, which is unable to bind to PI3K, reduces *in vitro* bone resorbing activity of osteoclasts by 80% (Miyazaki *et al.*, 2004) and fails to restore either the basal or the M-CSF-induced motility of Cbl-deficient osteoclasts, in contrast to the complete rescue by wild-type Cbl (Sanjay, unpublished observation), suggesting that the binding of PI3K to Cbl is required for normal osteoclast migration and bone resorption.

$\alpha_v\beta_3$-Dependent Activation of Syk and Vav

Another more recently reported signaling mechanism downstream of the β_3 integrin-induced activation of Src involves the nonreceptor tyrosine kinase Syk and the Rho family guanine nucleotide exchange factor Vav (Zou *et al.*, 2007). Following the RANK ligand-induced phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on DAP12 and FcRγ, two receptors that are required for the fusion of mononuclear preosteoclasts to form active multinucleated osteoclasts (Koga *et al.*, 2004; Mocsai *et al.*, 2004), Syk binds via its tandem SH2 domains to the phosphorylated ITAMs (Mocsai *et al.*, 2004). Integrin engagement induces the activation of Src, which then binds to and phosphorylates the ITAM-associated Syk, activating Syk (Zou *et al.*, 2007). The activated Syk in turn phosphorylates and activates Vav3 (and possibly Vav1 and Vav2, which are also expressed in osteoclasts) (Faccio *et al.*, 2005). The Vav proteins are known to regulate cytoskeleton organization by activating Rho family GTPases, especially Rac, as well as by Rho family-independent mechanisms (Hornstein *et al.*, 2004). The importance of this pathway in osteoclasts is indicated by the findings that deletion of any of the genes that encode DAP12, FcRγ, Syk, and Vav3 interferes with the formation of multinuclear osteoclasts and bone resorption (Faccio *et al.*, 2005; Kaifu *et al.*, 2003; Koga *et al.*, 2004; Mocsai *et al.*, 2004).

Although Cbl proteins have not been demonstrated to modulate this signaling pathway in osteoclasts, they interact with and downregulate Syk and Vav in a number of cellular systems (Bustelo *et al.*, 1997; Lupher *et al.*, 1998; Miura-Shimura *et al.*, 2003). Interestingly, the deletion of Cbl-b uncouples tyrosine phosphorylation of Vav1 in T-cells from the requirement for co-stimulation by CD28 (Bachmaier *et al.*, 2000; Chiang *et al.*, 2000), suggesting that a similar effect of the absence of Cbl-b on Vav activation downstream of DAP12 and FcRγ in osteoclast precursors and mature osteoclasts might contribute to the enhanced osteoclast activity and low bone mass in the Cbl-b$^{-/-}$ mice.

Thus, integrins expressed by the osteoclast, particularly the $\alpha_v\beta_3$ integrin, play critical roles in osteoclast biology. These include adhesion itself as well as mediating outside-in signaling, which ensures proper organization of the cytoskeleton, and inside-out signaling, which modulates the affinity of the receptors for their substrates. These two regulatory modes are essential in ensuring the assembly and disassembly of the adhesion structures (podosomes), a cyclic process that is necessary for efficient cell motility.

CALCITONIN AND THE CYTOSKELETON

Because of its potent inhibitory effects on osteoclast activity, calcitonin has long been recognized as a potential therapeutic agent for the treatment of diseases that are characterized by increased bone resorption, such as osteoporosis, Paget's disease, and late-stage malignancies. Signaling mechanisms downstream of the calcitonin receptor have therefore been of great interest. A comprehensive discussion of calcitonin-induced signaling appears elsewhere in this volume, and we focus here on what is known of the interaction of calcitonin-activated signaling events with attachment-related signaling.

In situ, calcitonin causes reduced contact of osteoclasts with the bone surface and altered osteoclast morphology (Holtrop *et al.*, 1974; Kallio *et al.*, 1972), whereas osteoclasts treated with calcitonin *in vitro* retract and become less mobile (Chambers and Magnus, 1982; Chambers *et al.*, 1984; Zaidi *et al.*, 1990). These effects suggest that some of the key targets of calcitonin signaling are involved in integrin-related signaling, cell attachment, and cytoskeletal function.

The calcitonin receptor, a G protein–coupled receptor that has been cloned from several species and cell types, couples to multiple heterotrimeric G proteins (G_s, $G_{i/o}$, and G_q; Chabre *et al.*, 1992; Chen *et al.*, 1998; Force *et al.*, 1992; Shyu *et al.*, 1996, 1999). Because it couples to multiple G proteins, the proximal signaling mechanisms that are activated by calcitonin include many classical GPCR-activated effectors, such as adenylyl cyclase and protein kinase A (PKA); phospholipases C, D, and A2; and protein kinase C (PKC). For technical reasons, much of the initial characterization of signaling downstream of the calcitonin receptor has been conducted in cells other than osteoclasts, particularly cell lines that express recombinant calcitonin receptor. In HEK 293 cells that express the calcitonin receptor, calcitonin induces the phosphorylation and activation of the extracellular signal-regulated kinases, Erk1 and Erk2 (Chen *et al.*, 1998; Raggatt *et al.*, 2000), via mechanisms that involve the $\beta\gamma$ subunits of pertussis toxin-sensitive G_i as well as pertussis toxin-insensitive signaling via phospholipase C, PKC, and elevated intracellular calcium ($[Ca^{2+}]_i$).

Treatment of the calcitonin receptor–expressing HEK 293 cells with calcitonin induces the tyrosine phosphorylation and association of three components of cellular adhesion complexes, FAK, paxillin, and HEF1, a member of the $p130^{Cas}$ family (Zhang *et al.*, 1999). Interestingly, the HEK 293 cells express both HEF1 and $p130^{Cas}$, but only HEF1 is affected by calcitonin treatment. These calcitonin-induced changes are independent of adenylyl cyclase/PKA and of pertussis toxin–sensitive mechanisms and appear to be mediated by the pertussis toxin-insensitive PKC/$[Ca^{2+}]_i$ signaling pathway and to require integrin attachment to the substratum and an intact actin cytoskeleton (Zhang *et al.*, 2000). Calcitonin also induces the phosphorylation of FAK and paxillin in osteoclasts (Zhang *et al.*, 2002), although the calcitonin-induced signaling mechanisms appear to differ to some degree in the osteoclasts and the HEK293 cells. Thus, the calcitonin-induced tyrosine phosphorylation of paxillin and HEF1 in the HEK 293 cells is enhanced by the overexpression of c-Src and strongly inhibited by the overexpression of a dominant negative kinase-dead Src, suggesting that c-Src is required at some point in the coupling mechanism (Zhang *et al.*, 2000). In contrast, the coupling occurs equally well in osteoclast-like cells derived from wild-type and $Src^{-/-}$ mice (Zhang *et al.*, 2002). Similarly, calcitonin treatment of the calcitonin receptor-expressing HEK293 cells increases the phosphorylation of Pyk2, the other member of the FAK family, but reduces Pyk2 phosphorylation in osteoclasts by way of a mechanism that appears to also disrupt attachment complexes and reduce osteoclast attachment (Shyu *et al.*, 2007; Zhang *et al.*, 2002). Finally, calcitonin was recently shown to reduce osteoclast motility, spreading and bone resorbing activity in part by inhibiting calpain activity via a protein kinase C-dependent mechanism (Marzia *et al.*, 2006).

CATHEPSIN K AS A NOVEL ANTIRESORPTIVE TARGET

Degradation of Bone Matrix

Cathepsin K (Cat K) is a member of the CA1 family of lysosomal cysteine proteases. This family comprises 11 human members (cathepsins B, C [J, dipeptidyl peptidase I], F, H, K [O-2], L, O, S, V [L2], W [lymphopain], X [P, Y, Z]), which share a common papain-like structural fold and a conserved active site Cys-Asn-His triad of residues (Rossi *et al.*, 2004; Turk *et al.*, 2001; Vasiljeva *et al.*, 2007; Yasuda *et al.*, 2005). These enzymes are synthesized as preproenzymes and are converted from the catalytically inactive zymogen to the active form in an acidic environment as found in the late endosome or lysosome. Traditionally, cysteine cathepsins were thought to exclusively function as nonspecific proteases in the lysosome. However, there is growing evidence that cathepsins are also secreted in the active form to acidic extracellular compartments. Collectively, cathepsins participate in important physiological as well as pathological processes, including activation of precursor proteins (proenzymes and prohormones), bone remodeling, MHC-II-mediated antigen presentation, keratinocyte differentiation, hair cycle morphogenesis, and apoptosis. They have also been implicated in tumor progression and metastasis, inflammatory diseases, arthrosclerosis, and periodontitis (Lipton, 2005; Stoka *et al.*, 2005; Vasiljeva *et al.*, 2007). The role of cathepsin K in regulating skeletal remodeling and in other bone diseases is discussed here (Lipton, 2005; Vasiljeva *et al.*, 2007; Wang and Bromme, 2005; Yasuda *et al.*, 2005).

Bone is approximately 60% calcium hydroxyapatite and approximately 40% proteins. Collagen type I, which forms trimeric helices that involve all but the extreme N- and C-terminal regions (telopeptides), accounts for about 90% of the bone matrix protein. Both dissolution of minerals and degradation of collagen I are required for normal bone resorption. Early studies on the roles of cysteine proteases in bone matrix degradation examined the effects of various nonselective cysteine protease inhibitors including leupeptin and E-64 (Everts *et al.*, 1988). Treatment of osteoclasts on bone with E-64 results in accumulation of undigested collagen fibrils in late endosome/lysosome vacuoles suggesting an important role of these enzymes in the degradation of matrix collagen (Delaisse *et al.*, 1984). Cat K cDNAs encoding proteins of 329 amino acid residues with 94% sequence homology were cloned from rabbit osteoclast cDNA and human osteoarthritic hip bone cDNA libraries, respectively, and originally designated cathepsin O-2 (Tezuka *et al.*, 1994; Inaoka *et al.*, 1995). Cat K is most closely related to Cat S (48% homology) and most distantly related to Cat B (20% homology; Turk *et al.*, 2001). More recently, Cat K mRNA or protein has been detected in other tissues, including embryonic lung and thyroid gland, macrophages, synovial fibroblasts, neonatal dermal fibroblasts, and activated chondrocytes, and in breast and prostate tumors (Buhling *et al.*, 2000; Littlewood-Evans *et al.*, 1997; Morko *et al.*, 2004; Runger *et al.*, 2007; Tepel *et al.*, 2000).

Cat K is unique in its ability to cleave at multiple sites within the native collagen trimer in both the nonhelical and the proteolytically resistant helical regions (Kafienah *et al.*, 1998). The human disease characterized by cathepsin K (*CSTK*) gene mutations is known as pyknodysostosis, a rare autosomal-recessive osteochondrodysplasia (Gelb *et al.*, 1996). These patients typically exhibit an osteopetrotic phenotype with skeletal dysplasia, including short stature, skeletal fragility, acro-osteolysis of the distal phalanges, clavicular dysplasia, delayed closure of the cranial sutures, and spondylolysis of the lumbar vertebrae (Schilling *et al.*, 2007). There are at least 15 known mutations in the *CSTK* gene that inactivate Cat K or result in early termination of protein synthesis (Fratzl-Zelman *et al.*, 2004; Gelb *et al.*, 1996; Schilling *et al.*, 2007). Targeted deletion of Cat K in mice reproduced the high bone mass phenotype of pyknodysostosis with normal numbers of differentiated osteoclasts on the bone surface, supporting the pivotal role of Cat K in osteoclast activity (Kiviranta *et al.*, 2005; Li *et al.*, 2006; Saftig *et al.*, 1998). Histological analysis revealed that osteoclasts from patients with pyknodysostosis or from Cat K–deficient mice exhibit a morphology similar to that of cells treated with E-64 (Everts *et al.*, 2003; Fratzl-Zelman *et al.*, 2004; Saftig *et al.*, 2000). Because of the lack of proteolytic activity, these cells contain large cytoplasmic vacuoles with undigested collagen and form demineralized matrix fringes on the bone surface (Fratzl-Zelman *et al.*, 2004). In contrast, overexpression of Cat K by its own promoter in transgenic mice resulted in an osteopenic phenotype associated with an increased rate of turnover in metaphyseal trabecular bone and increased porosity of diaphyseal cortical bone (Kiviranta *et al.*, 2001; Morko *et al.*, 2005a). Pharmacological inhibition of Cat K both in *in vitro* osteoclast-based bone resorption assays and in animals and humans results in a reduction in bone resorption and an increase in bone mass, consistent with the premise that Cat K is a promising therapeutic target for the treatment of diseases involving inappropriately rapid bone turnover, including osteoporosis, Paget's disease, arthritis, and metastatic bone disease (Lipton, 2005; Vasiljeva *et al.*, 2007).

Regulation of CSTK Expression

Consistent with the critical role of receptor activator of NFκB (RANK) ligand (RANKL) in the induction of osteoclast differentiation from myeloid precursors (Asagiri and Takayanagi, 2007), RANKL stimulates Cat K mRNA and protein expression in human osteoclasts and in isolated mature rat osteoclasts (Corisdeo *et al.*, 2001; Shalhoub *et al.*, 1999). RANKL appears to stimulate the transcription of the Cat K gene via a number of TRAF6-mediated mechanisms. The activation of NFκB and p38 and the induction of c-Fos expression all promote Cat K gene expression (Asagiri and Takayanagi, 2007; Troen, 2006). NFκB and c-Fos induce the expression of NFATc1 (NFAT2), which activates Cat K expression. Overexpression of NFATc1 markedly stimulates the rat Cat K promoter. Phosphorylation of NFATc1 by p38 kinase further enhances *CTSK* gene expression and promoter activity.

Activated p38 kinase also induces phosphorylation of the microphthalmia transcription factor (Mitf; Matsumoto *et al.*, 2004), a member of the helix-loop-helix (HLH) leucine zipper family. Mutations in the microphthalmia gene (mi/mi) selectively affect osteoclast development and/or function and lead to osteopetrosis (Motyckova and Fisher, 2002). Dominant negative mutations of Mitf exhibit osteopetrosis and lack Cat K mRNA and protein. Mitf binds directly to three E-box motifs in the human *CTSK* promoter, and mutation of any one of these significantly impairs stimulation of promoter activity by Mitf (Steingrimsson *et al.*, 2002). Furthermore, Mitf and PU.1, another transcription factor mediating early osteoclastogenesis (Tondravi *et al.*, 1997), synergistically potentiate NFATc1-induced stimulation of human *CTSK* promoter activity.

Other physiological activators or inhibitors of osteoclast bone resorption also stimulate or inhibit Cat K gene expression (Troen, 2006). Some of these known regulators are various members of the AP-1 transcription factor family, osteoprotegerin (OPG), estrogen, parathyroid hormone (PTH), interleukin (IL)-1α, IL-6, or tumor necrosis factor (TNF)-α. However, it is unclear whether the regulation of Cat K gene expression is simply correlated with osteoclast differentiation or activation or if these factors are intrinsically capable of regulating Cat K synthesis, thereby leading to enhancement of bone resorption in the mature osteoclast. Thus, there have been tremendous advances in our understanding of the effect of Cat K inhibition on bone remodeling.

THERAPEUTIC IMPLICATIONS

Inhibition of Bone Resorption without Affecting Bone Formation

Under normal conditions, bone resorption and formation are tightly coupled events. Although bisphosphonates, estrogen/SERMs, and more recently anti-RANKL reduce bone resorption via inhibition of osteoclastogenesis or survival, the rate of bone formation subsequently falls, thereby reducing the long-term therapeutic effect. To support this line of evidence on the coupling of bone resorption and formation, PTH action on bone formation seems to require osteoclast activation. In PTH-treated ovariectomized (OVX) rats, co-treatment with either estrogen or risedronate reduced histomorphometrically assessed bone formation parameters (Wronski *et al.*, 1993). When sheep were treated with PTH for 3 months, the anabolic effects were significantly reduced when tiludronate was coadministered (Delmas *et al.*, 1995). This evidence from animal studies is supported by recent clinical studies, which showed that treatment of osteoporosis patients with alendronate significantly blunted the anabolic response to PTH (Black *et al.*, 2003; Finkelstein *et al.*, 2003; Khosla, 2003).

Recent evidence suggests that a number of agents that inhibit osteoclastic bone resorption while preserving osteoclast secretory activity may have less effect on bone formation. Evidence from genetically distinct osteopetrotic phenotypes suggests that activated osteoclasts could be the source of a factor that promotes bone formation. Most forms of osteopetrosis (as in *c-fos*$^{-/-}$ mice, for example, which cannot generate osteoclasts; Grigoriadis *et al.*, 1994) are characterized by decreased bone formation in addition to defective bone resorption, due to the coupling processes. In contrast, humans with autosomal dominant osteopetrosis, due to inactivating mutations in the chloride-7 channel (ClC-7) or in ATP6i, have increased osteoclast numbers, but deficient activity due to the decreased acidification of resorption lacunae (Cleiren *et al.*, 2001; Taranta *et al.*, 2003). Bone formation appeared normal in these patients, but without true age-matched controls and with limited biopsies available, the significance of this observation was uncertain (de Vernejoul and Benichou, 2001). In mice, disruption of the ClC-7 chloride channel or the a3 V-ATPase subunit also results in the inhibition of bone resorption accompanied by a significant increase in osteoclast numbers but no change in the rate and extent of bone formation (Karsdal *et al.*, 2005; Xu *et al.*, 2007). Similarly, there are several mouse lines in which the Cat K gene has been deleted; all exhibit defects in bone resorption with normal or enhanced osteoclast numbers (Chen *et al.*, 2007; Gowen *et al.*, 1999; Kiviranta *et al.*, 2005; Li *et al.*, 2006; Saftig *et al.*, 1998). Histomorphometric analysis of cancellous regions of femoral bones from Cat K-deficient mice shows an increased bone formation rate. An intermediate effect was observed for the female heterozygotes (Kiviranta *et al.*, 2005; Li *et al.*, 2006). This suggests that the osteoclasts may still be generating factor(s) that promote bone formation, even though they do not resorb bone because of a failure to remove bone matrix.

More recently, pharmacological evaluation of Cat K inhibitors in OVX animals has provided evidence to support the hypothesis that Cat K inhibition has less effect on bone formation than bisphosphonates. In an OVX-rabbit model, both a Cat K inhibitor (L-006235) or alendronate (ALN) completely blocked the bone loss observed in vehicle-treated animals. ALN also reduced the bone formation rate of both cancellous and endocortical bone. In contrast, no reduction in bone formation rate was observed at either site in the L-006235 group (Pennypacker *et al.*, 2006). Similarly, studies of the effects of Cat K inhibitors (balicatib, relacatib) in ovariectomized cynomolgus monkeys studies suggest that Cat K inhibitors may suppress both resorption and formation in cancellous bone, but may stimulate periosteal cortical bone deposition (Novartis, 2003; Stroup *et al.*, 2005, 2001), possibly by transiently increasing plasma levels of PTH (Stroup *et al.*, 2006a).

Similar observations of the effects of balicatib on bone formation markers in humans have also been reported. A 1-year dose-ranging study of balicatib in postmenopausal women found a 61% decrease in serum and urinary collagen crosslinks (a marker of bone resorption) at the highest dose. Serum osteocalcin and bone-specific alkaline phosphatase, markers of bone formation, were similar to placebo after 1 year of treatment (Adami *et al.*, 2006). This apparent decoupling of bone resorption and bone formation, based on bone turnover markers, distinguishes Cat K inhibitors from other antiresorptives such as bisphosphonates, denosumab, and SERMs, all of which suppress markers for both resorption and formation. Increases in bone mineral density were also observed at the highest dose, providing the first clinical evidence that this mechanism is effective at increasing bone mineral density (BMD) in humans. The clinical validation of the effect of Cat K inhibition on bone mineral density, plus the provocative data suggesting a decoupling of bone resorption and formation provide a compelling framework for further development of Cat K inhibitors for the treatment of osteoporosis.

Other Indications for the Therapeutic Use of Cat K Inhibitors in Bone-related Disease

There is mounting evidence that Cat K may also play a role in other pathologies. In addition to the known high expression of Cat K in osteoclasts, its expression has been documented more recently in a number of other tissues including, but not limited to, cartilage (Morko *et al.*, 2004), atherosclerotic plaques (Lutgens *et al.*, 2006; Platt *et al.*, 2007), adipose tissue (Xiao *et al.*, 2006), lung (Buhling *et al.*, 2000), and skin (Runger *et al.*, 2007). However, levels in these tissues are generally orders of magnitude lower than that in osteoclasts.

Although the ability of Cat K inhibitors to prevent the progression of osteoarthritis (OA) in preclinical models has not yet been reported, they may be beneficial in the treatment of OA via either of two mechanisms (Felson and Neogi, 2004). First, OA has recently been viewed as a whole-organ disease, and not a disease of only articular cartilage. In this view, subchondral bone may play a key role in the triggering and progression of OA. Increased bone turnover and microfracture could increase the stiffness of subchondral bone, transmitting increased stress to the cartilage thus increasing degradation of the cartilage. Furthermore, the formation of osteophytes, or bone spurs, in the joints of OA patients is likely related to increased endochondral bone formation. Second, Cat K has been demonstrated to efficiently degrade collagen type II, the major matrix constituent of cartilage (Kafienah *et al.*, 1998). This enzyme is thus suggested to play a direct role, along with metalloproteinases, in articular cartilage degradation (Wang and Bromme, 2005; Yasuda *et al.*, 2005). Increased Cat K expression has been documented in cartilage samples from human OA patients as well as in synovial tissue and articular chondrocytes (Hou *et al.*, 2002; Morko *et al.*, 2004). The observation that overexpression of CatK under its own promoter in transgenic mice results in spontaneous development of synovitis and cartilage degradation (Morko *et al.*, 2005b) further strengthens the argument that Cat K plays a role in the development of OA.

Another potentially useful application of Cat K inhibitors may be in treating metastatic bone disease, which is characterized by very high levels of bone turnover in proximity to the tumor (Rodan, 2003; Roodman, 2004). Bone resorption inhibitors such as bisphosphonates represent the current standard of care for the treatment of bone metastases, primarily due to breast, prostate or multiple myeloma, and it has been proposed that other strong antiresorptives such as Cat K inhibitors could also be useful in the treatment of bone metastases. Support for this possibility comes from the observation that treatment with a Cat K inhibitor significantly reduced the area of breast cancer-mediated osteolytic lesions in the tibia following the implantation of human breast cancer cells into nude mice (Stroup *et al.*, 2006b). In a separate study, an additive effect of a Cat K inhibitor and zoledronic acid on the reduction of tumor-induced osteolysis was found (Waltham *et al.*, 2006). Similarly, treatment with a Cat K inhibitor both prevented and diminished the progression of cancer growth in bone when prostate cancer cells were injected into the tibia of SCID mice (Lu *et al.*, 2005).

Integrin-Related Signaling

The findings that gene deletion of $\alpha_v\beta_3$ integrin and its downstream effectors, as well as the use of inhibitors of this integrin, results in inhibition of bone resorption *in vivo* in rodent models point to these molecules as potential therapeutic targets for osteoporosis therapy. $\alpha_v\beta_3$ integrin is highly and somewhat selectively expressed in osteoclasts across species. $\beta_3^{-/-}$ mice appear to have normal development and growth, besides the defects related to $\alpha_v\beta_3$ integrin associated osteopetrosis and $\alpha_{IIb}\beta_3$ integrin-associated bleeding. Orally active RGD mimetics have been reported to successfully block bone resorption in rodents without notable adverse effects. Although early clinical studies have uncovered some side effects, particularly in skin, it is not clear if this is an effect of the specific compound or of the entire class. However, the possibilities still exist of developing safe and effective therapeutic agents for osteoporosis based on interfering with the interaction of the osteoclast $\alpha_v\beta_3$ integrin with its physiological ligands. Moreover, antagonists to integrin receptors may play a role in preventing the homing of metastatic cancer cells to the skeleton (Zhao *et al.*, 2007).

Calcitonin

Although the mechanism and action of calcitonin in blocking osteoclast function are not fully understood, the relative selective expression of the calcitonin receptor in osteoclasts and its well-accepted role in regulating osteoclastic cytoskeleton have made calcitonin an attractive therapeutic agent for many years. Human, pig, salmon, and eel calcitonin has been used in both injected and intranasal form in the treatment of osteoporosis and Paget's disease. However, calcitonin-induced downregulation of the receptors in osteoclasts has been observed, resulting in the hormone-induced resistance of osteoclasts to inhibition of bone resorption. It therefore remains to be seen whether this problem can be overcome for therapeutic application of this class of receptor. Identification of the downstream signaling pathway of calcitonin receptor would further our understanding of how calcitonin blocks bone resorption and regulates calcium hemostasis. As discussed here, it is possible that many of the effects of calcitonin on the osteoclast are due to its ability to generate intracellular signals that interfere with the normal regulation of the cytoskeleton, adhesion, and/or cell motility, thereby converging on similar functional targets as the integrin signaling pathways.

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Chapter 13

Apoptosis of Bone Cells

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INTRODUCTION

Apoptosis was defined by Kerr and Wyllie as a series of morphological changes in nuclear chromatin and cytoplasm, leading to specific and controlled deletion of cells, that is, the functional opposite of mitosis (Kerr *et al.*, 1972; Wyllie *et al.*, 1980). It is now established that apoptosis is controlled by a variety of physiological and pathological factors and is involved in the control of cell number not only during development but also during the repair and maintenance of regenerating epithelial tissues such as skin, cornea, the alimentary canal, and the hematopoietic system. Since the previous version of this chapter appeared in 2002, it has become increasingly clear that apoptosis plays an important role in the growth and maintenance of the skeleton. Moreover, dysregulation of apoptosis is a feature of the most common diseases of the skeleton and may in fact represent a therapeutic target.

After briefly describing general features of apoptosis and its regulation, we will concentrate on the occurrence and control of apoptosis in chondrocytes, osteoclasts, osteoblasts, and osteocytes. We will place emphasis on the role of apoptosis of osteoclasts and osteoblasts during remodeling by the basic multicellular unit (BMU), the role of osteocyte apoptosis in adaptation to mechanical strain, and the significance of bone cell apoptosis in the pathophysiology of bone disease and its treatment.

GENERAL FEATURES AND REGULATION OF APOPTOSIS

Apoptotic cells exhibit several specific morphological changes including cytoplasmic shrinkage due to dehydration, nuclear chromatin condensation, DNA degradation, cytoplasmic blebbing, loss of contact with neighboring cells, detachment from the extracellular matrix, and fragmentation into so-called apoptotic bodies (Kerr *et al.*, 1972; Fig. 1). Phosphatidylserine, normally present only in the inner leaflet of the plasma membrane, is transferred to the outer leaflet and is recognized by phagocytes that quickly ingest the apoptotic bodies, ensuring that the process occurs without surrounding tissue edema or recruitment of inflammatory cells. Thus, apoptotic cells vanish without a trace in a manner so efficient that they are often difficult to find in typical tissue sections. The entire process, from initiation to the complete disappearance of the cell, lasts anywhere from 2 or 3 hours up to 1 or 2 days depending on the cell type (Bursch *et al.*, 1990; Pompeiano *et al.*, 1998). Apoptotic cells are interspersed with viable cells. In contrast, cell death due to necrosis occurs in clumps of adjacent cells. Unlike apoptotic cells, necrotic cells are characterized by increased intracellular water and cloudy swelling of the cytoplasm, calcium precipitation, and zones of surrounding hyperemia and inflammatory cell infiltration (Anderson, 1966).

Apoptosis is the default fate of most nucleated cells and is triggered by two distinct pathways: the extrinsic pathway, which is activated by death receptor signaling, and the intrinsic pathway, which is initiated by loss of mitochondrial integrity (Kroemer *et al.*, 2007). As summarized in Figure 2, both pathways culminate in the activation of a family of normally latent proteolytic enzymes called caspases that, once activated, cleave specific intracellular proteins, leading to the morphological changes that characterize apoptosis (Kumar, 2006). There are two types of caspases. The initiator caspases −2, −8, −9, and −10 undergo autoactivation following recruitment to the signaling complexes that form in response to extrinsic or intrinsic stimulation. The proteolytically active forms of the initiator caspases then cleave and activate the latent effector caspases −3, −6, and −7, which execute the death program.

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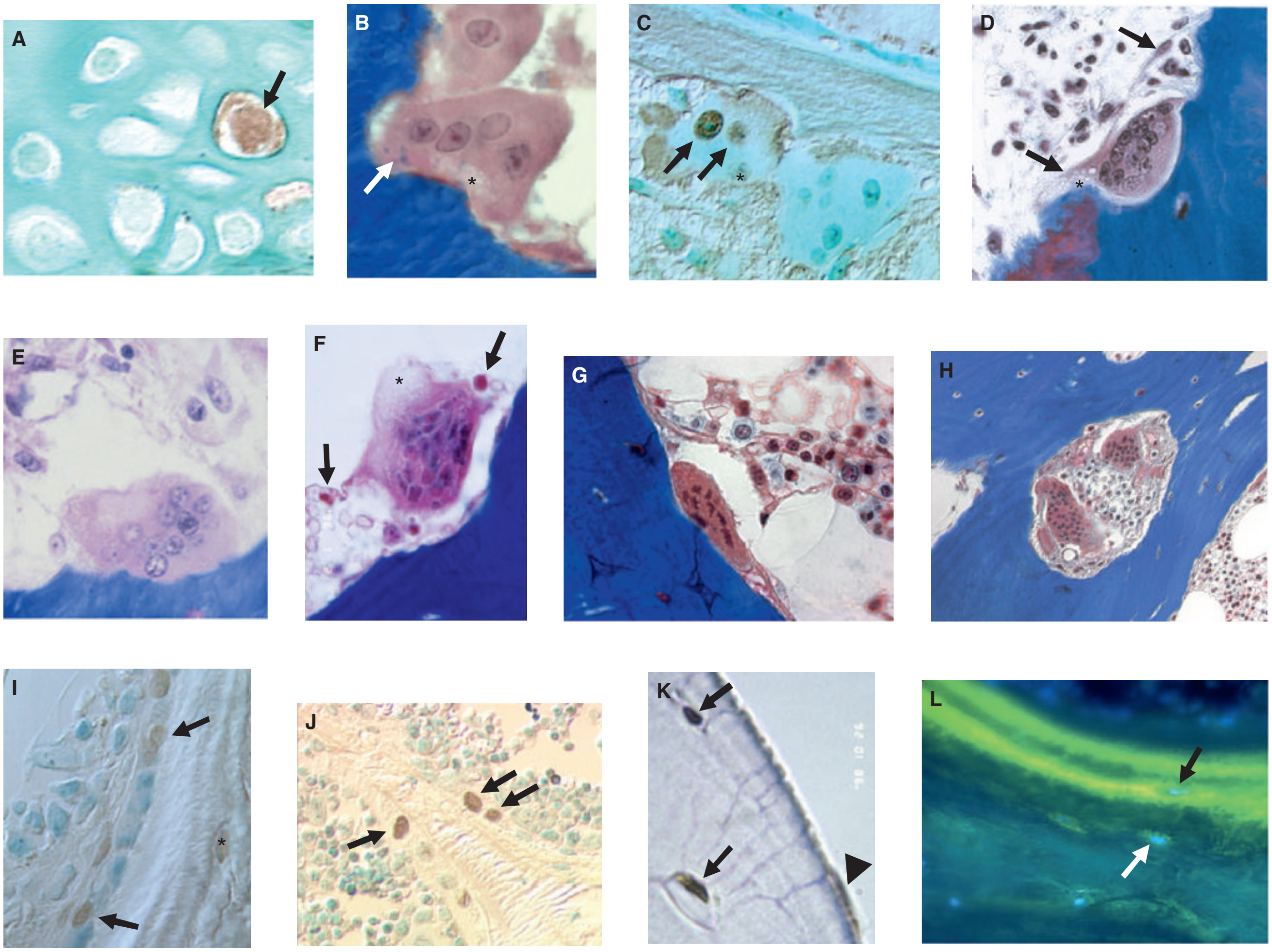


FIGURE 1 Apoptosis of bone cells. **A**, Chondrocyte apoptosis with positive ISEL staining, shrinkage, and nuclear fragmentation is noted (*arrow*). Toluidene blue counterstain, ×630. **B**, A normal osteoclast is shown with a distinct sealing zone and lysosomal-rich clear area (*) at the bone surface. Note the prominent nucleoli. Tiny fragments of blue resorbed mineralized bone can be seen inside the cell (*arrow*). Masson stain, ×1000. **C**, Human osteoclast apoptosis in a dialysis patient with secondary hyperparathyroidism. With just two distinct apoptotic nuclei (*arrows*), the upper left osteoclast remains attached to bone but in situ nick-end labeling defines the cell as apoptotic. Another nuclear profile does not exhibit staining (*). The osteoclast on the right has no ISEL staining and is viable. ISEL staining with toluidene blue counterstain, ×630. **D**, A morphological sign of osteoclast apoptosis is blebbing (*arrows*) and vacuolization (*). The changes were induced by alendronate treatment of Paget's disease of bone. Blebbing is a later event during the process of apoptosis than the ISEL staining shown in **C.** Masson stain, ×400. **E**, In normal osteoclasts, the nuclei are polarized away from the bone surface and the clear zone is immediately adjacent to the ruffled border (see **B**). However, in the osteoclast shown here, the clear zone is away from, and the nuclei are adjacent to, the bone surface. This section was taken from a patient with postmenopausal osteoporosis treated with alendronate. This extent of abnormal osteoclast architecture is a later feature of apoptosis than blebbing and vacuolization shown in panel **D**. Masson stain, ×400. **F**, End-stage alendronate-induced human osteoclast apoptosis in Paget's disease of bone. The osteoclast has detached from the bone surface, contracted, and the nuclei are condensed and fragmented. Prominent cytoplasmic blebbing and vacuolization at the top of the cell (*) and formation of apoptotic bodies at the left and right sides can be seen (*arrows*). This stage of osteoclast apoptosis is rarely seen because of the fleeting existence of apoptotic osteoclasts before they are swept away by phagocytosis. Masson stain, ×630. **G**, This osteoclast is still attached to the bone surface, but the nuclei are pyknotic and the clear zone is absent in this section from a patient receiving long-term treatment with alendronate. Nuclear detail in the surrounding bone marrow is intact. Masson stain, ×630. **H**, Giant osteoclasts, detached from the bone surface and containing 15 to 30 nuclei, are noted after long-term treatment with alendronate in postmenopausal osteoporosis. Masson stain, ×400. **I**, Apoptotic osteoblasts are identified by the brown ISEL staining (arrows) and nuclear condensation in this section taken from a patient receiving long-term treatment with prednisone. An apoptotic osteocyte is present at the right side of the photomicrograph (*). The apoptotic osteoblasts are interspersed with unaffected viable (blue) members of the osteoblast team. Counterstained with methyl green and viewed by Nomarski differential interference contrast microscopy, ×630; modified from Weinstein et al. (1998). **J**, Murine osteoblast apoptosis on cancellous bone. Normal blue-green osteoblasts are interspersed between the brown apoptotic cells (*arrows*). ISEL staining with toluidene blue counterstain, ×250. **K**, Chronic glucocorticoid therapy causes the accumulation of markedly pyknotic apoptotic osteocytes (*arrows*) and lining cells (*arrowhead*) in this section of a femoral head obtained during total hip replacement because of glucocorticoid-induced osteonecrosis. Note that the lacunar-canalicular system now links dead cells. ISEL staining with toluidene blue counterstain and viewed by Nomarski differential interference contrast microscopy, ×1600; modified from Weinstein and Manolagas (2000). **L**, Transiliac biopsy from a patient with glucocorticoid-induced osteoporosis stained with Höescht dye. Note the apoptotic condensed osteocytes recently buried in (*black arrow*) and below (*white arrow*) a new packet of bone outlined by yellow tetracycline labeling. Epifluorescent photomicrograph, ×630. (See plate section)

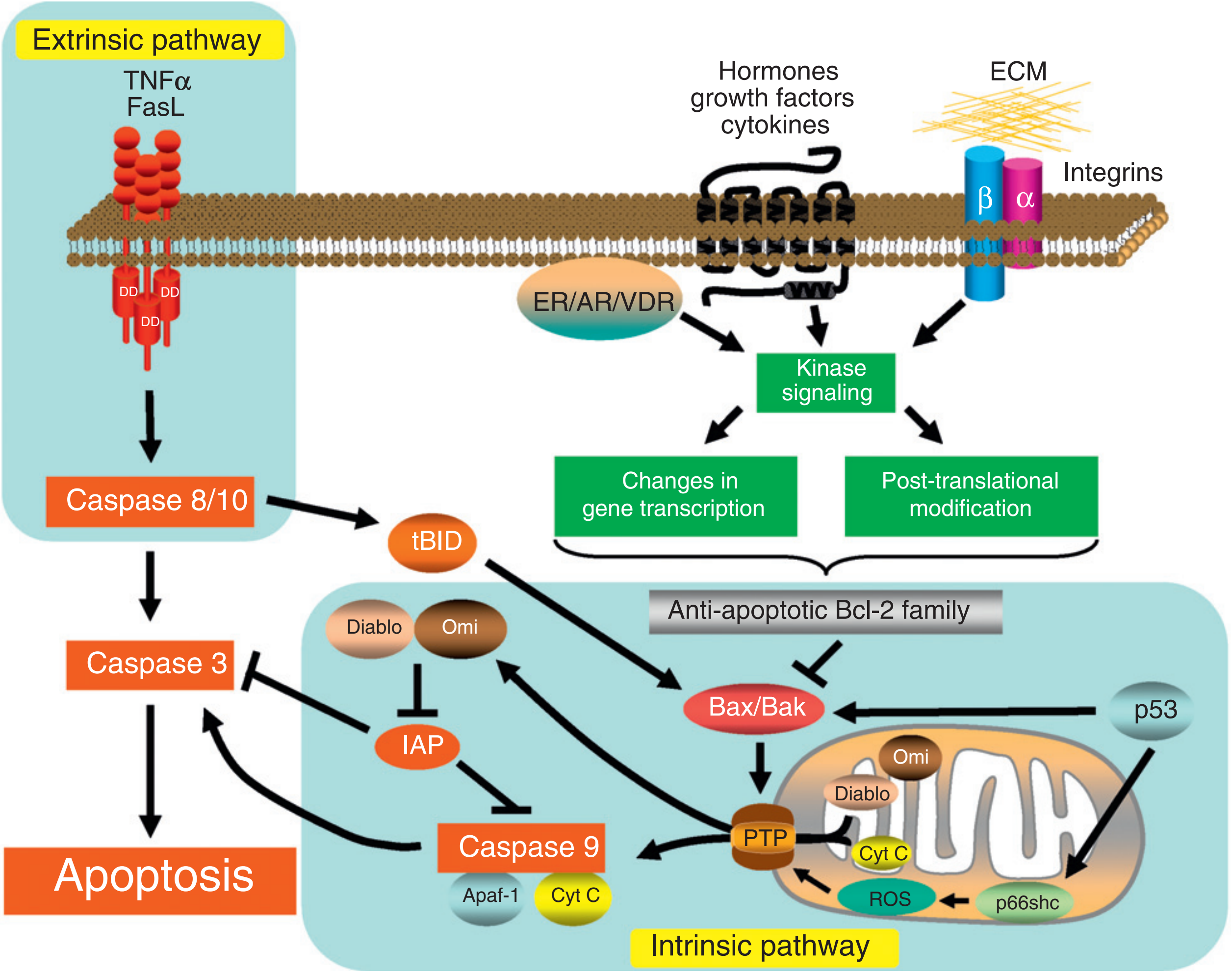

Extrinsic pathway
TNFα
FasL
DD
DD
DD
Caspase 8/10
Caspase 3
Apoptosis
Hormones
growth factors
cytokines
ECM
Integrins
β
α
ER/AR/VDR
Kinase signaling
Changes in gene transcription
Post-translational modification
tBID
Anti-apoptotic Bcl-2 family
Diablo
Omi
IAP
Bax/Bak
p53
Omi
Diablo
PTP
Cyt C
Caspase 9
Apaf-1
Cyt C
ROS
p66shc
Intrinsic pathway

FIGURE 2 Basic mechanisms of the regulation of apoptosis. See text for details. AR, androgen recepto; ; Apaf-1, apoptotic protease activating factor-1; cytc, cytochrome c; DD, death domain; ECM, extracellular matrix; ER, estrogen receptor; IAP, inhibitor of apoptosis protein; PTP, permeability transition pore; VDR, vitamin D receptor. (See plate section)

Activation of Apoptosis

The extrinsic pathway is activated by death receptors residing at the cell surface. These receptors comprise a family of transmembrane proteins, including Fas, and receptors for tumor necrosis factor (TNF)-α and TNF-related apoptosis-inducing factor (TRAIL), which possess a death domain in the intracellular portion of the receptor. Binding of cognate ligands promotes assembly of the death inducing signaling complex at the death domain, resulting in recruitment and activation of caspase-8 or caspase-10 (see Fig. 2). This pathway has been studied mainly in cells of the immune system and is responsible for deletion of autoreactive T-cells. TNF-α also activates antiapoptotic pathways that are mediated by NFκB—a phenomenon that may be important for osteoclast survival, as will be discussed later.

The intrinsic pathway represents the most common apoptotic mechanism in vertebrates and is activated by an increase in the permeability of the outer mitochondrial membrane (Kroemer *et al.*, 2007). The integrity of this membrane is controlled by the pro- and antiapoptotic members of the Bcl-2 family of proteins (Kim *et al.*, 2006). As illustrated in Figure 3, the level of pro-apoptotic sensor Bcl-2 proteins is increased by diverse stimuli, including genotoxic agents, locally produced growth factors, cytokines, systemic hormones, oxidative stress, diminished ATP levels, and loss of kinase-mediated survival pathways. The increase in these sensor proteins overcomes the inhibitory actions of antiapoptotic Bcl-2 family proteins, culminating in the opening of the permeability transition pore of the outer mitochondrial membrane by the proapoptotic Bcl-2 family members Bax and Bak. This results in the release of cytochrome *c*, Omi, and Diablo proteins from the space between the outer and inner mitochondrial membranes into the cytoplasm (see Fig. 2). Death signals also activate p66^{Shc}, a protein that interferes with the electron transport

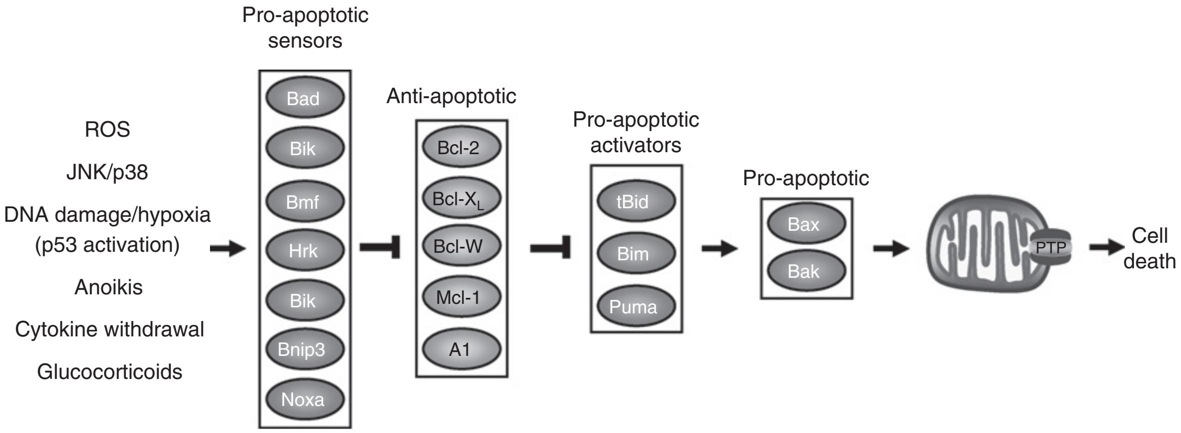


FIGURE 3 Control of mitochondrial membrane permeability by members of the Bcl-2 family of proteins. Apoptosis is triggered when the level of proapoptotic "sensor" members of the Bcl-2 family of proteins exceeds that of the antiapoptotic Bcl-2 family members. Proapoptotic stimuli increase the synthesis of "sensor" Bcl-2 proteins, reduce their sequestration or proteasomal degradation, and decrease the synthesis of antiapoptotic Bcl-2 proteins (Willis and Adams, 2005). The "sensor" Bcl-2 proteins activate apoptosis by binding to, and thereby inhibiting the function of, antiapoptotic Bcl-2 proteins, which normally prevent the actions of members of the proapoptotic "activator" Bcl-2 family (Kim et al., 2006). The resulting increase in the level of "activator" Bcl-2 proteins facilitate the homo-oligomerization of a third set of proapoptotic Bcl-2 proteins, Bax and Bak, which bind to the outer mitochondrial membrane and open the permeability transition pore (PTP). Modified from Galonek and Hardwick (2006).

machinery of the inner mitochondrial membrane and generates reactive oxygen species (ROS) that also contribute to permeabilization of the outer mitochondrial membrane (Giorgio *et al.*, 2005). Upon release of cytochrome *c* into the cytoplasm, it interacts with apoptotic protease activating factor-1 (Apaf-1) which causes the activation of caspase-9 (Kumar, 2006). Omi and Diablo promote apoptosis by blocking the activity of members of the inhibitor of apoptosis (IAP) family, which normally prevent caspase activation. A connection between the extrinsic and intrinsic pathways is provided by caspase-8 cleavage of Bid to generate a truncated form (tBid) that increases the permeability of the outer mitochondrial membrane (see Fig. 2).

Cells are kept alive by hormones and locally produced growth factors and cytokines that activate various kinase cascades leading to increased synthesis of antiapoptotic members of the Bcl-2 and IAP proteins and/or sequestration or degradation of proapoptotic sensor Bcl-2 proteins. Survival signals are also generated by the interaction of integrins with extracellular matrix proteins, which promote the formation of focal adhesion complexes within the cell comprising cytoskeletal proteins, focal adhesion kinase (FAK), and members of the Src family of kinases. Phosphorylation of FAK activates the PI3-kinase and the MAP kinase cascades, which prevent apoptosis.

Detection and Quantification of Apoptosis

Quantification of cells undergoing apoptosis in tissues has relied mainly on methods that detect the presence of degraded DNA (Gavrieli *et al.*, 1992; Wijsman *et al.*, 1993; Ansari *et al.*, 1993). These methods are known by a number of acronyms that are often used interchangeably, the most common being TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick-end-labeling) and ISEL (in situ end-labeling) (see Figs. 1A, C, I–K). The procedure involves attachment of a labeled nucleotide to hydroxyl groups at the 3′ end of DNA using either terminal deoxynucleotidyl transferase or the Klenow fragment of DNA polymerase I. Use of the Klenow enzyme detects cells with fewer DNA strand breaks, and labeling with this enzyme is less susceptible to artifact because it lacks exonuclease activity (Wijsman *et al.*, 1993). The incorporated labeled nucleotides are usually visualized with peroxidase staining in tissue sections. The technique is sensitive enough to detect the few DNA strand breaks that occur in the earliest stages of apoptosis (Pompeiano *et al.*, 1998). As in any quantitative histologic procedure, detection of degraded DNA in apoptotic cells requires standardization and the use of appropriate negative and positive controls during each staining procedure (Jilka *et al.*, 2007). Table 1 lists guidelines for obtaining reproducible estimates of apoptotic osteoblasts.

Other methods have been developed for detection of apoptotic cells in tissues and/or in culture, including histologic or enzymatic detection of active caspases, staining of DNA with fluorescent dyes such as acridine orange, Höescht dyes (see Fig. 1L), and propidium iodide, as well as detection of phosphatidylserine on the cell surface (Bellido and Plotkin, 2007). Fragmented nuclei can also be detected in cells transfected with green fluorescent protein containing a nuclear localization sequence—a useful tool for studying apoptosis in cells co-transfected with genes of interest for mechanistic studies (Bellido and Plotkin, 2007).

TABLE I Guidelines for the Detection of Apoptotic Osteoblasts

1. Section thickness must be less than 10 μm to allow access of reagents into all cells.
2. Exposure of fragmented DNA in nondecalcified sections after removal of the plastic is best accomplished by pretreatment with in 10 mM citrate buffer using laboratory-grade microwave irradiation (such as the H2800 Microwave Processor, Energy Beam Sciences, Inc., East Granby, Conn.) followed by incubation with pepsin or proteinase K that are free of contaminating nuclease activity (Negoescu *et al.*, 1996). Treatment must be brief to prevent loss of tissue morphology and to avoid false positive results.
3. Identically fixed and processed bone tissue serves as the best positive control. Weaned breast tissue (included as a positive control in apoptosis kits) is not useful for optimizing staining of apoptotic osteoblasts because it is easier to obtain positive staining in this tissue than in bone (Tidball and Albrecht, 1998).
4. Efforts to increase the sensitivity of the staining procedure by increasing the duration of (a) proteinase pretreatment, (b) exposure to the transferase or Klenow enzyme, or (c) incubation with reagents to intensify color development can lead to nonspecific staining that can go unrecognized unless a standard specimen with a known coefficient of variation for osteoblast apoptosis is available for comparison (Labat-Moleur *et al.*, 1998).
5. Morphological changes such as discretely condensed chromatin and nuclear fragmentation or peripheral beading on the nuclear membrane should accompany the positive staining. Apoptotic osteoblasts should be juxtaposed to osteoid and interspersed with normal osteoblasts and show a cuboidal morphology to distinguish them from nearby marrow and lining cells (see Figs. 1I, J).

APOPTOSIS OF CHONDROCYTES DURING ENDOCHONDRAL OSSIFICATION

Chondrocyte apoptosis plays a major role in the shaping of long bones during development. In the growth plate, chondrocytes undergo a series of highly regulated transformations leading to the replacement of the cartilage template by bone. During this process, known as endochondral ossification, chondrocytes proliferate, produce type II collagen, and form a columnar cell layer. Subsequently they undergo cell cycle arrest, differentiation, and hypertrophy, which is followed by mineralization of the surrounding matrix. At this point, the chondrocytes die by apoptosis and, after blood vessel invasion, the cartilage is replaced with bone (Gibson *et al.*, 1995).

Apoptotic hypertrophic chondrocytes are easily recognized at the lateral margins of the growth plate with TUNEL or ISEL staining (see Fig. 1A). Although some investigators have reported that ISEL- or TUNEL-positive chondrocytes are rare (Roach *et al.*, 2004), classical ISEL-positive chondrocytes are so commonly found that they are useful as internal positive controls for apoptosis staining of other bone cells.

Chondrocyte apoptosis may begin well before the dramatic and massive death that characterizes hypertrophic chondrocytes as some chondrocytes exhibit TUNEL staining during the proliferative stage (Cruickshank *et al.*, 2005). As chondrocytes progress from the late proliferative stage toward the mature stage, there is a decrease in the permeability of the mitochondrial membrane and the level of Bcl-2, whereas binding of Bax to mitochondria increases (Amling *et al.*, 1997; Pucci *et al.*, 2007). These changes are associated with increased sensitivity to proapoptotic stimuli, such as elevated concentrations of inorganic phosphate (Pucci *et al.*, 2007). ROS are elevated in hypertrophic chondrocytes and may be involved in the initiation of apoptosis, because administration of the antioxidant *N*-acetylcysteine (NAC) reduces ROS and inhibits hypertrophy and apoptosis of these cells (Morita *et al.*, 2007).

Parathyroid hormone related peptide (PTHrP) and Indian hedgehog (Ihh) play central roles in the regulation of endochondral ossification and are involved in the maintenance of chondrocyte survival during this process (Kronenberg, 2003). Ihh is secreted by chondrocytes that have just stopped proliferating and it stimulates the synthesis of PTHrP, which restrains apoptosis (Lee *et al.*, 1996; Amizuka *et al.*, 1996; Weir *et al.*, 1996). As chondrocytes become more distant from the site where PTHrP is produced during hypertrophy, PTHrP-induced survival signaling diminishes. The antiapoptotic effect of PTHrP on chondrocytes is mediated in part by increased Bcl-2 expression (Amling *et al.*, 1997) and by Nkx3.2, a member of the NK family of homeoprotein transcriptional regulators (Provot *et al.*, 2006). Expression of Nkx3.2 is high in proliferating chondrocytes, but diminishes thereafter. NKX3.2 is absent in PTHrP-deficient mice. Interestingly, the antiapoptotic activity of Nkx3.2 appears unrelated to its function as a transcriptional regulator. Nkx3.2 enhances chondrocyte survival in a cell-autonomous fashion by activating NFκB, apparently in a ligand independent fashion (Park *et al.*, 2007). The mechanism by which NFκB inhibits apoptosis in chondrocytes is unknown, but in other tissues it upregulates the expression of antiapoptotic members of the Bcl-2 family, as well as members of the inhibitors of apoptosis family of proteins, such as c-IAP and XIAP (Dutta *et al.*, 2006).

A second factor that restrains chondrocyte apoptosis is the transcription factor hypoxia-inducible factor 1α (HIF-1α). At normal oxygen tension HIF-1α levels are low because proline hydroxylases utilize molecular oxygen to hydroxylate specific proline residues in HIF-1α, which results in its degradation by proteasomes (Wenger *et al.*, 2005). Under the low oxygen tension condition that prevails at the center of the growth plate, HIF-1α levels are high, leading to increased synthesis of several factors that prevent apoptosis including Bcl-2, glycolytic enzymes that maintain the level of ATP, and vascular endothelial growth factor (VEGF; Schipani *et al.*, 2001; Cramer *et al.*, 2004). VEGF induces vascularization and restrains chondrocyte apoptosis as indicated by increased apoptosis in the center of the proliferative and upper hypertrophic zones of the growth plate in mice with chondrocyte-specific deletion of VEGF (Zelzer *et al.*, 2004). Proline hydroxylase levels are increased in hypertrophic chondrocytes, suggesting that the consequent decline of HIF-1α levels contributes to apoptosis of these cells (Terkhorn *et al.*, 2007). This notion is supported by *in vitro* studies showing that diminished levels of HIF-1α in mature chondrocytes are associated with increased sensitivity to the proapoptotic effects of ROS (Terkhorn *et al.*, 2007).

Wnt proteins influence cell proliferation, differentiation, and survival in many tissues by binding to Frizzled, and low-density lipoprotein receptor-related protein 5 (LRP5) or LRP6 co-receptors. This causes accumulation of β-catenin in the nucleus where it activates the TCF/LEF family of transcription factors (Bodine and Komm, 2006). Wnt/β-catenin-induced signaling is involved in the maintenance of chondrocyte viability because deletion of β-catenin specifically in chondrocytes results in a dramatic increase in apoptosis of differentiated chondrocytes (Mak *et al.*, 2006). Although less severe, this growth plate phenotype resembles that of mice lacking HIF-1α or VEGF in chondrocytes. The increased chondrocyte death of mice lacking β-catenin in chondrocytes is rescued by induction of hedgehog signaling, suggesting that hedgehog-induced survival signaling is downstream of Wnt/β-catenin activation.

OSTEOCLAST APOPTOSIS

The life span of osteoclasts in cancellous bone has been estimated to be as long as 11 to 15 days in dogs (Jaworski *et al.*, 1981) and as short as 1 to 2 days at the end of lactation in rats (Miller and Bowman, 2007). Yet the lifetime of the BMU in either of these species, or in humans, is much longer, lasting several weeks to months (Parfitt, 1996). This has two important implications for the function of the BMU. First, for orderly progression of the BMU there must be a constant supply of new osteoclasts to replace those lost by apoptosis. Second, osteoclast life span controls the depth of resorption. Indeed, prolongation of osteoclast life span causes teams of osteoclasts to resorb to greater depths than normal, leading to the perforation of trabeculae and loss of connectivity (Parfitt *et al.*, 1996).

Compared to other bone cell types, osteoclasts are rare: 10-fold fewer than osteoblasts and 100-fold fewer than osteocytes. This fact hinders the detection of apoptotic osteoclasts in bone sections unless overall osteoclast number is increased and/or apoptosis is stimulated. The earliest morphological abnormalities seen in apoptotic osteoclasts are prominent cytoplasmic blebs (see Fig. 1D). Next, the ruffled border is disrupted (see Figs. 1E, G), the nuclei become pyknotic, and the cell detaches from the bone surface (see Figs. 1F–H; Weinstein and Manolagas, 2000). In mice treated with high levels of bisphosphonates (BPs), all of the nuclei exhibit condensed chromatin and TUNEL staining, as well as more intense cytoplasmic staining for tartrate-resistant acid phosphatase due to cytoplasmic contraction (Hughes *et al.*, 1995). Osteoclasts in which all of the nuclei are stained with TUNEL have also been observed in bone of estrogen-treated rats (Faloni *et al.*, 2007) and in mice after lactation (Ardeshirpour *et al.*, 2007). With the more sensitive ISEL method that permits visualization of the early stages of DNA degradation, some osteoclast nuclei are stained but others are not (see Fig. 1C).

As will be described later, numerous factors regulate osteoclast apoptosis. The regulation of osteoclast life span represents an elegant and sensitive mechanism by which rapid changes in osteoclast number, and thus bone resorption, can be accomplished. For example, stimulation of osteoclast apoptosis may rapidly reduce bone resorption once increased demand for calcium has ended, such as at the end of lactation. Conversely, a rapid increase in the demand for bone resorption, such as dietary calcium deficiency, may be met in part by inhibition of osteoclast apoptosis.

Fas ligand stimulates osteoclast apoptosis. This may be an important signal for the control of osteoclast number, because mice lacking Fas have increased osteoclasts and lower bone mass (Wu *et al.*, 2003). High extracellular calcium concentrations, similar to those that occur locally during bone resorption, can also induce osteoclast apoptosis *in vitro* (Lorget *et al.*, 2000), possibly via activation of the calcium sensing receptor (Mentaverri *et al.*, 2006). Detachment of osteoclasts *in vitro* induces apoptosis, presumably by disrupting integrin-mediated survival signaling (Villanova *et al.*, 1999). Calcitonin inhibits the resorptive activity of osteoclasts and also promotes detachment from the bone surface. However, it does not induce apoptosis due to compensatory cAMP-mediated survival signaling (Selander *et al.*, 1996).

Osteoclast differentiation depends on production of macrophage colony-stimulating factor (M-CSF) and RANKL by stromal cells, and these cytokines, as well as TNF-α and IL-1, also promote osteoclast survival (Fuller *et al.*, 1993; Lacey *et al.*, 2000). Many of these cytokines utilize the same signaling pathways to accomplish this function, and the details of these pathways have been recently reviewed (Xing and Boyce, 2005). Therefore, the present

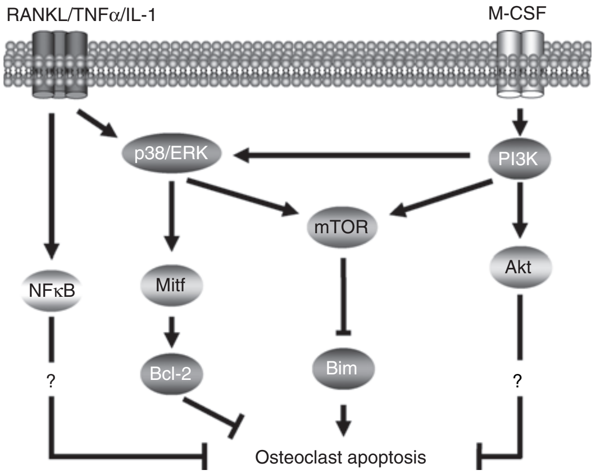


FIGURE 4 Summary of cytokine-activated pathways that promote osteoclast survival. Although RANKL, TNF-α, and IL-1 bind to distinct receptors, a generic receptor is shown in the diagram. See text for details. Akt, thymoma viral proto-oncogene; ERK, extracellular signal-regulated kinase; IL-1, interleukin-1; M-CSF, macrophage colony-stimulating factor; Mitf, microphthalmia-associated transcription factor; mTOR, mammalian target of rapamycin; NFκB, nuclear factor-kappa B; RANKL, receptor activator of NFκB ligand; PI3K, phosphoinositide-3 kinase; TNFα, tumor necrosis factor α.

discussion will focus on the common aspects of these survival pathways, which are illustrated in Figure 4.

M-CSF, RANKL, TNF-α, and IL-1 each activate extracellular signal-regulated kinases (ERKs) that activate antiapoptosis signaling in osteoclasts, as blockade of ERK activation blocks the ability of IL-1 or TNFα to promote osteoclast survival (Miyazaki *et al.*, 2000; Lee *et al.*, 2001). Phosphatidylinositol 3-kinase (PI3K) activity is also required for the anti-apoptotic action of each of these cytokines (Wong *et al.*, 1999; S. E. Lee *et al.*, 2001; Z. H. Lee *et al.*, 2002; Fukuda *et al.*, 2005). While some evidence suggests that PI3K promotes osteoclast survival via activation of the survival kinase Akt (Lee *et al.*, 2001; Lee *et al.*, 2002; Gingery *et al.*, 2003), a more recent study employing short-hairpin RNAs (shRNAs) to reduce Akt indicates that Akt is required for osteoclast differentiation but not for survival (Sugatani and Hruska, 2005). Mammalian target of rapamycin (mTOR) is a target of PI3K activity, and activation of this kinase is required for the antiapoptotic actions of M-CSF, RANKL, and TNF-α in osteoclasts (Glantschnig *et al.*, 2003). Thus, although the role of Akt in cytokine-mediated osteoclast survival remains to be clarified, each of these cytokine activated pathways appears to converge on mTOR activation as a critical determinant of their ability to promote osteoclast survival.

The transcription factor NFκB is activated by RANKL, TNF-α, and IL-1 and has been shown to oppose cell death induced by TNF-α in a variety of systems (Beg and Baltimore, 1996; Varfolomeev and Ashkenazi, 2004). Suppression of NFκB subunit expression via antisense RNA blocks the ability of IL-1 to support osteoclast survival (Jimi *et al.*, 1998), and decoy oligonucleotides that block NFκB binding to DNA induce osteoclast apoptosis *in vitro* and *in vivo* (Penolazzi *et al.*, 2003, 2004, 2006). Nonetheless, osteoclast precursors lacking NFκB subunits have normal survival (Xing *et al.*, 2002) and inhibition of NFκB activation via a dominant-negative IKK2 did not affect the ability of IL-1 to promote osteoclast survival (Miyazaki *et al.*, 2000). The reasons underlying these different results are unclear but may be related to off-target actions of some methods used to suppress NFκB activation (Miyazaki *et al.*, 2000).

The downstream effectors of prosurvival signaling in osteoclasts are best characterized in the case of M-CSF. M-CSF activates the Mitf transcription factor, which then stimulates transcription of Bcl-2 (Hershey and Fisher, 2004). The importance of this pathway is illustrated by the osteopetrotic phenotype of mice lacking either Mitf or Bcl-2 (McGill *et al.*, 2002), and the rescue of the osteopetrotic phenotype of M-CSF-deficient mice by overexpression of Bcl-2 (Lagasse and Weissman, 1997). The pro-apoptotic Bcl-2 family member Bim is also involved in the regulation of osteoclast survival as M-CSF promotes the proteasomal degradation of this factor (Akiyama *et al.*, 2003).

Besides cytokines, lipopolysaccharide (LPS) stimulates bone resorption (Abu-Amer *et al.*, 1997). LPS is a strong inducer of all of the cytokines mentioned earlier. However, LPS promotes survival of differentiated osteoclasts *in vitro* in the presence of OPG or IL-1 receptor antagonists, or in cells derived from TNF-α receptor-deficient mice, via activation of both the NFκB and PI3K pathways (Suda *et al.*, 2002). Thus, LPS can promote osteoclast survival both indirectly via stimulation of cytokines and directly via activation of survival pathways.

Sex steroids, glucocorticoids and BPs also influence osteoclast apoptosis as listed in Table II. Their effects will be discussed in detail in Section VII.

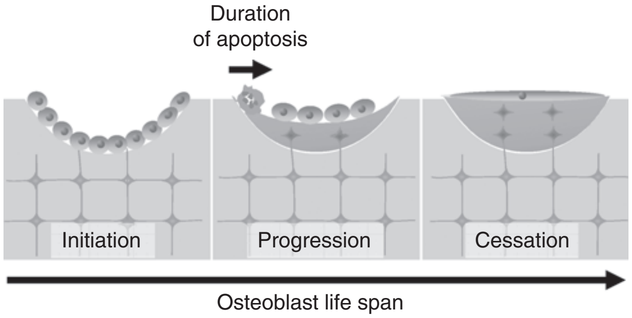
OSTEOBLAST APOPTOSIS

Apoptosis, the means of culling osteoblast number, appears to begin at the early stages of osteoblast differentiation and continues throughout all stages of the working life of this cell. Thus, apoptotic mesenchymal progenitors have been detected near the primary spongiosa of developing chick and rabbit long bones (Palumbo *et al.*, 2003), the osteogenic front of developing murine calvarial bone (Rice *et al.*, 1999), at sites of fracture healing (Landry *et al.*, 1997; Li *et al.*, 2002), and near the newly forming bone that develops during distraction osteogenesis—a process in which long bones are cut and gradually stretched, resulting in *de novo* bone formation within the expanding gap (Li *et al.*, 2003).

Apoptotic osteoblasts with positive TUNEL or ISEL staining are first recognized by russet-brown nuclear

TABLE II Changes in Bone Cell Apoptosis during Pathologic Bone Remodeling and Response to Therapy

Condition/ Treatment	Osteoclast Apoptosis	Osteoblast Apoptosis	Osteocyte Apoptosis	Bone Mass	Bone Strength
Sex steroid deficiency	↓	↑	↑	↓	↓
Glucocorticoid excess	↓	↑	↑	↓	↓
Aging	?	↑	↑	↓	↓
Immobilization	?	↑	↑	↓	↓
Intermittent PTH	?	↓	↓	↑	↑
Bisphosphonates	↑	↓	↓	↑	↑



$$\frac{\text{Duration of apoptosis}}{\text{Osteoblast life span}} = \frac{\text{\% TUNEL positive cells}}{\text{\% of osteoblasts that die}}$$

FIGURE 5 Estimation of osteoblast apoptosis. A team of osteoblasts assembles at the floor of the resorption cavity at the beginning of the bone formation process (“initiation”). During matrix synthesis (“progression”), some osteoblasts become entombed as osteocytes and some die by apoptosis. When matrix synthesis ceases (“cessation”), all that remains of the original team of osteoblasts are osteocytes and lining cells. The equation allows calculation of the fraction of osteoblasts that die by apoptosis based on the observed prevalence of osteoblasts exhibiting degraded DNA as detected by TUNEL or ISEL, osteoblast life span, and the length of time that apoptotic osteoblasts exhibit staining for TUNEL or ISEL. Osteoblast life span can be determined by dividing the wall width (the histologic measure of the amount of bone made by a team of osteoblasts) by the mineral apposition rate. For osteoblasts in cancellous bone of normal adult mice this is about 12 days (Weinstein *et al.*, 1998). It is unknown how long the DNA fragmentation phase of apoptosis lasts in dying osteoblasts, but in liver cells the duration of this particular phase is 2 to 3 hours when visualized with the terminal deoxynucleotidyl transferase method (Bursch *et al.*, 1990). There is no reason to expect that this phenomenon would substantially vary among cell types. Therefore, if the prevalence of murine osteoblast apoptosis is 0.6%, it can be calculated that 60% to 90% of osteoblasts die by apoptosis, the rest becoming osteocytes or lining cells.

pigmentation and small balls of chromatin rimming the nuclear membrane (see Figs. 1I, J). Later, cell shrinkage becomes evident, but detachment from the bone surface appears to be a late phenomenon and probably a fleeting one due to rapid phagocytosis after detachment (Weinstein *et al.*, 1998, 2004). In vertebral cancellous bone of adult mice, 0.5% to 1.0% of osteoblasts exhibit DNA strand breaks when analyzed with the terminal deoxynucleotidyl transferase enzyme (Jilka *et al.*, 1998; Silvestrini *et al.*, 1998; Weinstein *et al.*, 1998). Using the more sensitive Klenow enzyme the prevalence of apoptotic osteoblasts increases to as much as 5% to 10%; see Fig. 1J). Longitudinal sections of lumbar vertebrae (L1–L4) of adult mice are particularly advantageous samples for quantifying osteoblast apoptosis because they typically contain 800 to 1200 osteoblasts for inspection. This situation allows statistically meaningful estimates of the prevalence of apoptosis.

How can a phenomenon of such low prevalence be important? The phase of apoptosis that can be detected by TUNEL staining represents only a tiny fraction of the cell’s life span, analogous to the brevity of a 6-month-long terminal illness as compared to the entire life of a 75-year-old human. Tidball and Albrecht have estimated that an entire tissue could die of apoptosis in 20 days if only 0.4% of the nuclei in the tissue were detectably apoptotic (using the terminal deoxynucleotidyl transferase enzyme; Tidball and Albrecht, 1998). This calculation is based on the assumption that there is a 2-hour period of detectability of the phenomenon, that apoptosis occurs at a constant rate, and that no mitosis occurs during this period. Based on the fact that in the snapshot of a biopsy the fraction of cells exhibiting apoptotic features is equal to the corresponding fraction of time spent in apoptosis, one can calculate the percentage of cells that undergo apoptosis in mice is 60% to 90% (Fig. 5). This estimate is very similar to the estimate of Parfitt (1990) for the number of osteoblasts that die by apoptosis in human bone.

Despite the seeming parity between humans and mice with respect to the proportion of osteoblasts that die, the actual prevalence of osteoblast apoptosis in human bone is much lower compared to mice. Indeed, no apoptotic osteoblasts were seen in transiliac biopsies taken from 12 normal subjects using the less sensitive TUNEL enzyme (Weinstein *et al.*, 1998). The more sensitive Klenow enzyme was used to search for apoptotic osteoblasts in biopsies from 23 placebo-treated post-menopausal women in another study (Weinstein *et al.*, 2003), but none were found (R. S. Weinstein, unpublished observations). The different prevalence of apoptotic osteoblasts in murine and human cancellous bone is due to the fact that human osteoblasts live longer and are fewer in number compared to their murine counterparts because bone turnover in humans is lower. Specifically, whereas the average osteoblast life span is about 12 days in normal adult mice (Weinstein *et al.*, 1998), the average life span of normal human osteoblasts is about 150 days (Parfitt *et al.*, 1997)—a 12.5-fold difference. Therefore, a 0.6% prevalence of osteoblast apoptosis in mice corresponds to a prevalence of 0.05% in humans. Furthermore, whereas the average bone formation rate in Swiss-Webster mice is about $0.150\,\mu m^2/\mu m$/day (Weinstein *et al.*, 1998), the corresponding value in healthy humans is about $0.038\,\mu m^2/\mu m$/day (Han *et al.*, 1997)—a 4-fold difference. There is no reason to suspect that the duration of the morphologic features of apoptosis is different in the two species. Likewise, in both species the rate of bone formation is inexorably proportional to the number of osteoblasts (Parfitt *et al.*, 1995). That is to say, the higher the number of osteoblasts present in a defined area of bone, the greater the chance to detect an apoptotic one. If the relationship between osteoblast number and bone formation rate is similar in the two species (once again there is no reason to suspect that it is different), the 12-fold difference in life span between them needs to be multiplied by the fold difference in bone formation rate. This simple calculation makes the chance of detecting an apoptotic osteoblast in a section of human bone about 50 times lower than in a section of murine bone.

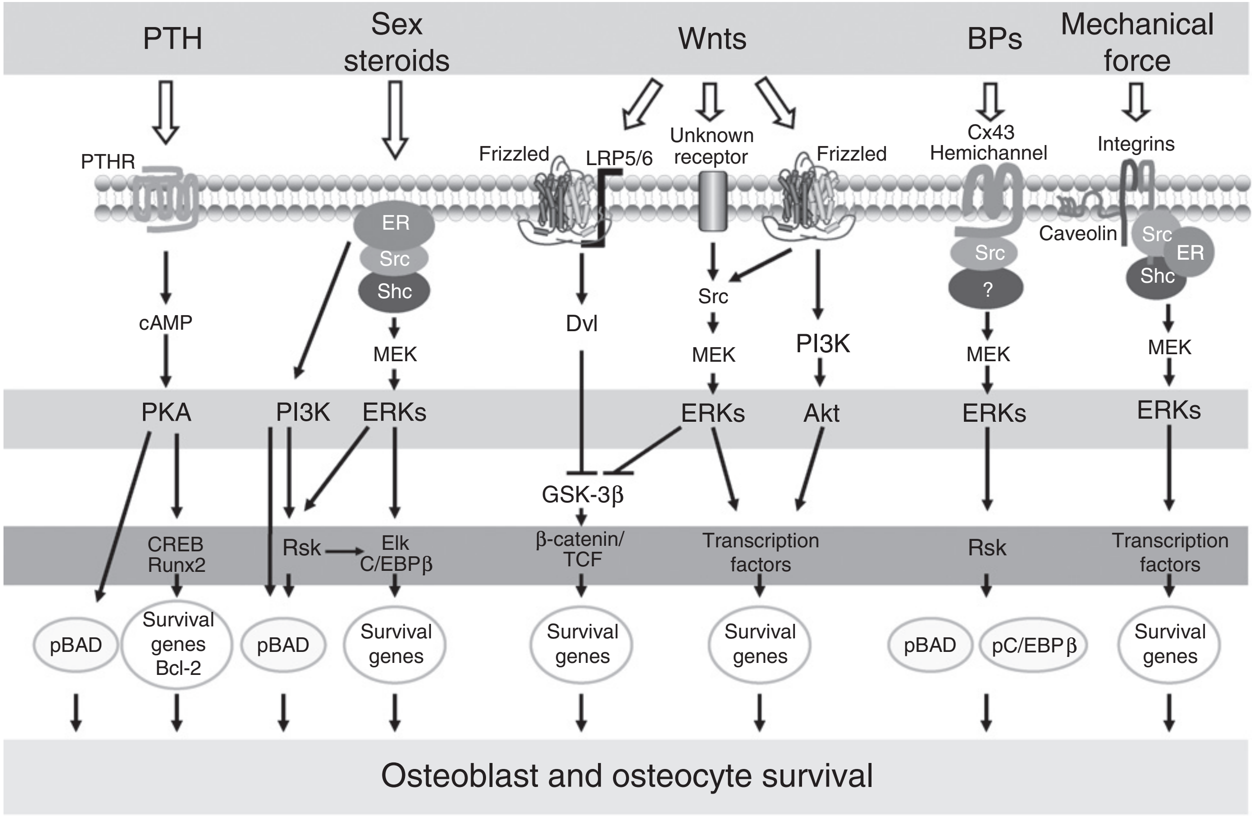
The total number of osteoblasts present in a longitudinal section obtained from a transiliac biopsy from humans is only 20 to 50 (Weinstein *et al.*, 2003) as compared to the 800 to 1200 cells present in a single longitudinal section of four murine vertebrae. If one were to estimate the prevalence of apoptosis relying on the inspection of 20–50 cells present in a section from a human biopsy, one will fail to find a single apoptotic cell most of the time because the phenomenon is predicted to occur in 0.01% to 0.1% of the entire osteoblast population. In other words, to achieve comparable statistical power for estimating the prevalence of osteoblast apoptosis in a human bone section as in a murine section, one would have to prepare 100 sections and stain them with the more sensitive Klenow enzyme. Such sections are usually 5 to 7 μm thick (the optimal usable thickness for staining and microscopy) and must be nonadjacent because the osteoblast height is 12 to 20 μm. Unfortunately, this is practically impossible to achieve with a normal-sized human transiliac biopsy of 7 mm diameter.

There are only two scientifically valid ways of estimating rare events such as apoptosis. The first is enumeration of a vast number of cells in which the sparse phenomenon may happen. The second is reliance on a simulation model based on a predetermined estimate of the probability of the rare event happening, which is the only practical option for human biopsies—perhaps with the exception of conditions, such as glucocorticoid excess, in which the prevalence of the phenomenon increases dramatically. But the sine qua non conditions under which such a model can be relied on is that the apoptotic events must be randomly spaced and independent of each other. Unfortunately, neither condition is certain. In fact, osteoblast apoptosis seems to occur in particular regions of bone. As elegantly explained in the case of the detection of another rare occurrence in bone samples—namely microcracks—frequency distributions are typically skewed with a few specimens exhibiting many events but most having only a few or none (Martin *et al.*, 2007). Therefore, if only a few apoptotic osteoblasts are identified in a specimen, it is a warning sign that the sample size is not large enough to estimate the prevalence of the phenomenon in the entire skeleton of an experimental group. An a priori estimate of the prevalence of apoptosis in advance of an experiment, together with the appropriate positive and negative controls, must be used to derive a reliable estimate of the required sample size.

Most if not all major regulators of skeletal homeostasis influence the apoptosis of osteoblasts as illustrated in Figure 6. Importantly, genetic factors that determine bone mass also control osteoblast apoptosis as evidenced by the inverse relationship between bone formation rate and osteoblast apoptosis in high bone mass C3H/HeJ mice, compared to low bone mass C57BL/6 mice (Sheng *et al.*, 2006). Osteoblast apoptosis is highly regulated during fracture repair and during the closing of skeletal defects in rodent models (Landry *et al.*, 1997; Olmedo *et al.*, 2000; Li *et al.*, 2002). Interestingly, in mice with partial deficiency in HIF-1α the callus of experimentally induced femoral fractures is larger and osteoblast apoptosis is decreased (Komatsu *et al.*, 2007). Thus, regulation of osteoblast apoptosis during fracture healing appears to play a role in modulating the pace of bone regeneration.

Bone morphogenetic proteins (BMPs) not only play an important role in osteoblast differentiation, but also induce apoptosis of mesenchymal osteoblast progenitors in interdigital tissues during the development of hands and feet (Zou and Niswander, 1996). Interestingly, BMP-2 induces apoptosis of cultured osteoblastic cells in a manner that is independent of its pro-differentiating effect (Hay *et al.*, 2004). Several other factors including Fas ligand, IL-1, and tumor necrosis factor, on the other hand, also stimulate apoptosis of cultured osteoblastic cells (Jilka *et al.*, 1998; Tsuboi *et al.*, 1999); but at present there is no evidence that they are involved in the regulation of osteoblast apoptosis *in vivo*.

Wnt signaling has a profound effect on bone as exemplified by the high bone mass phenotype of mice and


PTH
Sex steroids
Wnts
BPs
Mechanical force
PTHR
Frizzled
LRP5/6
Unknown receptor
Frizzled
Cx43 Hemichannel
Integrins
ER
Src
Shc
Caveolin
Src
ER
Shc
Src
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cAMP
MEK
Dvl
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Transcription factors
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Survival genes Bcl-2
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Survival genes
Survival genes
pBAD
pC/EBPβ
Survival genes
Osteoblast and osteocyte survival

FIGURE 6 Antiapoptotic signaling pathways activated by hormones, cytokines, and mechanical forces in osteoblasts and osteocytes. Akt, thymoma viral proto-oncogene; BP, bisphosphonates; c/EBPβ, CCAAT/enhancer binding protein β; CREB, cAMP response element-binding; Dvl, disheveled; ER, estrogen receptor; Elk, ETS oncogene family; ERK, extracellular signal-regulated kinase; LRP5/6, low-density lipoprotein receptor-related protein 5 or LRP6; MEK, ERK kinase; PI3K, phosphoinositide-3 kinase; PKA, protein kinase A; PTH, parathyroid hormone; PTHR, PTH receptor; rsk, ribosomal S6 kinase; Runx2, runt related transcription factor 2; TCF, T cell factor.

humans with activating mutations of LRP5, which acts as a co-receptor with members of the Frizzled protein family of receptors for Wnt ligands (Bodine and Komm, 2006). Besides their well-established role in the proliferation and differentiation of osteoblast progenitors, Wnts are involved in the control of osteoblast and osteocyte apoptosis (see Fig. 6). Thus, the increased bone formation exhibited by mice lacking the Wnt antagonist secreted frizzled related protein-1 (SFRP-1) is associated with decreased osteoblast and osteocyte apoptosis (Bodine *et al.*, 2004). Likewise, the prevalence of osteoblast and osteocyte apoptosis is decreased in mice expressing the high bone mass activating mutation of LRP5 (G171V; Babij *et al.*, 2003). This LRP5 mutant exhibits reduced ability to bind sclerostin (Ellies *et al.*, 2006)—the Wnt antagonist specifically secreted by osteocytes (Bellido *et al.*, 2005). Consistent with this, *in vitro* studies have shown that sclerostin induces osteoblast apoptosis *in vitro* (Sutherland *et al.*, 2004). As will be discussed later, the ability of PTH and mechanical loading to reduce sclerostin synthesis could contribute to their ability to stimulate osteoblast differentiation and prolong osteoblast life span (Bellido *et al.*, 2005; Robling *et al.*, 2006).

Consistent with this *in vivo* evidence, direct activation of Wnt signaling *in vitro* prevents apoptosis of uncommitted C2C12 osteoblast progenitors and more differentiated MC3T3-E1 and OB-6 osteoblastic cell models (Almeida *et al.*, 2005). Thus, Wnt3a and Wnt1, which activate the so-called canonical pathway leading to β-catenin mediated transcription, as well as Wnt5a that does not activate β-catenin, were all able to prevent apoptosis. Remarkably, both classes of Wnts activate Src/ERK and PI3/AKT kinases, which are required for the antiapoptotic effect of these ligands. The mechanism by which Wnts activate these survival kinases is unknown. However, it might result from Wnt association with other receptors, such as Ryk—an atypical member of the receptor tyrosine kinase family that binds Wnts and can either form a complex with Frizzled to activate canonical Wnt signaling or transduce noncanonical Wnt signals via Frizzled-independent pathways that involve downstream activation of ERKs (Katso *et al.*, 1999).

Despite evidence that Wnt signaling suppresses osteoblast apoptosis, the reduced osteoblast number in mice lacking LRP5 is apparently not associated with increased osteoblast apoptosis (Kato *et al.*, 2002), although osteoblastic calvaria cells isolated from such mice do exhibit increased apoptosis (Clement-Lacroix *et al.*, 2005). Likewise, osteoblast apoptosis is not reduced when canonical Wnt signaling is increased by deletion of Axin2, an intracellular inhibitor of the canonical Wnt signaling pathway (Yu *et al.*, 2005). Moreover, reduction of β-catenin levels does not affect osteoblast numbers, suggesting a lack of an effect on osteoblast apoptosis (Holmen *et al.*, 2005; Glass *et al.*, 2005). These findings are consistent with *in vitro* evidence that expression of an active form of β-catenin alone does not prevent osteoblast apoptosis, and that β-catenin-mediated transcription was only permissive for the antiapoptotic effects of canonical Wnts but it was dispensable for the antiapoptotic action of the noncanonical Wnt5a (Almeida *et al.*, 2005). Thus, activation of Src/ERK and PI3K/Akt pathways (and potentially other signaling molecules) appears to be primarily responsible for the anti-apoptotic effects of Wnts on osteoblasts.

PTHrP, produced by early osteoblast progenitors (Walsh *et al.*, 1995) as well as osteocytes (Chen *et al.*, 2005b), is an important member of the locally produced cytokines and growth factors that regulate osteoblast apoptosis (Miao *et al.*, 2004, 2005). Specific deletion of PTHrP in cells of the osteoblast lineage caused a reduction in bone mass that was associated with increased osteoblast apoptosis (Miao *et al.*, 2005). Other factors include transforming growth factor-β (TGF-β), insulin-like growth factor-I (IGF-I), fibroblast growth factor-2 (FGF-2), and interleukin-6 (IL-6) type cytokines (Jilka *et al.*, 1998; Bellido *et al.*, 1998; Hurley *et al.*, 1999; Grey *et al.*, 2003). *In vivo* evidence that TGF-β provides tonic antiapoptotic signaling is provided by the decreased bone mass associated with increased osteoblast apoptosis in mice lacking Smad-3, which mediates TGFβ-signaling (Borton *et al.*, 2001). Additional evidence is provided by studies of mice lacking PTEN, a phosphatase that dephosphorylates PI(3,4,5)trisphosphate generated by PI3Kinase—a survival pathway activated by many growth factors including IGF-I. Mice with deletion of PTEN in osteoblasts exhibit high bone mass, and removal of PTEN from cultured osteoblasts attenuates their apoptosis, although the prevalence of osteoblast apoptosis in bone of PTEN- deficient mice was not determined (Liu *et al.*, 2007).

Interaction of cultured osteoblastic cells with the extracellular matrix generates antiapoptotic signals via integrins (Globus *et al.*, 1998; Verderio *et al.*, 2003). Moreover, mice expressing a matrix metalloproteinase (MMP)-resistant mutant of type I collagen exhibit increased osteoblast and osteocyte apoptosis. Thus, MMP-mediated release of growth factors from the extracellular matrix or exposure of cryptic integrin binding sites in the matrix must also contribute to antiapoptotic signaling in these cells (Zhao *et al.*, 2000).

Some of the pathways affecting osteoblast apoptosis discussed earlier are regulated by systemic hormones and pharmacotherapeutic agents as listed in Table 2 and discussed in Section VII.

OSTEOCYTE APOPTOSIS

The Osteocyte Network

Osteocytes are former osteoblasts entombed individually in lacunae of the mineralized matrix. They display multiple (50 on average) cytoplasmic dendritic processes that radiate from the cell body. These processes run along narrow canaliculi and are linked by gap junctions with processes of neighboring osteocytes, the lining cells, and osteoblasts at the bone surface, as well as cellular elements of the bone-marrow endothelial cells of the marrow vasculature (Marotti *et al.*, 1990). Osteocytes uniquely synthesize proteins such as sclerostin that are capable of modulating osteoblast and osteoclast differentiation. Thus, osteocytes are ideally suited for sensing and responding to mechanical strain and provide a means by which bone adjusts to load by changing in mass, shape, or microarchitecture, and a means for initiating repair of microdamage (Martin *et al.*, 1998). The crucial role of osteocytes in the adaptation of bone to reduced mechanical strain is supported by recent evidence that mice in which osteocytes have been ablated fail to lose bone in response to hindlimb unloading (Tatsumi *et al.*, 2007).

Whereas osteoblasts and osteoclasts are relatively short-lived and transiently present on only a small fraction of the bone surface, osteocytes are long-lived and present throughout the skeleton. Unlike osteoblasts, which begin to die by apoptosis almost as soon as they are born, most osteocytes remain alive until the bone in which they reside is replaced. Nonetheless, some osteocytes do die by apoptosis, because the prevalence of the phenomenon under physiologic conditions is 2% to 5% when visualized with ISEL (Aguirre *et al.*, 2006). Apoptotic osteocytes are deep brown colored with TUNEL or ISEL, are markedly pyknotic, and occupy an eccentric position with their lacunae (see Fig. 1K). The pyknosis is particularly noteworthy when stained by DNA-specific bisbenzimide dyes such as Hoechst 33258 (Weinstein *et al.*, 2000; see Fig. 1L). Osteocyte apoptosis is cumulative because osteocytes are isolated from macrophages, and it requires extensive degradation to small molecules to dispose of the cells through the narrow canaliculi. Indeed, degraded DNA can be detected in osteocyte lacunae of necrotic human bone long after the initial apoptotic insult (Weinstein *et al.*, 2000; see Fig. 1K).

Significance of Osteocyte Apoptosis and Its Regulation by Mechanical Strain

Physiological levels of load imposed on bone *in vivo* decrease the number of apoptotic osteocytes (Noble *et al.*, 2003). On the other hand, lack of mechanical stimulation induced by unloading of bone leads to an increased number of hypoxic osteocytes (Basso and Heersche, 2006) as well as apoptotic osteocytes (Aguirre *et al.*, 2006). The hypoxic effect is reversed by loading, suggesting that mechanical forces facilitate oxygen diffusion and thereby osteocyte survival (Dodd *et al.*, 1999). Osteocytes interact with the extracellular matrix (ECM) in the pericellular space through discrete sites in their membranes, which are enriched in integrins and vinculin (Gohel *et al.*, 1995; Aarden *et al.*, 1996), as well as through transverse elements that tether osteocytes to the canalicular wall, as elegantly demonstrated by Schaffler and co-workers (You *et al.*, 2004). Fluid movement in the canaliculi resulting from mechanical loading might induce ECM deformation, shear stress, and/or tension in the tethering elements. The resulting changes in circumferential strain in osteocyte membranes might be converted into intracellular signals by integrin clustering and integrin interaction with cytoskeletal and catalytic proteins at focal adhesions (Giancotti, 1997). Indeed, physiological levels of mechanical strain imparted by stretching or pulsatile fluid flow prevent apoptosis of cultured osteocytes (Bakker *et al.*, 2004; Plotkin *et al.*, 2005a).

Mechanical forces are transduced into intracellular signals through integrins and a signalsome comprising actin filaments, microtubules, FAK, and Src kinases, resulting in activation of the ERK pathway and attenuation of osteocyte apoptosis (Plotkin *et al.*, 2005a; see Fig. 6). Intriguingly, a ligand-independent function of the estrogen receptor (ER) is indispensable for mechanically induced ERK activation in both osteoblastic and osteocytic cells (Aguirre *et al.*, 2007). This observation is consistent with reports that mice lacking the ERα and ERβ exhibit a poor osteogenic response to loading (Lee *et al.*, 2004a).

The disruption of the integrity of the osteocyte network that results from apoptosis serves as a signaling mechanism for bone's ability to self-repair and perhaps also to adapt to mechanical strains in a spatially defined pattern. Thus, in ulnae of adult rats, osteocyte apoptosis induced by bone fatigue is localized to regions of bone that contain microcracks and occurs in proximity to the subsequent osteoclastic resorption (Verborgt *et al.*, 2000, 2002). Likewise, osteocyte apoptosis induced by osteotomy in chicken radii temporally precedes an increase in osteoclasts (Clark *et al.*, 2005). Moreover, within 3 days of tail suspension, mice exhibit an increased incidence of osteocyte apoptosis in both trabecular and cortical bone (Aguirre *et al.*, 2006). This change is followed 2 weeks later by increased osteoclast numbers and cortical porosity, reduced trabecular and cortical width, and decreased spinal bone mineral density and vertebral strength. Importantly, whereas in ambulatory animals apoptotic osteocytes are randomly distributed, in unloaded mice apoptotic osteocytes are preferentially sequestered in endosteal cortical bone, the site that was subsequently resorbed. Furthermore, in rabbit tibia midshaft, osteocyte apoptosis and density exhibit a quantitative regional and linear association with remodeling (Hedgecock *et al.*, 2007). The conclusion from all these lines of evidence is that diminished mechanical forces eliminate signals that maintain osteocyte viability, thereby leading to apoptosis. Dying osteocytes in turn become the beacons for osteoclast recruitment to the vicinity and the resulting increase in bone resorption and bone loss (Manolagas, 2006). This contention is supported by the recent evidence that inducible and specific killing of osteocytes is sufficient to initiate bone resorption in mice (Tatsumi *et al.*, 2007).

The nature of the osteoclastogenic signals generated by the apoptotic osteocytes are, at this stage, only a matter of conjecture. Nonetheless, the oxygen deprivation that occurs with disuse enhances the expression of HIF-1α in osteocytes (Gross *et al.*, 2001). This transcription factor is a potent inducer of the angiogenic and osteoclastogenic factor VEGF. It is unknown whether increased HIF-1α also protects some anoxic osteocytes from apoptosis as it does in chondrocytes of the growth plate, or whether it promotes apoptosis in osteocytes as it does in other cell types by stabilizing p53 (Greijer and van der Wall, 2004).

Other mechanism(s) by which reduced mechanical forces trigger osteocyte apoptosis have been proposed. A deficit in nitric oxide (NO) production has been suggested as a potential culprit (Burger *et al.*, 2003). Consistent with this hypothesis, mechanical stimulation increases the production of NO by osteocytes (Klein-Nulend *et al.*, 1995; Pitsillides *et al.*, 1995; Zaman *et al.*, 1999). Mechanical stimulation of chicken and canine bone also increases the production of prostaglandin E_2 (Rawlinson *et al.*, 1991; Ajubi *et al.*, 1996), an agent known to inhibit bone cell apoptosis (Machwate *et al.*, 2001), raising the possibility that prostaglandins and perhaps other autocrine/paracrine soluble factors are also involved in the maintenance of osteocyte viability.

Similar to osteoblasts, signals for osteocyte survival may also be provided by the ECM itself as indicated by the increase in the prevalence of osteocyte apoptosis in transgenic mice expressing collagenase-resistant collagen type-I (Zhao *et al.*, 2000). It is thought that exposure of cryptic sites of ECM proteins by MMPs is required for the maintenance of cell–ECM interactions that result in "outside-in" integrin signaling that preserves osteocyte (and osteoblast) viability. This scenario is consistent with the observation that physiological levels of mechanical strain promote survival of osteocytic cells via a mechanism mediated by integrins (Plotkin *et al.*, 2005a). Besides mechanical forces, systemic hormones and pharmacotherapeutic agents affect osteocyte apoptosis in a manner analogous to osteoblasts (see Table 2) using similar pathways (see Fig. 6), as discussed in Section VII.

Several clinical conditions, ranging from complete motor paralysis to temporary immobilization such as bed rest, cause different degrees of bone loss, named disuse osteopenia (Bikle *et al.*, 1997, 2003). Bone loss is also a consequence of spaceflights and represents a significant hindrance for long-term space flying (Bikle *et al.*, 1997). The evidence that mechanical forces regulate the life span of osteocytes raises the possibility that preservation of the function of the osteocyte network by maintaining osteocyte viability represents a powerful means for the prevention of the dramatic bone loss that ensues with weightlessness.

IMPLICATIONS FOR THE PATHOGENESIS AND TREATMENT OF BONE DISEASE

The pathophysiology of osteoporosis is due to excessive osteoclasts relative to the need for bone remodeling, too few osteoblasts relative to the need created by bone resorption, or a combination of both conditions (Manolagas, 2000). In addition to derangements in the genesis of bone cells, conditions leading to loss of bone mass and strength are associated with prolonged osteoclast life span and shortened osteoblast and osteocyte life span (summarized in Table 2). Not surprisingly, chronic diseases of the joint are also associated with dysregulated apoptosis.

Degenerative and Inflammatory Joint Disease

Osteoarthritis is characterized by progressive loss of articular cartilage leading to inflammation, subchondral bone formation, and the development of osteophytes at the joint margins. A prominent histologic feature of the cartilage in osteoarthritis is a decrease in the number of chondrocytes, which are normally responsible for the repair and regeneration of articular cartilage (Hashimoto *et al.*, 1998; Kim and Blanco, 2007). With increasing severity of osteoarthritis, there is continued loss of chondrocytes associated with increased numbers of apoptotic chondrocytes. Although it is unclear whether apoptosis represents the event that initiates the development of osteoarthritis, available evidence supports the notion that chondrocyte death and matrix loss are part of a vicious cycle leading to disease progression (Kim and Blanco, 2007).

In vitro studies have shown that chondrocyte apoptosis is stimulated by factors known to be present in osteoarthritic lesions including nitric oxide, ROS, TNF-α, Fas ligand, and TRAIL (Lotz *et al.*, 1999; Lee *et al.*, 2004b). Loss of matrix attachment, increased mechanical force, and increased levels of pyrophosphate also stimulate chondrocyte apoptosis (Johnson *et al.*, 2001; Islam *et al.*, 2002). That apoptosis plays a fundamental role in the development of the disease is indicated by the ability of caspase inhibitors to reduce both chondrocyte apoptosis and the severity of osteoarthritic changes seen in appropriate animal models (D'Lima *et al.*, 2006).

Rheumatoid arthritis is characterized by joint destruction due to accumulation of synovial fibroblasts and macrophages that produce proinflammatory cytokines and matrix degradative enzymes, as well as lymphocytes that invade the area to produce additional cytokines. Interestingly, it has been proposed that the synovial fibroblast hyperplasia is the result of insufficient apoptosis (Liu and Pope, 2003). Indeed, induction of apoptosis of these cells with Fas ligand (Yao *et al.*, 2000) or TRAIL (Terzioglu *et al.*, 2007) reduced inflammation and associated joint destruction. Chondrocyte viability was unaffected, presumably due to the lack of vascularization.

RANKL is produced by synovial cells and T cells in the arthritic joint. It has recently been shown that this cytokine plays a important role in the development and recruitment of osteoclasts that resorb the adjacent bone surfaces, resulting in destruction and weakening of the joint (Takayanagi, 2005). TNF-α is also increased and may prolong osteoclast life span and stimulate osteoblast apoptosis in rheumatic joints either directly, or indirectly by stimulating the synthesis of the Wnt antagonist Dkk1 (Diarra *et al.*, 2007).

Osteoporosis Associated with Sex Steroid Deficiency

Sex steroids preserve bone mass by attenuating bone turnover and maintaining a focal balance between the rate of bone formation and resorption. Attenuation of bone turnover is the result of suppressive effects of sex steroids on the generation of both osteoblast and osteoclast progenitor cells. Maintenance of a focal balance between bone resorption and formation is due to an antiapoptotic action of estrogens and androgens on osteoblasts/osteocytes and a proapoptotic effect on osteoclasts (Manolagas, 2000). Estrogen deficiency also increases the prevalence of osteocyte apoptosis in humans (Tomkinson *et al.*, 1997), rats (Tomkinson *et al.*, 1998), and mice (Kousteni *et al.*, 2001). Increased osteocyte apoptosis and the resulting disruption of the osteocyte network may contribute to bone fragility that follows estrogen deficiency.

Sex steroids act directly on osteoblasts to induce survival signals via a kinase-mediated action of the ER, independent of the DNA binding actions of the receptor (Kousteni *et al.*, 2001; see Fig. 6). Extensive *in vitro* studies have shown that the antiapoptotic effects of 17β-estradiol (E_2) on osteoblastic and osteocytic cells are mediated by transient activation of the Src/Shc/ERKs, as well as modulation of the PI3K and JNK signaling pathways. Moreover, activation of cytoplasmic kinases by estrogens in osteoblast cell culture and murine bone promotes the activation of key transcription factors downstream of ERKs such as Elk-1, CCAAT enhancer binding protein-b (C/EBPβ), cyclic adenosine monophosphate-response element binding protein (CREB), and the suppression of c-Jun (Kousteni *et al.*, 2003). And, activation of these transcription factors is required for the antiapoptotic effects of estrogens. The evidence that direct receptor–DNA interaction is dispensable for the regulation of osteoblast apoptosis by E_2 is further supported by evidence that mice lacking the DNA-binding function of the ERα (NERKI) show increased osteoblast apoptosis following ovariectomy, whereas replacement with E_2 prevents this effect (M. Almeida, unpublished observations).

In contrast to the antiapoptotic effects of estrogens and androgens on osteoblasts and osteocytes, these hormones exert proapoptotic effects on mature osteoclasts and their precursors. This latter effect is associated with reduced expression of IL-1R1 mRNA and increased IL-1 decoy receptor expression (Sunyer *et al.*, 1999). In murine bone marrow cocultures, the proapoptotic effect of estrogen is mediated by TGF-β produced by osteoblastic cells (Hughes *et al.*, 1996). The proapoptotic actions of E_2 on osteoclasts may also involve E_2-induced downregulation of cytokines such as M-CSF, TNF-α, and RANKL that promote osteoclast survival (Eghbali-Fatourechi *et al.*, 2003; Syed and Khosla, 2005), and increased synthesis of OPG that blocks the actions of RANKL (Hofbauer *et al.*, 1999).

E_2 has direct proapoptotic effects on osteoclasts mediated by ERKs (Chen *et al.*, 2005a). E_2-induced ERK phosphorylation in osteoclasts was sustained for at least 24 hours following exposure to the hormone, in contrast to its transient effect on ERK phosphorylation in osteoblast and osteocytic cells. Interestingly, the proapoptotic effect of E_2 on osteoclasts was abrogated by conversion of sustained ERK phosphorylation to transient (Chen *et al.*, 2005a). Moreover, 4-estren-3α,17β-diol (estren), a synthetic ligand of the ER or androgen receptor that reproduces the nongenotropic effects of classical sex steroids with minimal effects on classical transcription, was as potent as E_2 or 5α-dihydrotestosterone (DHT) not only in protecting osteoblasts/osteocytes from apoptosis but also in promoting osteoclast apoptosis (Kousteni *et al.*, 2002). Conversely, 1,2,5-tris(4-hydroxylphenyl)-4-proylpyrazole, an ER ligand with potent classical transcriptional activity but minimal effects on ERK or JNK kinases, did not affect osteoclast (or osteoblast) apoptosis. Moreover, osteoclasts generated from the bone marrow of mice with ERα lacking the ability to bind to DNA ($\mathrm{ER}\alpha^{\mathrm{NERKI}/-}$) also underwent apoptosis upon E_2 treatment (Kousteni *et al.*, 2005). Taken together, the foregoing evidence indicates that the proapoptotic effect of sex steroids on osteoclasts, similar to their antiapoptotic effect on osteoblasts and osteocytes, results from a kinase-mediated mechanism of receptor action.

Antioxidant actions of sex steroids are critical for their effects on the life span of osteoclasts and osteoblasts. Loss of sex steroids promotes an increase in oxidative stress in bone that is rescued by treatment with E_2, DHT, or the antioxidant NAC. Importantly, NAC was as effective as E_2 or DHT in preventing the decrease in bone mineral density (BMD; Lean *et al.*, 2003), as well as the increase in osteoblast and osteocyte apoptosis caused by gonadectomy (Almeida *et al.*, 2007). The proapoptotic effect of both sex steroids on osteoclasts is associated with increased levels of glutathione reductase (GSR), consistent with evidence that osteoclasts utilize reactive oxygen species for survival signaling (Almeida *et al.*, 2007). GSR defends against oxidative damage by acting in concert with glutathione peroxidase and glutathione to convert peroxides into harmless alcohols (Dickinson and Forman, 2002). Thus, drugs such as buthionine sulfoximine (BSO) or diethylmaleate (DEM) that deplete the cells of glutathione abrogate E_2- or DHT-induced osteoclast apoptosis *in vitro* (Almeida *et al.*, 2007). Further, the effects of E_2 or DHT on GSR levels in osteoclasts were abrogated by specific inhibitors of Src and MEK kinases. BSO or DEM also abrogated the antiapoptotic effects of E_2 or DHT on osteoblastic OB-6 cells and calvaria cells.

The mechanism by which sex steroids modulate the redox status of osteoblasts is unclear; however, it seems that increased GSR synthesis is not involved (M. Almeida *et al.*, unpublished observation). Instead, E_2 or DHT appears to suppress H_2O_2-induced $\mathrm{p66}^{\mathrm{shc}}$ phosphorylation (required for transduction of oxidant stress signals into apoptosis) via activation of the Src/ERK signaling (Almeida *et al.*, 2007). Thus, stimulation of osteoclast and attenuation of osteoblast apoptosis by E_2 involve nonprotein thiol metabolism and are the result of actions mediated via cytoplasmic kinases and probably downstream transcriptional control (Kousteni *et al.*, 2003).

Glucocorticoid-Induced Osteoporosis

Growth retardation due to glucocorticoid excess is characterized by increased apoptosis of both proliferating and terminal hypertrophic chondrocytes resulting in decreased width of the growth plate (Chrysis *et al.*, 2003). The increase in chondrocyte apoptosis is accompanied by increased immunoreactivity for caspase-3 and decreased immunoreactivity for the antiapoptosis proteins, Bcl-2 and Bcl-X, as well as decreased immunoreactivity for PTHrP.

Glucocorticoid excess also increases the prevalence of osteoblast and osteocyte apoptosis in both adult murine vertebrae and human iliac bone (Weinstein *et al.*, 1998; Gohel *et al.*, 1999; see Figs. 1I–L). This may explain, at least in part, the decrease in osteoblast number and bone formation rate caused by glucocorticoid excess. Glucocorticoid-induced osteoporosis in humans is often complicated by the *in situ* death of portions of bone, associated with abundant apoptotic osteocytes juxtaposed to the subchondral fracture crescent—a ribbon-like zone of collapsed trabeculae (Weinstein *et al.*, 2000). Osteocytes with condensed chromatin have been observed between the tetracycline labeling that demarcates sites of bone formation, indicating that these cells died immediately after entombment (see Fig. 1L). In this situation, osteocyte apoptosis represents a cumulative and unrepairable defect that would disrupt the mechanosensory osteocyte–canalicular network and prevent repair of microfractures. This situation would promote collapse of the femoral head and explain the correlation between total steroid dose and the incidence of avascular necrosis of bone (Felson and Anderson, 1987), as well as the occurrence of osteonecrosis after cessation of steroid therapy.

The proapoptotic effect of glucocorticoids on osteoblasts and osteocytes is direct as overexpression of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), an enzyme that inactivates glucocorticoids, specifically in osteoblasts and osteocytes prevents the steroid-induced increase in apoptosis (O'Brien *et al.*, 2004). Moreover, osteoblast number, osteoid area, and bone formation rate are significantly higher in glucocorticoid-treated 11β-HSD2 transgenic mice compared to glucocorticoid-treated wild-type controls. Overexpression of 11β-HSD2 also preserves osteocyte viability and bone strength despite loss of bone mass (O'Brien *et al.*, 2004), suggesting that osteocytes contribute to bone strength independently of bone mass. Although the mechanism(s) involved have not been established, disruption of the lacunocanalicular system that occurs with osteocyte apoptosis can lead to changes in bone material properties (Weinstein *et al.*, 2006).

Glucocorticoid-stimulated apoptosis of cultured osteoblastic and osteocytic cells is strictly dependent on the glucocorticoid receptor (GR; Gohel *et al.*, 1999; Plotkin *et al.*, 2007b), and osteoblast-specific deletion of the GR prevents glucocorticoid-induced osteoblast apoptosis (Tuckermann *et al.*, 2005). Strikingly, in an osteocytic cell model (MLO-Y4) the proapoptotic effect of glucocorticoids is preceded by cell detachment due to interference with FAK-mediated survival signaling generated by integrins. In this mechanism, Pyk2 (a member of the FAK family) becomes phosphorylated and subsequently activates proapoptotic JNK signaling (Plotkin *et al.*, 2007b). In addition, the proapoptotic actions of glucocorticoids may involve suppression of the synthesis of locally produced antiapoptotic factors including IGF-I and IL-6 type cytokines, as well as MMPs (Canalis and Delany, 2002), and stimulation of the proapoptotic Wnt antagonist SFRP-1 (Wang *et al.*, 2005).

As in the case of sex steroids, the effects of glucocorticoids on osteoclast life span are the opposite of that on osteoblasts (see Table 2). However, the rat appears to be an exception as there is a glucocorticoid-induced increase in cancellous bone mass and decrease in osteoclasts (King *et al.*, 1996), possibly due to increased apoptosis (Dempster *et al.*, 1997). In mice receiving excess glucocorticoids, osteoclast progenitors are reduced, but cancellous osteoclast number does not decrease, presumably because of the ability of glucocorticoids to prolong osteoclast life span (Jia *et al.*, 2006). This effect may account for the early transient increase in bone resorption in patients with exogenous or endogenous hyperglucocorticoidism. The prosurvival effect of glucocorticoids on osteoclasts is powerful enough to antagonize the proapoptotic effect of BPs (Weinstein *et al.*, 2002).

The antiapoptotic actions of glucocorticoids on osteoclasts are also direct, because mice expressing 11β-HSD2 specifically in osteoclasts exhibit a reduction in osteoclast number following administration of glucocorticoids, consistent with suppressive effects of the steroid on progenitors and prevention of steroid-induced survival signaling in osteoclasts (Jia *et al.*, 2006). In accord with this decrease in osteoclast number, the loss of bone density observed in wild-type mice was strikingly prevented in these transgenic mice. Nevertheless, the expected glucocorticoid-induced decrease in osteoblast number and bone formation and increasein the prevalence of osteoblast apoptosis were still observed.

Age-Related Involutional Osteoporosis

Age-related loss of bone mass and strength is an invariable feature of human biology. Recent population-based longitudinal studies demonstrate that substantial trabecular bone loss begins as early as the 20s in young adult women and men, long before any hormonal changes (Riggs *et al.*, 2008). In bone of aging dogs, there is an accumulation of microdamage (Frank *et al.*, 2002), and in humans there is a decline in osteocyte density (Vashishth *et al.*, 2000; Qiu *et al.*, 2002) accompanied by decreased prevalence of osteocyte-occupied lacunae (Qiu *et al.*, 2003), an index of premature osteocyte death. Furthermore, osteocyte death is associated with hip fractures in aging humans (Dunstan *et al.*, 1990). In view of the evidence discussed earlier for the role of osteocytes in microfracture repair, age-related loss of osteocytes due to apoptosis could be partially responsible for the disparity between bone quantity and quality that occurs with aging (Hui *et al.*, 1988).

Both female and male C57BL/6 mice lose bone strength and mass progressively between the ages of 4 and 31 months. These changes are temporally associated with a decreased rate of remodeling as evidenced by decreased osteoblast and osteoclast numbers and decreased bone formation rate, as well as decreased wall width and increased osteoblast and osteocyte apoptosis (Almeida *et al.*, 2007). Increased levels of ROS may contribute to this increase in apoptosis as evidenced by a temporal linkage with increased ROS and decreased activity of GSR in the bone marrow, and a corresponding increase in the phosphorylation of p53 and $p66^{Shc}$. In addition, aged mice exhibit a twofold increase in serum corticosterone, adrenal weight, and bone mRNA for 11β-HSD1, the enzyme that amplifies glucocorticoid action (Weinstein *et al.*, unpublished data). Thus, local amplification of endogenous glucocorticoids may also contribute to increased osteoblast and osteocyte apoptosis.

Increased production of PPARγ-activating oxidized lipids could also contribute in view of evidence that this transcription factor exerts tonic suppression of bone formation (Akune *et al.*, 2004; Klein *et al.*, 2004) and that administration of the PPARγ ligand rosiglitazone to mice decreases bone formation and increases the number of apoptotic osteoblasts (Soroceanu *et al.*, 2004). Finally, the decline in physical activity with old age could stimulate apoptosis of osteoblasts and osteocytes because of reduced skeletal loading.

Bone Anabolism by Intermittent PTH

Daily injections of parathyroid hormone (PTH) increase bone mass and reduce the incidence of fracture in postmenopausal women, in elderly men, and in women with glucocorticoid-induced osteoporosis (Hodsman *et al.*, 2005). In humans receiving daily injections of PTH for 28 days (Lindsay *et al.*, 2006) or for 12 to 24 months (Ma *et al.*, 2006), most of the increase in bone formation occurred within preexisting BMUs. New bone formation also took place on surfaces adjacent to the BMU, perhaps due to spillage of osteoblasts outside the boundary of the bone surface being remodeled. Thus, PTH overrides the mechanisms that normally limit the number of osteoblasts in the BMU to that needed to replace the bone previously excavated by osteoclasts. The increase in osteoblast number appears to be due to both increased osteoblast differentiation and decreased osteoblast apoptosis (Jilka, 2007). In view of the prodifferentiating and prosurvival effects of locally produced PTHrP discussed earlier (Miao *et al.*, 2005), it is possible that daily injections of PTH simply add to existing osteogenic signals arising from PTHrP-induced activation of PTHR1.

Studies in mice have shown that increased survival is a major contributor to the increase in osteoblast number caused by intermittent PTH. Daily administration of 3 to 300 ng/g/d of PTH for 28 days to adult mice caused a dose-dependent increase in the BMD of the spine and hindlimb that was associated with a reduction in osteoblast apoptosis at both skeletal sites (Bellido *et al.*, 2003). Moreover, the same doses of PTH that inhibited osteoblast apoptosis also increased osteoblast number, bone formation rate, and the amount of cancellous bone. The prevalence of osteoblast apoptosis in this experiment was inversely correlated with circulating osteocalcin, bone formation rate, and osteoblast number. A decline in the number of apoptotic osteoblasts was detected after only two injections. After four injections, there was a 50% reduction in the prevalence of apoptotic osteoblasts, and osteoblast number increased two-fold.

In contrast, a 50% increase in apoptotic osteoblasts was observed in the primary spongiosa of the distal femur of 1-month-old rats after 7 and 21 days, but not after 28 days, of PTH injections (Stanislaus *et al.*, 2000). Nevertheless, these investigators also reported that the activity of caspases 2, 3, and 7 was dramatically decreased in extracts of the femoral metaphyseal bone, which included both primary and secondary spongiosa. This result is consistent with an antiapoptotic effect of daily hormone injections on osteoblasts in the secondary spongiosa, and perhaps on the preosteoblasts that are prevalent in the metaphysis during growth (Onyia *et al.*, 1997).

In a recent study of transiliac biopsies from postmenopausal women receiving daily injections of PTH for 28 days, Lindsay *et al.* (2007) found an increase in osteoblast apoptosis in cancellous bone, as well as a positive correlation between osteoblast apoptosis and bone formation rate. It seems highly unlikely that a species difference can account for this incongruent result. The absolute number of apoptotic osteoblasts could increase with the stimulation of osteoblastogenesis in response to intermittent PTH as Lindsay *et al.* suggest, yet the prevalence of apoptosis could well be unaffected or reduced when expressed per number of osteoblasts. However, osteoblast apoptosis expressed per millimeter of osteoblast surface, as was done in the study of Lindsay *et al.*, does not permit estimation of prevalence because it ignores the variation in osteoblast size that occurs during their transition from plump cuboidal, at the beginning of the refilling of a resorption cavity, to bricklike rectangular, near the end of the process (Parfitt, 1990). Other methodologic issues could also be responsible for the discrepancy (Jilka *et al.*, 2007). Indeed, as discussed earlier, apoptotic osteoblasts in humans are quite rare. Thus, it may be necessary to examine the role of apoptosis in the anabolic effect of intermittent PTH in humans under conditions in which the baseline prevalence of the phenomenon is substantially increased (e.g., glucocorticoid excess) to facilitate detection of the effects of pharmacologic manipulation.

PTH and PTHrP inhibit apoptosis in cultures of rat, murine and human osteoblastic cells (Jilka *et al.*, 1998; Bellido *et al.*, 2003; Chen *et al.*, 2002) via cAMP-activated protein kinase A and inactivation of the proapoptotic protein Bad, as well as increased transcription of survival genes such as Bcl-2 (Bellido *et al.*, 2003; see Fig. 6). The increased synthesis of survival genes requires cAMP response element-binding protein and Runx2. Besides PTH and PTHrP, other hormones that stimulate cAMP production in osteoblastic cells, including calcitonin and prostaglandin E, inhibit apoptosis of osteoblastic cells (Plotkin *et al.*, 1999; Machwate *et al.*, 2001).

In cultured osteoblastic cells, the antiapoptotic effect of PTH lasts only about 6 hours because of PTH-induced activation of proteasomal degradation of Runx2 (Bellido *et al.*, 2003) and termination of PKA-mediated gene expression downstream of receptor desensitization by protein kinase inhibitor γ (Chen *et al.*, 2007). Consistent with short-lived PTH-induced survival signaling, a reduction in osteoblast apoptosis is not observed after 6 days of continuous elevation of PTH in mice by infusion, or after

2 days of hormone elevation caused by feeding a calcium-deficient diet (Bellido *et al.*, 2003). Hence, the decrease in apoptotic osteoblasts seen in mice receiving intermittent PTH appears to be due to short bursts of survival signaling resulting in repeated delays of apoptosis.

Locally produced factors may also mediate the effects of intermittent PTH on osteoblast apoptosis. Experiments with genetically modified mice have shown that the anabolic effect of intermittent PTH depends upon growth factors with anti-apoptotic properties, such as IGF-I and FGF-2, that are known to be increased by intermittent PTH administration (Miyakoshi *et al.*, 2001; Bikle *et al.*, 2002; Yamaguchi *et al.*, 2005; Hurley *et al.*, 2006). Moreover, PTH decreases the expression of Wnt antagonists SFRP-2 and sclerostin, suggesting that intermittent PTH increases Wnt signaling, which not only stimulates differentiation but also inhibits apoptosis as discussed earlier. Consistent with this notion, daily PTH injections increased vertebral cancellous bone in male wild-type mice, but not in male LRP5-null mice (Kharode *et al.*, 2006); nevertheless, the anabolic response to PTH in cortical bone is not affected by LRP5 deficiency (Kharode *et al.*, 2006; Sawakami *et al.*, 2006; Iwaniec *et al.*, 2007).

The Therapeutic Efficacy of Bisphosphonates

Bisphosphonates greatly attenuate the bone loss induced by glucocorticoid excess, sex steroid deficiency, and immobilization, and they are also used for the treatment of multiple myeloma and Paget's disease, as well as the management of the skeletal complications of cancer (Lipton, 2005). Decreased osteoclast progenitor development, decreased osteoclast recruitment, impairment of osteoclast function, and promotion of apoptosis of mature osteoclasts (see Figs. 1C–H) each contribute to the antiresorptive actions of BPs (Fleisch, 1998).

Nonamino- and aminobisphosphonates both lead to increased osteoclast apoptosis, although by distinct molecular mechanisms (Rogers, 2004). The nonamino derivatives, such as etidronate, clodronate and tiludronate, are metabolized to nonhydrolyzable ATP-like molecules. Intracellular accumulation of these metabolites inhibits the ATP-dependent enzyme adenine nucleotide translocase, a component of the mitochondrial permeability transition pore, leading to increased permeability of the outer mitochondrial membrane, caspase 3 activation, and osteoclast apoptosis (Rogers, 2004). This proapoptotic effect of non-aminobisphosphonates appears to be the primary mechanism for inhibition of resorption, because inhibition of caspases abolishes the antiresorptive effect of clodronate and etidronate *in vitro* (Halasy-Nagy *et al.*, 2001).

On the other hand, aminobisphosphonates, such as alendronate, risedronate, and zoledronate, suppress bone resorption and induce osteoclast apoptosis by inhibiting farnesyl diphosphate synthase (FPPS), an enzyme of the mevalonate metabolic pathway crucial for posttranslational prenylation and membrane targeting of proteins (Rogers, 2004). Accumulation of unprenylated molecules, in particular small GTPases that control cytoskeletal reorganization and vesicular trafficking, is an important cause of the decreased resorption, because bypassing aminobisphosphonate-dependent inhibition of FPPS restores resorption (Halasy-Nagy *et al.*, 2001). Loss of actin rings as well as defective ruffled border formation, trafficking of lysosomal enzymes and transcytosis of resorption products induced by aminobisphosphonates is eventually followed by osteoclast apoptosis. However, apoptosis may not be the mechanism responsible for the anti-resorptive activity of aminobisphosphonates. Indeed, unlike the case with non-amino- bisphosphonates, the anti-resorptive action of alendronate and risedronate is unaffected by caspase inhibitors (Halasy-Nagy *et al.*, 2001). This may explain observations from human studies that the reduction of bone resorption by aminobisphosphonates occurs in the face of unchanged osteoclast number (Chavassieux *et al.*, 1997, 2000).

Long-term treatment of human and nonhuman primates with BPs increases wall thickness, an index of focally increased osteoblast numbers or activity, resulting in more complete refilling of resorption cavities (Storm *et al.*, 1993; Balena *et al.*, 1993; Chavassieux *et al.*, 1997). This evidence raises the possibility that BPs do more than simply reduce remodeling space and that they also have a positive effect on this particular aspect of bone formation. Albeit, overall bone formation declines, as measured by serum markers, because of the decreased rate of remodeling. Moreover, the antifracture efficacy of BPs is disproportionate to their effect on bone mass (Cummings *et al.*, 2002), suggesting an additional effect on bone strength unrelated to effects on bone resorption or bone formation. In addition, the initial increase in bone resorption characteristic of glucocorticoid-induced bone loss is not prevented by BPs (Weinstein *et al.*, 2002). This evidence indicates that the actions of BPs on osteoclasts do not explain all aspects of their bone protective effects.

A series of studies has revealed that BPs prevent osteoblast and osteocyte apoptosis *in vitro* and *in vivo* (Plotkin *et al.*, 1999; Kogianni *et al.*, 2004; Follet *et al.*, 2007), providing a potential explanation for the increased wall thickness associated with BP therapy. Prevention of apoptosis of osteocytes and osteoblasts by BPs is exerted via activation of ERKs via a novel paradigm of signal transduction (Plotkin *et al.*, 1999; see Fig. 6). Specifically, hexameric connexin-43 (Cx43) hemichannels are essential transducers of the ERK-activating/antiapoptotic effects of BPs; however, the gap junctions formed by two Cx43 hemichannels are not. The signal-transduction property of Cx43 requires not only the pore-forming domain of Cx43, but also the C-terminal portion of the protein, which is physically associated with the kinase Src. Consistent with this, Src expression and activity, as well as its interaction with Cx43, are indispensable for the antiapoptotic effects of BPs on osteoblasts and osteocytes. Unlike most ERK-activating stimuli that require nuclear functions of ERKs leading to gene transcription, BPs promote survival through ERK functions that are restricted to the cytoplasm. Such extranuclear functions require the activity of the kinase Rsk, the subsequent inactivation of the proapoptotic protein Bad, and the creation of a functional caspase inhibitory domain in C/EBPβ independent of its transcriptional effects (Plotkin *et al.*, 2005b). This evidence adds Cx43 to the list of transmembrane proteins capable of transducing survival signals in response to extracellular cues and raises the possibility that Cx43 may serve in this capacity for endogenously produced molecules or even other drugs.

Prevention of osteocyte apoptosis by BPs represents yet another potential mechanism for the inhibition of bone remodeling by these agents, beyond the well-established direct inhibition of osteoclast development and function. As discussed earlier, osteocyte apoptosis may initiate a targeted remodeling response. Therefore, prevention of osteocyte apoptosis by BPs might inhibit remodeling indirectly by decreasing osteoclast recruitment.

SUMMARY

Apoptosis of chondrocytes, osteoclasts, osteoblasts, and osteocytes plays a critical role in the development of the skeleton and in the maintenance of bone mass and strength throughout life. Dysregulation of bone cell apoptosis contributes to joint disease, as well as to the development of osteoporosis that occurs with sex steroid deficiency, glucocorticoid excess, and aging. Furthermore, apoptosis may be an important therapeutic target because the beneficial effects of intermittent PTH, as well as BPs, are associated with a reduction in osteoblast and osteocyte apoptosis. Future innovations that facilitate detection of this important event in human bone, as well as a deeper understanding of the factors and signaling pathways that control bone cell survival, should provide a better appreciation of the role of apoptosis in bone physiology, pathophysiology, and treatment. And, they may lead to new and improved therapeutic strategies. This potential is highlighted by recent work from our group showing that aminobisphosphonates that lack antiresorptive/antiremodeling activity, but retain the antiapoptotic property of BPs on osteoblast and osteocytes, preserve bone strength (Plotkin *et al.*, 2007a).

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Chapter 14

Skeletal Gene Expression in Nuclear Microenvironments

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INTRODUCTION

Skeletal development and bone remodeling require stringent control of gene activation and suppression in response to physiological cues. The fidelity of skeletal gene expression necessitates integrating a broad spectrum of regulatory signals that govern the commitment of osteoprogenitor stem cells to the bone cell lineage and proliferation and differentiation of osteoblasts, as well as maintenance of the bone phenotype in osteocytes residing in a mineralized bone extracellular matrix. To accommodate the requirements for short-term developmental and sustained phenotypic expression of cell growth and bone-related genes, it is necessary to identify and functionally characterize the promoter regulatory elements as well as cognate protein–DNA and protein–protein interactions that determine the extent to which genes are transcribed. However, it is becoming increasingly evident that the catalogue of regulatory elements and proteins is insufficient to support transcriptional control in the nucleus of intact cells and tissues. Rather, gene regulatory mechanisms must be understood within the context of the subnuclear organization of nucleic acids and regulatory proteins.

There is growing appreciation that transcriptional control, as it is operative *in vivo*, requires multiple levels of nuclear organization. It is essential to package 2.5 yards of DNA as chromatin within the limited confines of the nucleus. Gene promoter elements must be rendered competent for protein/DNA and protein/protein interactions in a manner that permits binding and functional activities of primary transcription factors as well as coactivators and co-repressors. Less understood but pivotally relevant to physiological control is the localization of the regulatory machinery for gene expression, replication, and repair at subnuclear sites where the macromolecular complexes that support DNA and RNA synthesis are localized.

This chapter focuses on contributions by several indices of nuclear architecture to the control of gene expression in bone cells. We present cellular, biochemical, molecular, genetic, and epigenetic evidence for linkages of developmental and tissue-specific gene expression with the organization of transcriptional regulatory machinery in subnuclear compartments. Using skeletal genes as a paradigm, this chapter addresses mechanisms that functionally organize the regulatory machinery for transcriptional

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activation and suppression as well as cell fate and lineage commitment during skeletal development and remodeling. It also provides evidence for consequences that result from perturbations in nuclear structure: gene expression interrelationships that are associated with skeletal disease and tumors that metastasize to bone.

GENE EXPRESSION WITHIN THE THREE-DIMENSIONAL CONTEXT OF NUCLEAR ARCHITECTURE: MULTIPLE LEVELS OF NUCLEAR ORGANIZATION SUPPORT FIDELITY OF GENE REGULATION

Although the mechanisms that control gene expression remain to be formally defined, there is growing awareness that the fidelity of gene regulation necessitates the coordination of transcription factor metabolism and the spatial organization of genes and regulatory proteins within the three-dimensional context of nuclear architecture. The components of nuclear organization include the sequence of gene regulatory elements, chromatin structure, and higher order organization of the transcriptional regulatory machinery into subnuclear domains. All of these parameters involve mechanisms that include transcription factor synthesis, nuclear import and retention, post-translational modifications of factors, and directing factors to subnuclear sites that support the organization and assembly of regulatory machinery for gene expression. Remodeling of chromatin and nucleosome organization to accommodate requirements for protein–DNA and protein–protein interactions at promoter elements are essential modifications for both activation of genes and physiological control of transcription. This is a key component of epigenetic control that mediates competency for gene activation or suppression and conveys phenotype and lineage commitment to progeny cells during mitosis. The reconfiguration of gene promoters and assembly of specialized subnuclear domains reflect the orchestration of both regulated and regulatory mechanisms. There are analogous and complex regulatory requirements for processing of gene transcripts. Here it has been similarly demonstrated that the regulatory components of splicing and export of messenger RNA to the cytoplasm are dependent on the architectural organization of nucleic acids and regulatory proteins. There is growing evidence that the focal localization of regulatory machinery in nuclear microenvironments supports the integration of regulatory signals in a manner that facilitates competency for physiological responsiveness.

From a biological perspective, each parameter of factor metabolism requires stringent control and must be linked to structure–function interrelationships that mediate transcription and processing of gene transcripts. However, rather than representing regulatory obstacles, the complexities of nuclear biochemistry and morphology provide the required specificity for physiological responsiveness to a broad spectrum of signaling pathways to modulate transcription under diverse circumstances. Equally important, evidence is accruing that modifications in nuclear architecture and nuclear structure–function interrelationships accompany and appear to be causally related to compromised gene expression under pathological conditions.

Multiple levels of genomic organization that contribute to transcription are illustrated schematically in Figure 1. Additional levels of nuclear organization are reflected by the subnuclear localization of factors that mediate transcription, processing of gene transcripts, DNA replication, DNA repair at discrete domains, and retention of regulatory factors with target gene promoters during mitosis to epigenetically maintain phenotype in progeny cells.

Sequence Organization: A Blueprint for Responsiveness to Regulatory Cues

Appreciation is accruing for the high density of information in both regulatory and mRNA-coding sequences of cell growth and phenotypic genes. The modular organizations of promoter elements provide blueprints for responsiveness to a broad spectrum of regulatory cues that support competency for transient developmental and homeostatic control as well as sustained commitments to tissue-specific gene expression. Overlapping recognition elements expand the options for responsiveness to signaling cascades that mediate mutually exclusive protein–DNA and protein–protein interactions. Splice variants for gene transcripts further enhance the specificity of gene expression. However, it must be acknowledged that the linear order of genes and flanking regulatory elements is necessary but insufficient to support expression in a biological context. There is a requirement to integrate the regulatory information at independent promoter elements and selectively utilize subsets of promoter regulatory information to control the extent to which genes are activated and/or suppressed.

Transcription of the osteocalcin (OC) gene is controlled by a modularly organized promoter with proximal basal regulatory sequences and distal hormone responsive enhancer elements (reviewed in Lian *et al.*, 2004). The OC gene is not expressed in nonosseous cells, nor is it transcribed in osteoprogenitor cells or early-stage proliferating osteoblasts. Following the postproliferative onset of osteoblast differentiation, transcription of the OC gene is regulated by Runx2 (Fig. 2). Maximal levels of transcription are controlled by the combined activities of the vitamin D response element, C/EBP site, AP-1 regulatory elements, and the OC box. Linear organization of the OC gene promoter reveals proximal regulatory elements that control basal and tissue-specific activity. These include the OC box for homeodomain protein binding and an osteoblast-specific complex, the TATA domain, a Runx site, and a C/EBP site. The distal promoter contains a vitamin D responsive

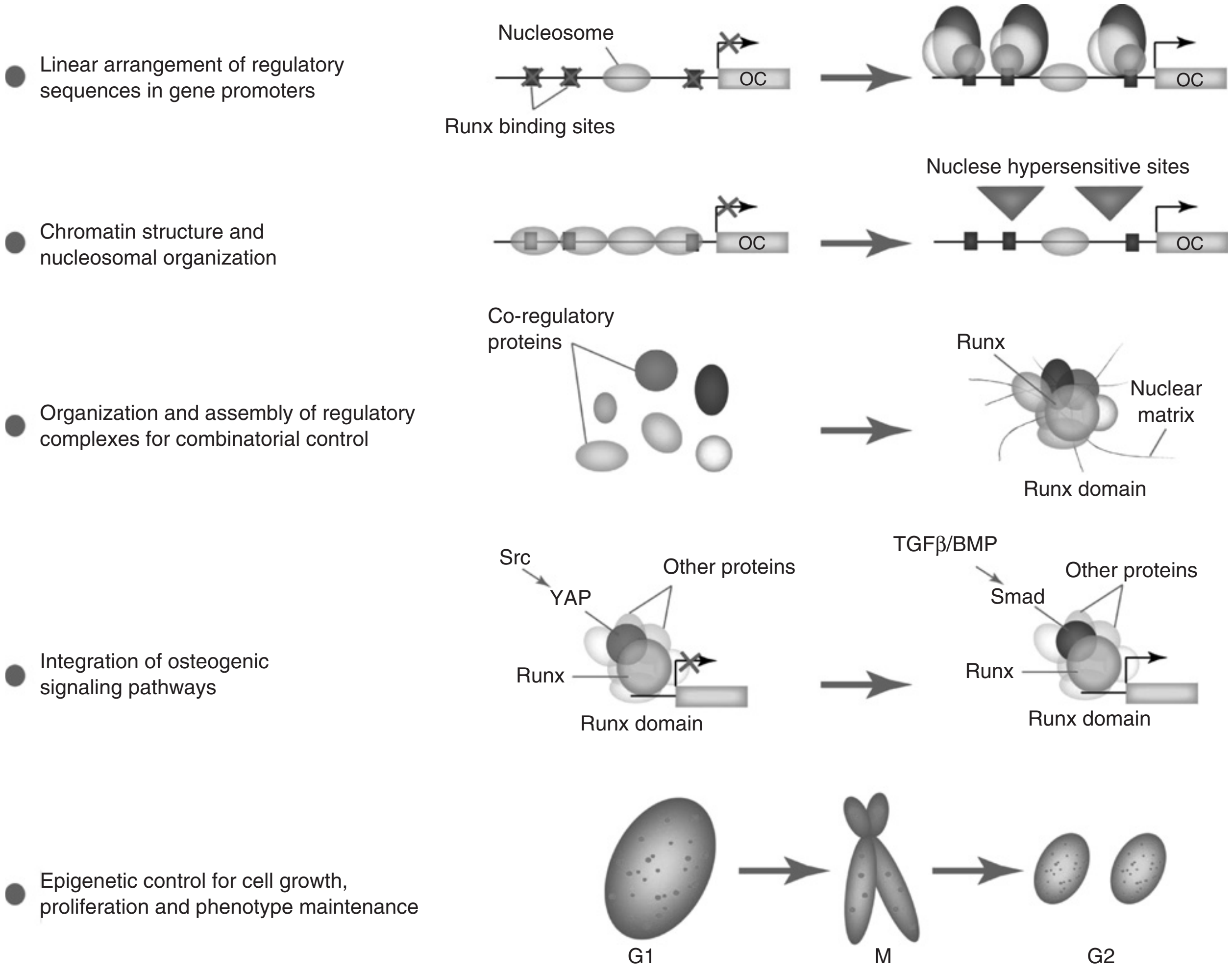

Linear arrangement of regulatory sequences in gene promoters
Nucleosome
Runx binding sites
OC
OC
Nuclese hypersensitive sites
Chromatin structure and nucleosomal organization
OC
OC
Co-regulatory proteins
Organization and assembly of regulatory complexes for combinatorial control
Runx
Nuclear matrix
Runx domain
Integration of osteogenic signaling pathways
Src
YAP
Other proteins
Runx
Runx domain
TGFβ/BMP
Smad
Other proteins
Runx
Runx domain
Epigenetic control for cell growth, proliferation and phenotype maintenance
G1
M
G2

FIGURE 1 Multiple levels of nuclear organization. The linear placement of DNA-regulatory elements in gene promoters constitutes the primary level of nuclear organization. The distance between these regulatory sites is intricately regulated by the packaging of DNA into nucleosomes and higher order chromatin structures. Scaffolding nuclear proteins, such as RUNX, provide structural platforms for the assembly of multiprotein supercomplexes to facilitate the combinatorial control of gene expression. Genes and macromolecular regulatory complexes together give rise to dynamic microenvironments within the nucleus. RUNX nuclear microenvironments contain various co-regulatory proteins that are involved in combinatorial control of gene activation, as well as repression, chromatin remodeling and cellular signaling. In addition, Runx nuclear microenvironments are equally partitioned during mitosis to epigenetically regulate cell growth and phenotypic properties.

enhancer element (VDRE) that is flanked by Runx sites. The control of transcription is dependent on protein–DNA interactions in the basal and upstream elements that are in part dependent on the accessibility of cognate regulatory sequences and additionally on the consequences of mutually exclusive protein–DNA interactions.

Chromatin Reorganization: Packaging Genomic DNA in a Manner that Controls Access to Genetic Information

Chromatin structure and nucleosome organization provide architectural linkages between gene organization and components of transcriptional control. Within the eukaryotic nucleus the DNA is organized into a highly structured nucleoprotein complex named chromatin. The nucleosome is the fundamental unit of chromatin and is composed of a 147-bp DNA fragment wrapped around a core of histone proteins (two each of histones H2A, H2B, H3, and H4; Ramakrishnan, 1997; Richmond and Davey, 2003). Chromatin structure and nucleosome organization reduce distances between regulatory sequences, contributing to cross talk between promoter elements, and render elements competent for interactions with positive and negative regulatory factors.

The presence of nucleosomes has been generally considered to block accessibility of most transcription factors to their cognate binding sequences. Moreover, gene activity is usually accompanied by alterations in the nucleosomal array, as evidenced by increased nuclease hypersensitivity at specific promoter and enhancer elements (Li *et al.*, 2007; Narlikar *et al.*, 2002). Thus, nuclease digestion has

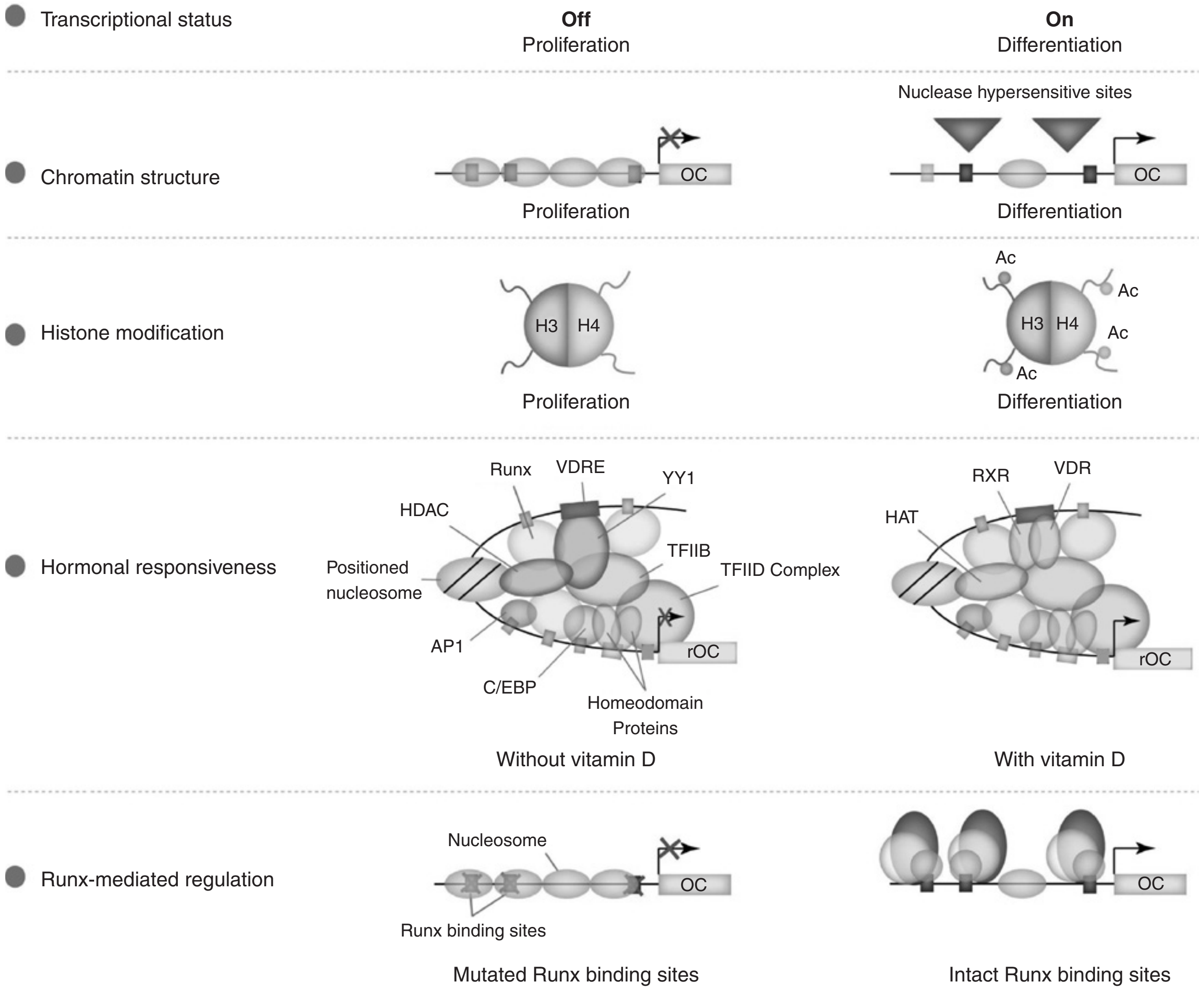

Transcriptional status
Off
Proliferation
On
Differentiation
Chromatin structure
Nuclease hypersensitive sites
OC
OC
Proliferation
Differentiation
Histone modification
H3
H4
Proliferation
Ac
Ac
Ac
Ac
H3
H4
Differentiation
Hormonal responsiveness
Runx
VDRE
YY1
HDAC
TFIIB
TFIID Complex
Positioned
nucleosome
AP1
rOC
C/EBP
Homeodomain
Proteins
Without vitamin D
RXR
VDR
HAT
rOC
With vitamin D
Runx-mediated regulation
Nucleosome
OC
Runx binding sites
OC
Mutated Runx binding sites
Intact Runx binding sites

FIGURE 2 Runx-dependent regulation of the bone specific osteocalcin gene at multiple levels of nuclear organization. Osteocalcin is actively transcribed during osteoblast differentiation. This transcriptional upregulation is accompanied by Runx-dependent chromatin remodeling, histone modifications, and steroid hormone responsiveness. Intact Runx binding sites are required for transcriptional regulation of the osteocalcin gene; mutations in Runx binding sites result in a closed chromatin conformation, presence of transcriptional inhibitory histone modifications, and recruitment of repressor complexes.

been widely used to probe spatial organization of chromatin both *in vitro* and *in vivo* based on the premise that chromatin accessibility to nuclease activity reflects chromatin access to nuclear regulatory molecules. Chromatin immunoprecipitation (ChIP) assays that utilized antibodies to covalently modified histones or transcription regulators provide the basis for defining the dynamic association of protein complexes with chromatin domains that exhibit particular patterns of epigenetic markers. Multiple studies have established that there are intrinsic differences in the nucleosome binding capacity among transcription factors. Although several transcription factors cannot bind when their sites are assembled into nucleosomes, others can recognize and interact with nucleosome-engaged binding elements (Li *et al.*, 2007; Narlikar *et al.*, 2002), albeit with different degrees of affinity.

Changes in chromatin organization have been documented under many biological conditions where modifications of gene expression are necessary for the execution of physiological control. Transient changes in chromatin structure accompany and are linked functionally to developmental and homeostatic-related control of gene expression. Long-term changes occur when the commitment to phenotype-specific gene expression occurs with differentiation. Superimposed on the remodeling of chromatin structure and nucleosome organization that renders genes transcriptionally active are additional alterations in the packaging of DNA as chromatin support steroid hormone-responsive enhancement or dampening of transcription.

A large family of protein complexes that promote transcription by altering chromatin structure in an ATP-dependent manner have been described (Becker and Horz, 2002; de la Serna *et al.*, 2006; Narlikar *et al.*, 2002; Peterson, 2002). Among them is the SWI/SNF subfamily, which is composed of several subunits and has been implicated in a wide range of cellular events, including gene regulation, cell cycle control, development, and differentiation (Becker and Horz, 2002; de la Serna *et al.*, 2006; Li *et al.*, 2007; Peterson, 2002). The mammalian SWI/SNF complexes contain a catalytic subunit that can be either BRG1 or BRM, each of which includes ATPase activity. Mutations in the ATPase domain of BRG1 or BRM that abrogate the ability of these proteins to bind ATP result in the formation of inactive SWI/SNF complexes (de la Serna *et al.*, 2000, 2001a, 2001b). Furthermore, expression of mutant BRG1 or BRM proteins in NIH3T3 cells impairs the ability of these cells to activate endogenous stress response genes in the presence of arsenite (de la Serna *et al.*, 2000) and to differentiate into muscle or adipocytic cells (de la Serna *et al.*, 2000, 2001a, 2001b; Salma *et al.*, 2004). In addition, we have recently shown that the presence of the mutant BRG1 protein in these NIH3T3 cell lines inhibits BMP2-induced differentiation into the osteoblast lineage (Young *et al.*, 2005). Similarly, expression of mutant BRG1 in osteoblastic cells exhibiting a differentiated phenotype inhibits the expression of genes associated with this terminally differentiated stage (Villagra *et al.*, 2006).

A highly debated feature of ATP-dependent chromatin remodelers is their ability to catalyze *in vitro* nucleosome mobilization along the DNA (sliding), to reposition the histone octamer to a nucleosome-free DNA, or to transiently expose nucleosomal DNA (Becker and Horz, 2002; de la Serna *et al.*, 2006; Li *et al.*, 2007; Narlikar *et al.*, 2002; Peterson, 2002). It has been proposed that the varied outcomes observed in these studies are related to the different experimental conditions used during the analyses. Although considerable scientific effort has been dedicated during the past few years to establishing the molecular mechanisms by which ATP-dependent, and in particular SWI/SNF, complexes remodel nucleosomal organization, only limited attention has been given to the contribution of the DNA sequence to chromatin structure and chromatin remodeling (Thastrom *et al.*, 2004). Our group has reported that promoter sequences can be critical components of the regulatory mechanisms that control chromatin remodeling and gene transcription (Gutierrez *et al.*, 2000, 2007a; Paredes *et al.*, 2002). Using as a model system the promoter region of the rat OC gene reconstituted *in vitro*, we demonstrated that SWI/SNF complexes induce histone octamer mobilization to preferential positions within the promoter sequence, leading to a nucleosomal organization that resembles that found in intact bone cells expressing the OC gene. This SWI/SNF-mediated preferential nucleosome positioning is primarily determined by the presence of both nucleosome positioning and nucleosome excluding sequences that are conveniently distributed within the proximal promoter domain of the OC gene (Gutierrez *et al.*, 2007a).

An increasing body of evidence also indicates that the activity of SWI/SNF chromatin remodeling complexes involves nucleosome eviction (Boeger *et al.*, 2004; Gutierrez *et al.*, 2007b; Korber *et al.*, 2006; Liu *et al.*, 2006; Reinke and Horz, 2003). Recent pulse-chase experiments show that there is a dynamic turnover of histones at transcriptionally active genes (reviewed by Clayton *et al.*, 2006). Similarly, histone H2A/H2B dimers are dynamically exchanged between nucleosomes (reviewed by Workman, 2006). Interestingly, the presence of a transcription factor bound to its target sequence enhances SWI/SNF-mediated nucleosome eviction (Gutierrez *et al.*, 2007b). Therefore, it has been proposed that interaction of transcriptional activators with their cognate elements is one of the first determinants leading to targeted remodeling by ATP-dependent complexes (Gutierrez *et al.*, 2007b). Consequently, the position of the cognate binding sites for these SWI/SNF-targeting factors, relative to the translational position of the nucleosomes within a given promoter, becomes a key element during the chromatin remodeling process that leads to transcription activation.

Histone modifications also play a major role in regulating chromatin structure and gene transcription in eukaryotic cells (for a recent review, see Kouzarides, 2007). These modifications may alter chromatin organization by disrupting intranucleosomal and/or internucleosomal histone–DNA interactions (e.g., histone acetylation). In addition, these post-translational modifications provide specific docking domains on the nucleosomal surfaces, enhance recognition by proteins, modify chromatin structure, and regulate transcription (e.g., histone methylation). An important breakthrough in addressing the physiological role of histone modifications experimentally came with the identification and cloning of enzymes that catalyze these modifications *in vivo* (Bernstein *et al.*, 2007; Kouzarides, 2007). Thus, it has been shown that nuclear histone modifying enzymes are critical during steroid hormone-dependent transcriptional activation.

Vitamin D, a sec-steroid, provides a paradigm for understanding molecular mechanisms involved in bone metabolism. Vitamin D directly regulates the expression of genes that support bone formation during development and bone remodeling throughout life. Therefore, osteoblast differentiation is a model for understanding developmental responsiveness to vitamin D (Christakos *et al.*, 2003; van Driel *et al.*, 2004).

Vitamin D exerts its genomic effects through the vitamin D receptor (VDR), which is a member of the superfamily of nuclear receptors (Rachez and Freedman, 2000; Xu and O'Malley, 2002). As in other nuclear receptors, binding of the ligand induces conformational changes in the C-terminal ligand-binding domain (LBD) of the VDR. The changes establish competency for VDR interaction with coactivators of the p160/SRC family, including SRC-1/NCoA-1, SRC-2/NCoA-2/GRIP/TIF2, and SRC-3/ACTR. These complexes are critical for transcriptional activation (Christakos *et al.*, 2003; Rachez and Freedman, 2000; Xu and O'Malley, 2002). p160/SRC coactivators form high-molecular-weight complexes by interacting with other coactivator proteins including p300, its related homologue CBP, and P/CAF (Goodman and Smolik, 2000). Moreover, p160/SRC coactivators have been shown to recruit CBP/p300 and P/CAF to ligand-bound nuclear receptors. Multiprotein complexes containing different activities are functionally linked to ligand-dependent transcriptional regulation (Rachez and Freedman, 2000). Coactivators such as SRC-3/ACTR, SRC-1/NCoA-1, CBP/p300, and P/CAF contain intrinsic histone acetyltransferase (HAT) activity. Therefore, protein complexes including independent HAT activities can be recruited to gene promoters by nuclear receptors in a ligand-dependent manner (Rachez and Freedman, 2000). Once bound to these promoters, the HAT activities contribute to chromatin remodeling events that increase access of additional regulatory factors to their cognate elements (Narlikar *et al.*, 2002).

The multisubunit DRIP (VDR-Interacting Protein) complex also binds to VDR in response to the ligand vitamin D (Rachez *et al.*, 1999). This interaction occurs through the LBD of VDR in the same manner as the p160/SRC coactivators, resulting in transcriptional enhancement (Rachez *et al.*, 2000). In contrast to p160/SRC coactivators, DRIP is devoid of HAT and other chromatin remodeling activities and interacts with nuclear receptors through a single subunit designated DRIP205, which anchors other subunits to the receptor LBD. Several of these subunits are also present in the Mediator complex, which interacts with the C-terminal domain (CTD) of RNA polymerase II, forming the holoenzyme complex (Kornberg, 2005). Therefore, the DRIP complex appears to function as a transcriptional coactivator by forming a molecular bridge between the VDR and the basal transcription machinery, reflecting the importance of three-dimensional promoter organization to regulatory activity.

In the past few years various investigators have shown that coactivator complexes including p160/SRC and DRIP are recruited to steroid hormone-regulated genes by nuclear receptors in a sequential and mutually exclusive manner (Burakov *et al.*, 2002; Kim *et al.*, 2005; Metivier *et al.*, 2003; Sharma and Fondell, 2002). The ordered association of transcriptional regulators exhibits binding kinetics with periods of 40 to 60 minutes. These results provided the basis for a model in which cyclical association of different coactivator complexes reflects the dynamics of the transcription activation process of nuclear receptor-regulated genes (Metivier *et al.*, 2006; Xu and O'Malley, 2002). Alternatively, recent reports indicate that occupancy at the target gene regulatory regions by nuclear receptor-associated coactivator complexes may also occur gradually and at a significantly lower rate (Oda *et al.*, 2003; Wang *et al.*, 2005). Thus, it has been shown that during keratinocyte differentiation, there is a specific utilization of p160/SRC or DRIP205/Mediator coactivator complexes to regulate vitamin D–dependent genes (Oda *et al.*, 2003). The proposed model indicates that both coactivator complexes have important roles during early stages of keratinocyte differentiation, but a subsequent decrease in major DRIP/Mediator components leads to a predominant role for p160/SRC in the later stages of differentiation.

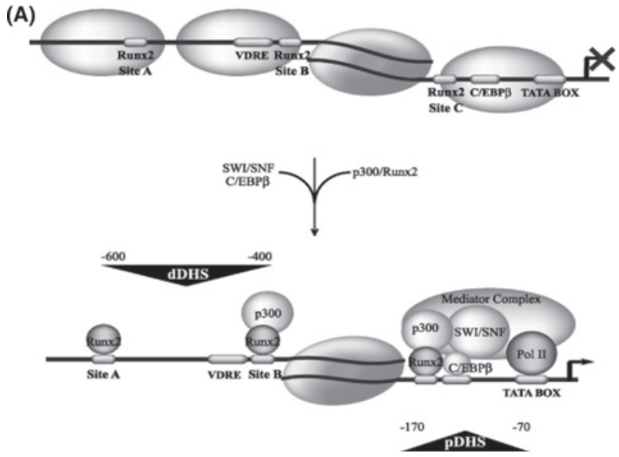
The bone-specific OC gene and skeletal-restricted Runx2 transcription factor serve as examples of obligatory relationships between nuclear structure and vitamin D–mediated physiological control of skeletal gene expression (Montecino *et al.*, 2007). It appears that there are similar relationships between nuclear organization and other bone-related vitamin D-responsive genes (e.g., osteopontin and 24-hydroxylase). However, we will largely confine our consideration of nuclear structure–gene expression relationships to the OC gene.

The rat OC gene encodes a 10-kDa bone-specific protein that is induced in osteoblasts with the onset of mineralization at late stages of differentiation (Owen *et al.*, 1990). Modulation of OC gene expression during bone formation and remodeling requires physiologically responsive accessibility of proximal and upstream promoter sequences to regulatory and co-regulatory proteins, as well as protein–protein interactions that integrate independent promoter domains. The chromatin organization of the OC gene illustrates dynamic remodeling of a promoter to accommodate requirements for phenotype-related developmental and vitamin D–responsive activity (Montecino *et al.*, 2007).

Transcription of the OC gene is controlled by modularly organized basal and hormone-responsive promoter elements (see Fig. 2), located within two DNase I–hypersensitive sites (distal site, positions −600 to −400; proximal site, positions −170 to −70) that are only nuclease accessible in bone-derived cells expressing this gene. A key regulatory element that controls OC gene expression is recognized by the VDR complex on ligand stimulation. This vitamin D responsive element (VDRE) is located in the distal region (Fig. 3A) of the OC promoter (positions −465 to −437) and functions as an enhancer to increase OC gene transcription (Montecino *et al.*, 2007). The retention of a nucleosome between the proximal and upstream enhancer domains reduces the distance between the basal regulatory elements and the VDRE and supports a promoter configuration that is conducive to protein–protein interactions between VDR-associated proteins and components of the RNA polymerase II–bound complex (see Fig. 3B). Interaction of the VDR at the distal promoter region of the OC gene requires nucleosomal remodeling (Montecino *et al.*, 1999; Paredes *et al.*, 2002).

Another key regulator of OC gene expression is the nuclear matrix-associated transcription factor Runx2, a member of the Runt homology family of proteins that has been shown to contribute to the control of skeletal gene expression (Lian *et al.*, 2004). Runx2 proteins serve as a scaffold for the assembly and organization of co-regulatory proteins that mediate biochemical and architectural control of promoter activity. The rat OC gene promoter contains three recognition sites for Runx2 interactions, site A (−605 to −595), site B (−438 to −430), and site C (−138 to −130). Mutation of all three Runx2 sites results in significantly reduced OC expression in bone-derived cells (Javed *et al.*, 1999). We have recently shown that within the OC gene promoter context there is a tight functional relationship between Runx2 and the vitamin D-dependent pathway (Paredes *et al.*, 2004). Runx2 and VDR are components of the same nuclear complexes, colocalize at punctate foci within the nucleus of osteoblastic cells, and interact directly in protein–protein binding assays *in vitro* (Paredes *et al.*, 2004). Additionally, mutation of the distal Runx2 sites A and B (which flank the VDRE; see Fig. 3) abolishes vitamin D–enhanced OC promoter activity (Paredes *et al.*, 2004). In contrast to most nuclear receptors, the VDR does not contain an N-terminal AF-1 transactivation domain and thus is unable to interact with coactivators through this region (Rachez and Freedman, 2000). Therefore, Runx2 plays a key role in the vitamin D–dependent stimulation of the OC gene promoter in osteoblastic cells by directly stabilizing binding of the VDR to the VDRE. Runx2 also allows recruitment of the coactivator p300 to the OC promoter (see Fig. 3(A), which results in upregulation of both basal and vitamin D–enhanced OC gene transcription (Sierra *et al.*, 2003). Based on these results, we have postulated that Runx2-mediated recruitment of p300 may facilitate the subsequent interaction of p300 with the VDR on ligand stimulation (Paredes *et al.*, 2004).

The rate of recruitment of p160/SCR-1 and DRIP coactivators to the OC gene in response to vitamin D has recently been studied (Carvallo *et al.*, 2007). It has been found that the VDR and SRC-1 rapidly and stably interact with the distal region of the OC promoter encompassing the VDRE (see Fig. 3B). The interaction of SRC-1 and VDR directly correlates with vitamin D–mediated transcriptional enhancement of the OC gene, increased association of the RNA polymerase complex, and vitamin D–stimulated histone H4 acetylation (Shen *et al.*, 2002).


(A)
Runx2 Site A
VDRE Runx2 Site B
Runx2 Site C
C/EBPβ
TATA BOX
SWI/SNF C/EBPβ
p300/Runx2
-600
-400
dDHS
Runx2
Site A
VDRE
Site B
p300
Runx2
Mediator Complex
p300
SWI/SNF
Runx2
C/EBPβ
Pol II
TATA BOX
-170
-70
pDHS

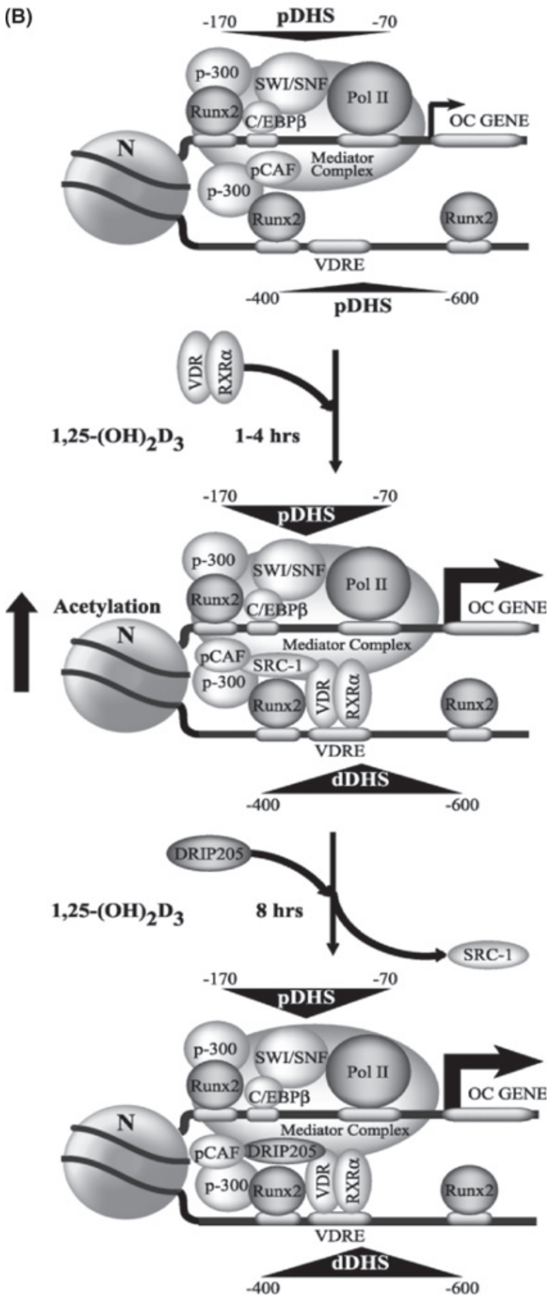

(B)
-170
pDHS
-70
p-300
SWI/SNF
Pol II
Runx2
C/EBPβ
OC GENE
N
Mediator Complex
pCAF
p-300
Runx2
Runx2
VDRE
-400
pDHS
-600
VDR
RXRα
1,25-(OH)2D3
1-4 hrs
-170
-70
pDHS
Acetylation
p-300
SWI/SNF
Pol II
Runx2
C/EBPβ
OC GENE
N
Mediator Complex
pCAF
SRC-1
p-300
Runx2
VDR
RXRα
Runx2
VDRE
dDHS
-400
-600
DRIP205
1,25-(OH)2D3
8 hrs
SRC-1
-170
-70
pDHS
p-300
SWI/SNF
Pol II
Runx2
C/EBPβ
OC GENE
N
Mediator Complex
pCAF
DRIP205
p-300
Runx2
VDR
RXRα
Runx2
VDRE
dDHS
-400
-600

FIGURE 3 The rat osteocalcin gene promoter and the chromatin remodeling process that accompanies basal and vitamin D–enhanced osteocalcin transcription in osteoblastic cells. The principal regulatory elements, cognate transcription factors and associated co-regulators bound to the osteocalcin (OC) gene promoter, are indicated. The filled circles represent nucleosomes assembled along the first 800 bp of the OC promoter and the arrow indicates the transcription start site and direction of transcription. The presence of the distal (dDHS, −600 to −400) and proximal (pDHS, −170 to −70) DNase I hypersensitive sites is also marked. **A**, Linear representation of the OC promoter. **B**, Proposed three-dimensional organization of the OC gene promoter when transcribing at basal or vitamin D–enhanced (after treatment of osteoblastic cells expressing OC with vitamin D for 4 and 8 hours) conditions. The size of the arrow indicates the level of transcription. The increase in histone acetylation that accompanies vitamin D–enhanced transcription is also marked.

Interestingly, DRIP205 was found to bind to the OC promoter only after several hours of continuous treatment with vitamin D, concomitant with release of SRC-1 (see Fig. 3B). Based on these results it has been postulated that this preferential recruitment of SRC-1 to the OC gene promoter is based on the specific distribution of regulatory elements at the distal region of the promoter. This organization may lead to the formation of a stable complex at the distal region that includes Runx2, p300, VDR, and SRC-1. Once established, this complex may mediate an increase in acetylation of histones H3 and H4 and directly stimulate the basal transcription machinery bound to an OC promoter actively engaged in transcription.

The general relevance of vitamin D–mediated chromatin-based mechanisms of promoter activity, accessibility, and crosstalk between regulatory domains is further illustrated by the vitamin D responsiveness of the osteopontin and 24-hydroxylase genes. Recent studies have established that vitamin D induces a rapid and cyclical association of the VDR/RXR heterodimer with the proximal mouse 24(OH)ase gene promoter in osteoblastic cells (Kim *et al.*, 2005). Vitamin D treatment also induces a rapid recruitment of coactivators such as p160/SRC and p300/CBP, which leads to acetylation of histone H4. DRIP205/Mediator is also recruited to the proximal promoter region concomitantly with the interaction of RNA polymerase II. Together, these results support a model in which highly dynamic association of the VDR with chromatin occurs during vitamin D–dependent induction of the 24(OH)ase gene in osteoblasts (Kim *et al.*, 2005).

Carlberg and co-workers have also monitored the spatio-temporal regulation of the human 24(OH)ase gene (Vaisanen *et al.*, 2005). They have evaluated 25 contiguous genomic regions spanning the first 7.7 kb of the human 24(OH)ase promoter and found that in addition to the proximal VDREs, three further upstream regions are associated with the VDR on vitamin D stimulation. Interestingly, only two of these regions contain sequences resembling known VDREs that are transcriptionally responsive to this hormone. The other VDR-associated upstream promoter region does not contain any recognizable classical VDRE that could account for the presence of the VDR protein. However, simultaneous association of the VDR, RXR, p160/SRC, and DRIP/Mediator coactivators, as well as RNA polymerase, was detected in all four vitamin D–responsive sequences after the addition of the ligand (Vaisanen *et al.*, 2005). Remarkably, despite participating in the same process, all four chromatin regions displayed individual vitamin D–dependent patterns of interacting proteins. Based on these results, the authors propose that these upstream vitamin D–responsive regions may have a role in the implementation of gene activation, as they raise their vitamin D–dependent histone H4 acetylation status earlier than that of the proximal promoter VDREs (Vaisanen *et al.*, 2005). It has also been suggested that the simultaneous communication of the individual promoter regions with the RNA polymerase II complex occurs through a particular three-dimensional organization of the chromatin at the 24(OH)ase promoter. This arrangement could be facilitating close contact between distal and proximal regulatory regions.

On the other hand, Pike and co-workers have described that on vitamin D treatment of osteoblastic cells, there is a rapid and cyclical association of the VDR with the osteopontin (OP) promoter (Kim *et al.*, 2005). This increased binding of the VDR parallels vitamin D–mediated transcriptional enhancement of the OP gene and additionally involves cyclical, sequential, and mutually exclusive recruitment of the coactivators p160/SRC, p300/CBP, and DRIP/Mediator. Interestingly and in contrast to the OC and 24(OH)ase genes, p160/SRC–p300/CBP binding does not result in increased histone H4 acetylation. These results further confirm that in osteoblastic cells different promoters are regulated by distinct mechanisms in response to vitamin D.

The transcription factor C/EBPβ is also a principal transactivator of the OC gene that binds within the proximal promoter region (−106 to −99; see Fig. 3A) and synergizes with Runx2 to enhance basal and tissue-specific OC gene transcription (Gutierrez *et al.*, 2002). C/EBPβ has been recently shown to recruit the SWI/SNF complex to the OC promoter (see Fig. 3A), where it is required for both formation of the proximal nuclease hypersensitive site and transcriptional activation of this gene in osteoblastic cells (Villagra *et al.*, 2006). Therefore, it is postulated that binding of C/EBPβ and Runx2 to the proximal promoter region of the OC gene in differentiated osteoblasts allows recruitment of both SWI/SNF and HAT-containing chromatin remodeling activities, and that together, these activities catalyze the changes in chromatin structure that facilitate transcription (see Fig. 3). Interestingly, both C/EBPβ and Runx2 are bound to the OC promoter in differentiated osteoblastic cells that are not transcribing the OC gene because of an inhibition of SWI/SNF-mediated chromatin remodeling (Villagra *et al.*, 2006). This result indicates that interaction of both factors with the OC promoter is, at least partially, independent of SWI/SNF activity and therefore prior to the formation of the proximal DNase I hypersensitive site.

Higher Order Nuclear Organization: Interrelationships of Transcriptional Regulatory Machinery with Nuclear Architecture

The necessity for both nuclear architecture and biochemical control to regulate gene expression is becoming increasingly evident. An ordered organization of nucleic acids and regulatory proteins to assemble and sustain macromolecular complexes that provide the machinery for transcription requires stringent, multistep mechanisms. Each component of transcriptional control is governed by responsiveness to an integrated series of cellular signaling pathways. Each gene promoter selectively exercises options for regulating factor interactions that activate or repress transcription. All transcriptional control is operative *in vivo* under conditions where, despite low representation of promoter regulatory elements and cognate factors, a critical concentration is essential for a threshold that can initiate sequence-specific interactions and functional activity.

Historically, there was a dichotomy between pursuit of nuclear morphology and transcriptional control. However, the growing experimental evidence indicating that components of gene regulatory mechanisms are associated architecturally strengthens the nuclear structure–function paradigm. Are all regulatory events that control gene expression linked architecturally? Can genetic evidence formally establish consequential relationships between nuclear structure and transcription? What are the mechanisms that direct genes and regulatory factors to subnuclear sites that support transcription? How are boundaries established that compartmentalize components of gene expression to specific subnuclear domains? Can the regulated and regulatory parameters of nuclear structure–function interrelationships be distinguished? These are key questions that must be addressed experimentally to validate components of gene expression that have been implicated as dependent on nuclear morphology. From a biological perspective, it is important to determine if breaches in nuclear organization are related to compromised gene expression in diseases that include cancer where incurred mutations abrogate transcriptional control.

Nuclear Matrix: A Scaffold for the Architectural Organization of Regulatory Complexes

The identification and *in situ* visualization of the nuclear matrix, together with the characterization of a chromosome scaffold, were bases for pursuing the control of gene expression within the three-dimensional context of nuclear architecture.

The anastomosing network of fibers and filaments that constitute the nuclear matrix supports the structural properties of the nucleus as a cellular organelle and accommodates modifications in gene expression associated with proliferation, differentiation, and changes necessary to sustain phenotypic requirements in specialized cells (Fig. 4; Bidwell *et al.*, 1994; Dworetzky *et al.*, 1990). Regulatory functions of the nuclear matrix include but are by no means restricted to DNA replication (Berezney and Coffey, 1975), gene location (Zeng *et al.*, 1997), imposition of physical constraints on chromatin structure that support formation of loop domains, concentration and targeting of transcription factors (Cai *et al.*, 2006; Dobreva *et al.*, 2006; van Wijnen *et al.*, 1993), RNA processing and transport of gene transcripts (Blencowe *et al.*, 1994), and post-translational modifications of chromosomal proteins, as well as imprinting and modifications of chromatin structure (Davie, 1997; Drobic *et al.*, 2006). Additional evidence for participation of the nuclear matrix in gene expression came from reports of qualitative and quantitative changes in the representation of nuclear matrix proteins during the differentiation of normal diploid cells and in tumor cells associated with a spectrum of cancers. More direct evidence for functional linkages between nuclear architecture and transcriptional control was provided by demonstrations that cell growth and phenotypic regulatory factors are nuclear matrix associated and by modifications in the partitioning of transcription factors between the nuclear matrix and the nonmatrix nuclear fraction when changes in gene expression occur (reviewed in Stein *et al.*, 2003; Zaidi *et al.*, 2007).

Contributions of the nuclear matrix to control of gene expression is further supported by involvement in regulatory events that mediate histone modifications, chromatin remodeling, and processing of gene transcripts (de la Serna *et al.*, 2006; Zink *et al.*, 2004). Instead of addressing chromatin remodeling and transcriptional activation as complex but independent mechanisms, it is biologically meaningful to investigate the control of genome packaging and expression as interrelated processes that are operative in relation to nuclear architecture. Taken together with findings that indicate important components of the machinery for both gene transcription and replication are confined to nuclear matrix-associated subnuclear domains, the importance of nuclear architecture to intranuclear compartmentalization of regulatory activity is being pursued.

Subnuclear Domains: Nuclear Microenvironments Provide a Structural and Functional Basis for Subnuclear Compartmentalization of Regulatory Machinery

An understanding of interrelationships between nuclear structure and gene expression necessitates knowledge of the composition, organization, and regulation of sites within the nucleus that are dedicated to DNA replication, DNA repair, transcription, and processing of gene transcripts. During the past several years there have been developments in reagents and instrumentation to enhance the resolution of nucleic acid and protein detection by *in situ* hybridization and immunofluorescence analyses. We are beginning to make the transition from descriptive *in situ* mapping of genes, transcripts, and regulatory factors to visualization of gene expression from the three-dimensional perspective of nuclear architecture. Figure 4 displays components of gene regulation that are associated with the nuclear matrix. Initially, *in situ* approaches were utilized primarily for the intracellular localization of nucleic acids. Proteins that contribute to control of gene expression were first identified by biochemical analyses. We are

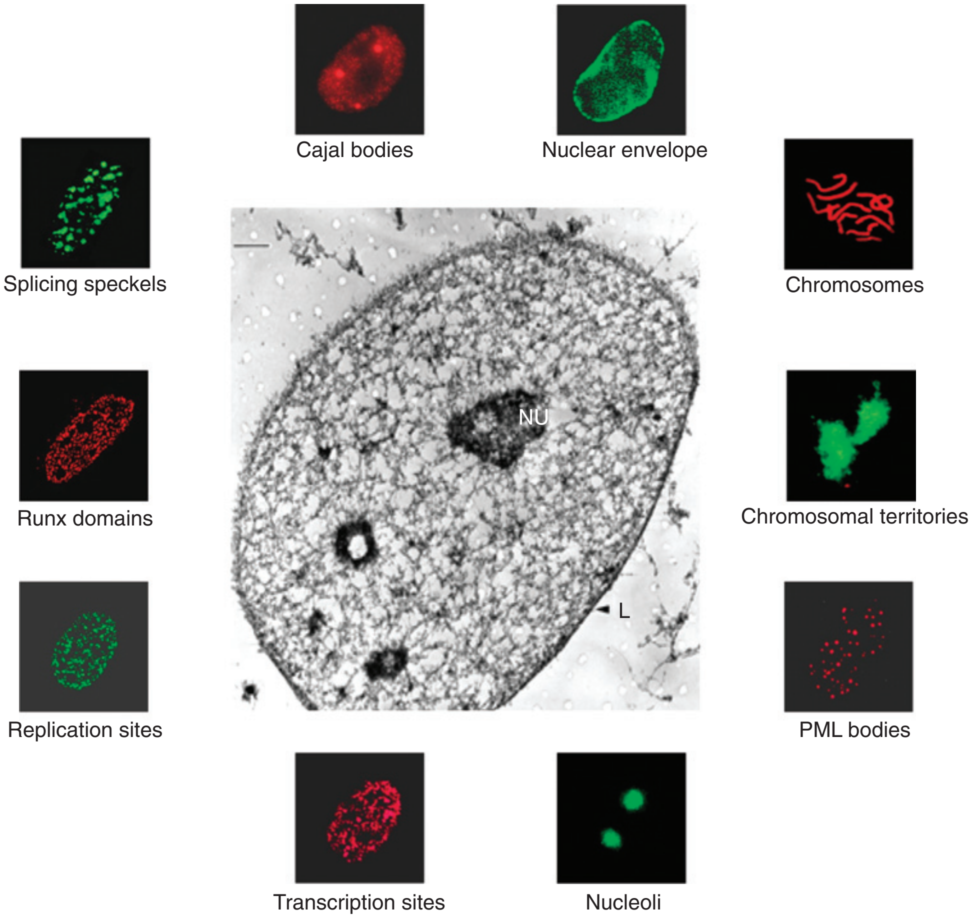


FIGURE 4 The nuclear architecture is functionally linked to the organization and sorting of regulatory information. Immunofluorescence microscopy of the nucleus *in situ* has revealed the distinct nonoverlapping subnuclear distribution of vital nuclear processes, including DNA replication sites; structural parameters of the nucleus (e.g., the nuclear envelope, chromosomes, and chromosomal territories); and Runx transcription factor domains for chromatin organization and transcriptional control of tissue-specific genes, as well as RNA synthesis and processing, involving, for example, transcription sites, SC35 domains, Cajal bodies, and nucleoli. These domains are associated with the nuclear matrix (the electron micrograph shown in the center). (See plate section)

now applying high-resolution *in situ* analyses for the primary identification and characterization of gene regulatory mechanisms under *in vivo* conditions.

We are increasing our understanding of the significance of nuclear domains to the control of gene expression. These local nuclear environments that are generated by the multiple aspects of nuclear structure are tied to the developmental expression of cell growth and tissue-specific genes. Historically, the control of gene expression and characterization of structural features of the nucleus were pursued conceptually and experimentally as minimally integrated questions. At the same time, however, independent pursuit of nuclear structure and function has occurred in parallel with the appreciation that several components of nuclear architecture are associated with parameters of gene expression or control of specific classes of genes.

For the most part, biochemical parameters of replication and transcription have been studied independently. However, paradoxically, from around the turn of the last century it was recognized that there are microenvironments within the nucleus where regulatory macromolecules are compartmentalized in subnuclear domains. Chromosomes and the nucleolus provided the initial paradigms for the organization of regulatory machinery within the nucleus. Also, during the past several decades, linkages have been established between subtleties of chromosomal anatomy and replication as well as gene expression. Regions of the nucleolus are understood in relation to ribosomal gene expression. The organization of chromosomes and chromatin is well accepted as a reflection of functional properties that support competency for transcription and the extent to which genes are transcribed. It has been generally recognized that chromosomes are nonrandomly organized in the interphase nucleus as chromosomal territories. Location of genes within chromosomal territories has been associated with transcriptional status (Cremer *et al.*, 2006). Recently, it has been shown that chromosomal territories intermingle with each other in the interchromatin space (Branco and Pombo, 2007). Chromosomal organization in the interphase nucleus may have implications for coordinated timing of replication at intranuclear sites for DNA duplication that appear to persist from one S-phase to the next (Sadoni *et al.*, 2004).

There is a broad-based organization of regulatory macromolecules within discrete nuclear domains. Examples of intranuclear compartmentalization now include but by no means are restricted to SC35 RNA processing sites, PML bodies, the structural and regulatory components of nuclear pores that mediate nuclear-cytoplasmic exchange (Moir *et al.*, 2000), coiled (Cajal) bodies, and replication foci, as well as defined sites where steroid hormone receptors and transcription factors reside (see Fig. 4; Glass and Rosenfeld, 2000; Leonhardt *et al.*, 2000; McNally *et al.*, 2000). The integrity of these subnuclear microenvironments is indicated by structural and functional discrimination between each architecturally defined domain. Corroboration of structural and functional integrity of each domain is provided by the uniqueness of the intranuclear sites with respect to composition, organization, and intranuclear distribution in relation to activity (Hirose and Manley, 2000; Kosak and Groudine, 2004; Lemon and Tjian, 2000; Taatjes *et al.*, 2004).

We are now going beyond mapping regions of the nucleus that are dedicated to replication and gene expression. We are gaining insight into interrelationships between the subnuclear organization of the regulatory and transcriptional machinery with the dynamic assembly and activity of macromolecular complexes that are required for biological control during development, differentiation, maintenance of cell and tissue specificity, homeostatic control, and tissue remodeling. Equally important, it is becoming evident that the onset and progression of cancer and neurological disorders are associated with and potentially functionally coupled with perturbations in the subnuclear organization of genes and regulatory proteins that relate to aberrant gene replication, repair, and transcription (reviewed in Zaidi *et al.*, 2007).

Intranuclear Trafficking to Subnuclear Destinations: Directing Skeletal Regulatory Factors to the Right Place at the Right Time

The traditional experimental approaches to transcriptional control have been confined to the identification and characterization of gene promoter elements and cognate regulatory factors. However, the combined application of *in situ* immunofluorescence together with molecular, biochemical, and genetic analyses indicates that several classes of transcription factors exhibit a punctate subnuclear distribution. This punctate subnuclear distribution persists after the removal of soluble nuclear proteins and nuclease-digested chromatin. We propose that the intranuclear organization of regulatory proteins could be linked functionally to their competency to affect gene expression (e.g., Guo *et al.*, 1995; Htun *et al.*, 1996; Nguyen and Karaplis, 1998; Stenoien *et al.*, 1998; Verschure *et al.*, 1999; Zeng *et al.*, 1997). Therefore, one fundamental question is the mechanism by which this compartmentalization of regulatory factors is established within the nucleus. This compartmentalization could be maintained by the nuclear matrix, which provides an underlying macromolecular framework for the organization of regulatory complexes (Berezney and Jeon, 1995; Berezney and Wei, 1998; Penman, 1995). However, one cannot dismiss the possibility that nuclear compartmentalization is activity driven (Misteli, 2000, 2004; Pederson, 2000; Spector, 2001).

Insight into architecture-mediated transcriptional control can be gained by examining the extent to which the subnuclear distribution of gene regulatory proteins affects their activities. We and others observed that members of the Runx/Cbfa family of hematopoietic and bone tissue-specific transcription factors (Banerjee *et al.*, 1997; Chen *et al.*, 1998; Merriman *et al.*, 1995; Zaidi *et al.*, 2001; Zeng *et al.*, 1997, 1998) exhibit a punctate subnuclear distribution and are associated with the nuclear matrix (Zeng *et al.*, 1997). Biochemical and *in situ* immunofluorescence analyses established that a 31-residue segment designated the nuclear matrix targeting signal (NMTS) near the C terminus of the Runx factor is necessary and sufficient to mediate association of these regulatory proteins to nuclear matrix-associated subnuclear sites at which transcription occurs (Zaidi *et al.*, 2001; Zeng *et al.*, 1997, 1998). The NMTS functions autonomously and can target a heterologous protein to the nuclear matrix. Furthermore, the NMTS is independent of the DNA-binding domain as well as the nuclear localization signal, both of which are in the N-terminal region of the Runx protein. The unique peptide sequence of the Runx NMTS (Zaidi *et al.*, 2001; Zeng *et al.*, 1997, 1998) and the defined structure obtained by x-ray crystallography (Tang *et al.*, 1998a, 1999) support the specificity of this targeting signal. These data are compatible with a model in which the NMTS functions as a molecular interface for specific interaction with proteins and/or nucleic acids that contribute to the structural and functional activities of nuclear domains. However, at present we cannot formally distinguish whether this interaction between the NMTS and its putative nuclear acceptor strictly reflects targeting or retention.

The idea that specific mechanisms direct regulatory proteins to sites within the nucleus is reinforced by the identification of targeting signals in the glucocorticoid receptor (Htun *et al.*, 1996; Tang *et al.*, 1998b; van Steensel *et al.*, 1995), PTHrP (Nguyen and Karaplis, 1998), the androgen receptor (van Steensel *et al.*, 1995), PIT1 (Stenoien *et al.*, 1998), SATB2 (Dobreva *et al.*, 2006), and YY1 (Guo *et al.*, 1995; McNeil *et al.*, 1998). These targeting signals do not share sequence homology with each other or with the Runx/Cbfa transcription factors. Furthermore, the proteins each exhibit distinct subnuclear distributions. Thus, a series of trafficking signals are responsible for directing regulatory factors to nonoverlapping sites within the cell nucleus. Collectively, the locations of these transcription factors provide coordinates for the activity of gene regulatory complexes.

The importance of architectural organization of regulatory machinery for bone-restricted gene expression is evident from the intranuclear localization of Runx2 co-regulatory proteins that control OC gene expression. For example, TLE/Groucho, a suppressor of Runx-mediated transcriptional activation, colocalizes with Runx2 at punctate subnuclear sites (Javed *et al.*, 2000). The Yes-associated protein represents another example of a Runx co-regulatory factor that is directed to Runx subnuclear sites when associated with Runx (Zaidi *et al.*, 2004).

Intranuclear targeting of regulatory factors is a multistep process, and we are only beginning to understand the complexity of each step. However, biochemical and *in situ* analyses have shown that at least two trafficking signals are required: The first supports nuclear import (the nuclear localization signal) and the second mediates interactions with specific sites associated with the nuclear matrix (the nuclear matrix-targeting signal). Given the multiplicity of determinants for directing proteins to specific destinations within the nucleus, alternative splicing of messenger RNAs might generate different forms of a transcription factor that are targeted to specific intranuclear sites in response to diverse biological conditions. Furthermore, the activities of transcription complexes involve multiple regulatory proteins that could facilitate the recruitment of factors to sites of architecture-associated gene activation and suppression.

Intranuclear Informatics

Recently, mathematical algorithms, designated intranuclear informatics, have been developed to identify and assign unique quantitative signatures that define regulatory protein localization within the nucleus (Young *et al.*, 2004). Quantitative parameters that can be assessed include nuclear size and variability in domain number, size, spatial randomness, and radial positioning (Fig. 5).

The significance and implication of intranuclear informatics can be shown by three distinct biological examples (see Figs. 5B–D). Regulatory proteins with different activities can be subjected to intranuclear informatics analysis, which assigns each protein a unique architectural signature. The overlap between the architectural signatures of different proteins is often correlated to their functional overlap. Alternatively, the subnuclear organization of a protein domain can be linked with subnuclear targeting, biological function, and disease. For example, Runx2 and its subnuclear targeting defective mutant (mSTD) show distinct architectural signatures, indicating that the biological activity of a protein can be defined and quantified as subnuclear organization. Finally, the data can be used to define functional conservation: For example this technique can be used to show that the postmitotic restoration of the spatially ordered Runx subnuclear organization is functionally conserved.

Integration of Osteogenic Signaling Pathways

Gene expression during skeletal development and bone remodeling is controlled by a broad spectrum of regulatory signals that converge at promoter elements to activate or repress transcription in a physiologically responsive manner. The subnuclear compartmentalization of transcription machinery necessitates a mechanistic explanation for directing signaling factors to sites within the nucleus where gene expression occurs under conditions that support integration of regulatory cues. The interactions of YAP and SMAD co-regulatory proteins with C-terminal segments of the Runx2 transcription factor permits assessment of requirements for recruitment of c-Src and BMP/TGF-mediated signals to skeletal target genes. Our findings indicate that nuclear import of YAP and SMAD co-regulatory factors is agonist dependent. However, there is a stringent requirement for fidelity of Runx subnuclear targeting for recruitment of these signaling proteins to active subnuclear foci transcriptionally. Our results demonstrate that the interactions and spatial-temporal organization of Runx and SMAD as well as YAP co-regulatory proteins are essential for the assembly of transcription machinery that supports expression or repression of skeletal genes (Zaidi *et al.*, 2004). Competency for intranuclear trafficking of Runx proteins has similarly been functionally linked with the subnuclear localization and activity of TLE/Groucho co-regulatory proteins (Javed *et al.*, 2000). These findings are consistent with Runx proteins serving as scaffolds for combinatorial interactions with co-regulatory proteins that contribute to biological control and a requirement for intranuclear trafficking to complete the transduction and implementation of regulatory signals that are requisite for physiological responsiveness.

Epigenetic Control of Cell Growth, Proliferation, and Sustained Phenotype

Postmitotic gene expression requires restoration of nuclear organization and assembly of regulatory complexes. In addition, skeletal development as well as osteoblast cell growth and function require stringent control of ribosomal biogenesis. Genetic alterations that deregulate ribosome production (e.g., Treacher Collins syndrome) result in craniofacial bone defects and growth retardation. Using *in situ* immunofluorescence microscopy, we have demonstrated that Runx2 remains associated with mitotic chromosomes at target gene loci to support lineage-specific transcription and is quantitatively distributed into postmitotic progeny cells (Young *et al.*, 2007b; Zaidi *et al.*, 2003). These findings suggest an epigenetic function of Runx2 at mitotic

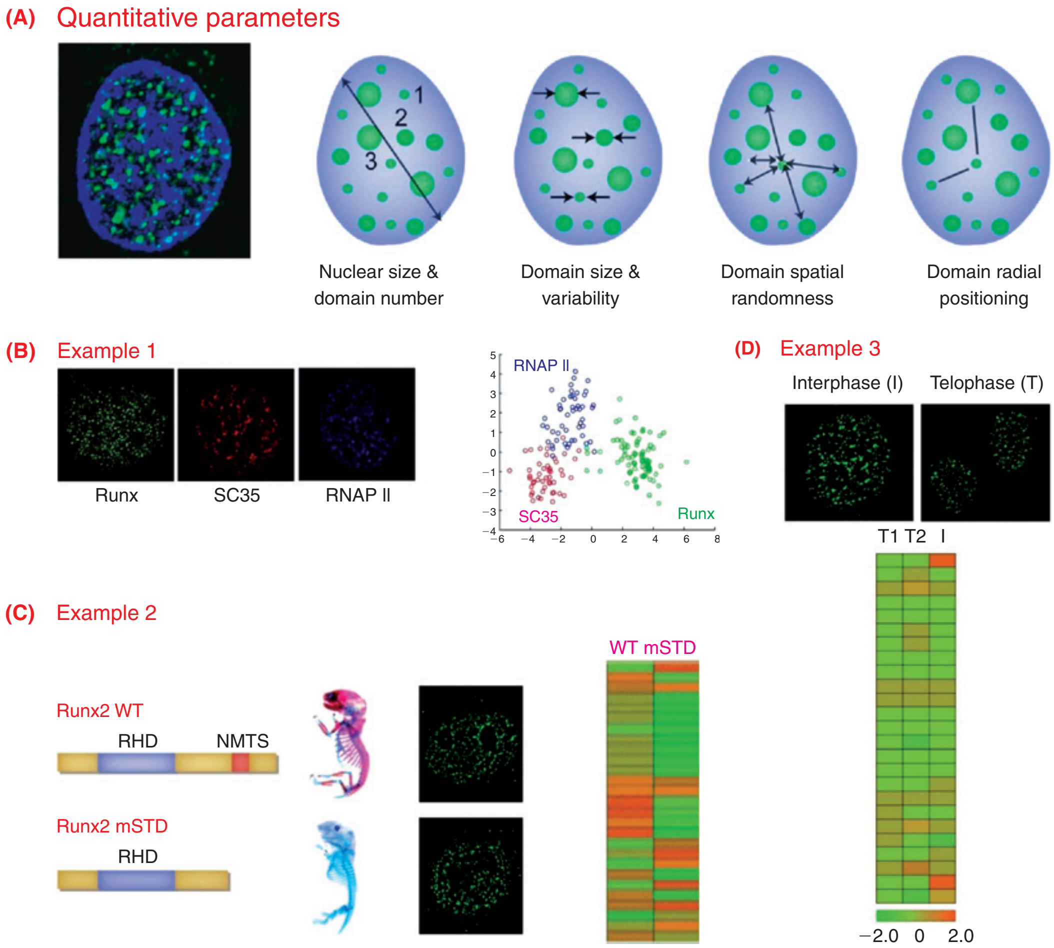


FIGURE 5 Intranuclear informatics. **A**, The conceptual framework for the quantitation of subnuclear organization by intranuclear informatics. The four main groups of parameters examined are based on inherent biological variability. **B**, Regulatory proteins with different activities can be subjected to intranuclear informatics analysis, which assigns each protein a unique architectural signature. The overlap between the architectural signatures of different proteins is often correlated with their functional overlap. Shown in **B** are Runx transcription factor (green), SC35 splicing protein (red), and RNA polymerase II (blue). These data obtained from intranuclear informatics can be presented in various forms such as a graph (shown in **B**) comparing two parameters: domain spatial randomness (on the *x*-axis) and domain radial positioning (on the *y*-axis). One hundred nuclei (each represented as one circle on the graph) co-stained for RUNX2, SC35, and RNA pol II were analyzed. As shown here, each of the three regulatory proteins (Runx, SC35, RNA polymerase II) exhibits distinct properties for the two parameters represented. We attribute these unique architectural signatures to distinct functional properties of these proteins. **C**, The subnuclear organization of Runx domains is linked with subnuclear targeting, biological function and disease. Biologically active RUNX2 and an inactive subnuclear targeting defective mutant of RUNX2 (mSTD) show distinct architectural signatures, indicating that the biological activity of a protein can be defined and quantified as subnuclear organization. The wild-type and mSTD RUNX2 proteins are schematically depicted in **C**. Alizarin red (bone) and alcian blue (cartilage) staining of skeletons from mice homozygous for wild-type and mSTD RUNX2 show a complete absence of mineralized bone in mSTD RUNX2 knock-in mice. Although both proteins exhibit similar patterns of subnuclear organization *in situ* in whole cell preparations (shown by the green fluorescence), intranuclear informatics shows that each protein exhibits a distinct architectural signature. All 28 quantitative parameters analyzed by intranuclear informatics are presented as hierarchical clusters. The green color represents the presence and red color represents absence of a specific nuclear feature. **D**, Postmitotic restoration of the spatially ordered subnuclear organization of Runx is functionally conserved. ROS 17/2.8 osteosarcoma cells were subjected to *in situ* immunofluorescence microscopy for endogenous RUNX2. RUNX2 is distributed at punctate subnuclear domains throughout the interphase and telophase nuclei. Subnuclear organization parameters were computed from deconvolved images for RUNX2 for interphase nuclei (I), and both progeny telophase nuclei, denoted arbitrarily as telophase nucleus 1 (T1) or telophase nucleus 2 (T2). A color map has been applied to the standardized data assigning red to higher values and green to lower values. (See plate section)

chromosomes. This epigenetic role of Runx2 at M/G1 transition is further confirmed by the association of Runx2 with phenotypic genes during mitosis (Fig. 6). In addition to controlling phenotypic genes at M/G1 transition, ribosomal RNA biosynthesis is also regulated by the bone-related Runx2 transcription factor that controls the proliferative potential of osteoprecursors and osteogenic lineage commitment (Fig. 7; Young *et al.*, 2007a). We find that reduction of Runx2 levels in human Saos-2 cells by siRNA modulates rRNA transcription, indicating that ribosomal gene production is indeed Runx2 responsive. Furthermore, a bioinformatics analysis reveals the presence of multiple Runx binding elements within regulatory regions of rRNA genes. Chromatin immunoprecipitation (ChIP) analysis

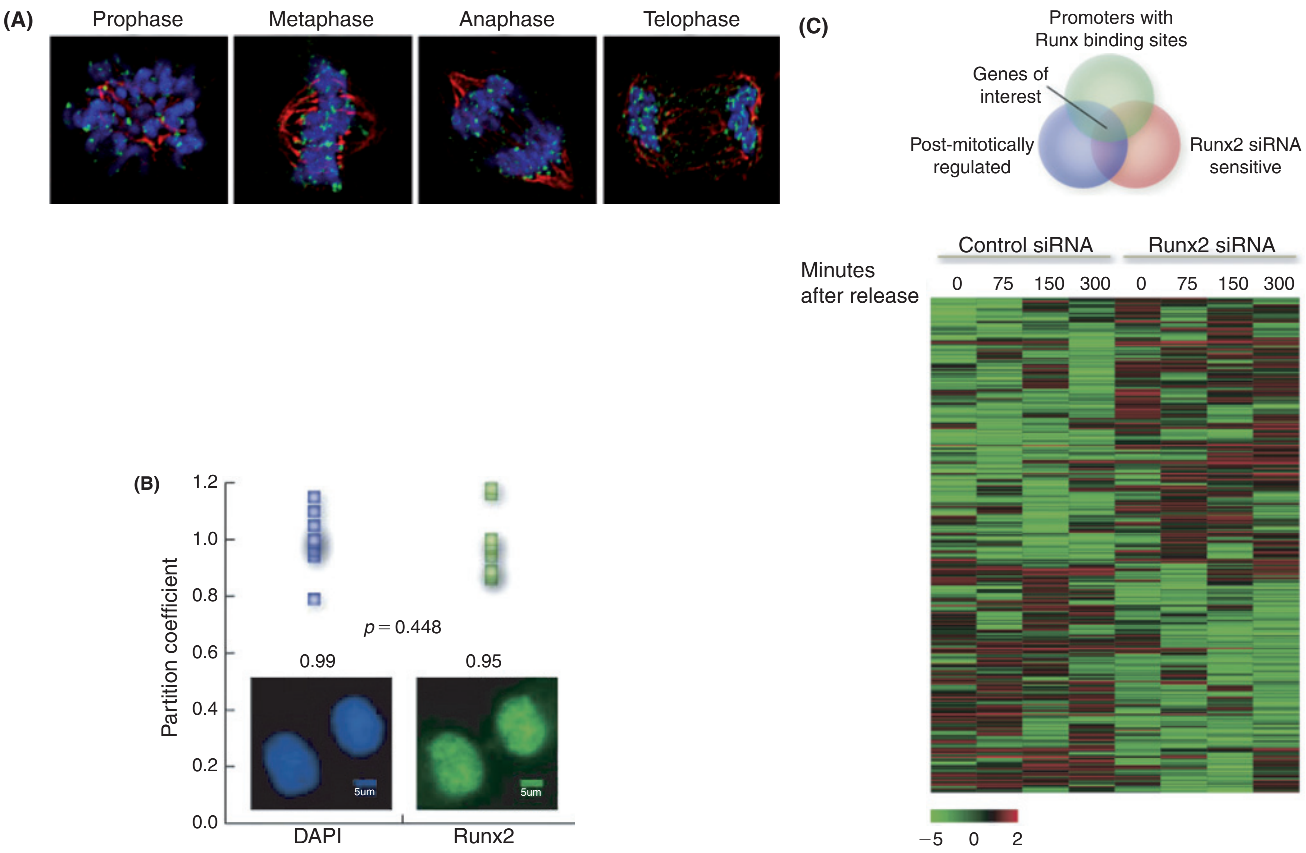


FIGURE 6 Runx2 confers phenotype commitment from parental to progeny cells by associating with phenotypic genes during mitosis. **A**, Asynchronously growing Saos-2 cells were fixed and stained for DNA using DAPI and for Runx2 using a rabbit polyclonal antibody. Mitotic cells were identified by chromosome morphology. High-resolution images obtained by three-dimensional deconvolution algorithms reveal that Runx2 (green) is localized in mitotic chromosomes. A subset of Runx2 colocalizes with the microtubules, labeled by α-tubulin staining (red). **B**, Runx2 protein partitions equivalently to progeny cells following cell division. A quantitative image analysis was applied to determine the relative levels of Runx in nuclei of the telophase cells ($n = 10$; *lower*). We defined a PC that reflects the ratio of integrated signal intensities between progeny nuclei. Runx2 exhibited a PC equivalent to that of DNA, demonstrating that this factors is equally segregated in progeny cells after cell division. Student's t test was performed to assess the significance of observed differences. **C**, Genome-wide identification of Runx2–sensitive gene expression patterns during the mitosis to G1 transition. Runx2 and control siRNA-treated cells were synchronized by nocodazole and mitotic shakeoff. Mitotic cells were isolated at shakeoff (0 hours), and remaining cells were replated and released for progression into G1 (1.25, 2.5, and 5 hours). Using an empirical Bayes linear modeling approach, we identified 500 genes significantly altered by siRNA treatment. A heat map illustrating hierarchical cluster analysis is shown. Two main clusters reflect genes that are repressed and activated by Runx2 knockdown. (See plate section)

establishes that Runx2 directly associates with ribosomal RNA genes. Both immunofluorescence and immunoelectron microscopy reveal that a subset of Runx2 is localized to nucleoli where ribosomal genes reside and ribosomal biogenesis occurs. Interestingly, we find that during the mitotic silencing of gene expression, Runx2 is localized at chromosomal foci that are associated with open chromatin at nucleolar organizing regions and colocalize with the RNA polymerase I transcription factor and upstream binding factor (UBF1). Functional linkage between Runx2 and ribosomal gene expression is further established by enhanced ribosomal RNA synthesis in primary cells isolated from the calvarial tissue of Runx2 null mice compared with wild-type Runx2 counterparts. Notably, induction of Runx2 in uncommitted mesenchymal cells directly represses ribosomal biogenesis, and this repression of ribosomal gene expression by Runx2 is associated with cell growth inhibition and expression of osteoblast-specific genes (Young *et al.*, 2007a, 2007b; Zaidi *et al.*, 2003). These results indicate that the function of Runx2 as a master regulator of osteoblast differentiation is coupled to its ability to modulate the anabolic function of osteoblasts.

ARCHITECTURAL REQUIREMENT OF NUCLEAR ORGANIZATION FOR FORMATION OF OSTEOLYTIC LESIONS BY METASTATIC TUMORS

A striking feature of tumor that metastasize to bone is the upregulation of skeletal genes that include OC, OP, and ON. Consequently, understanding of the regulatory

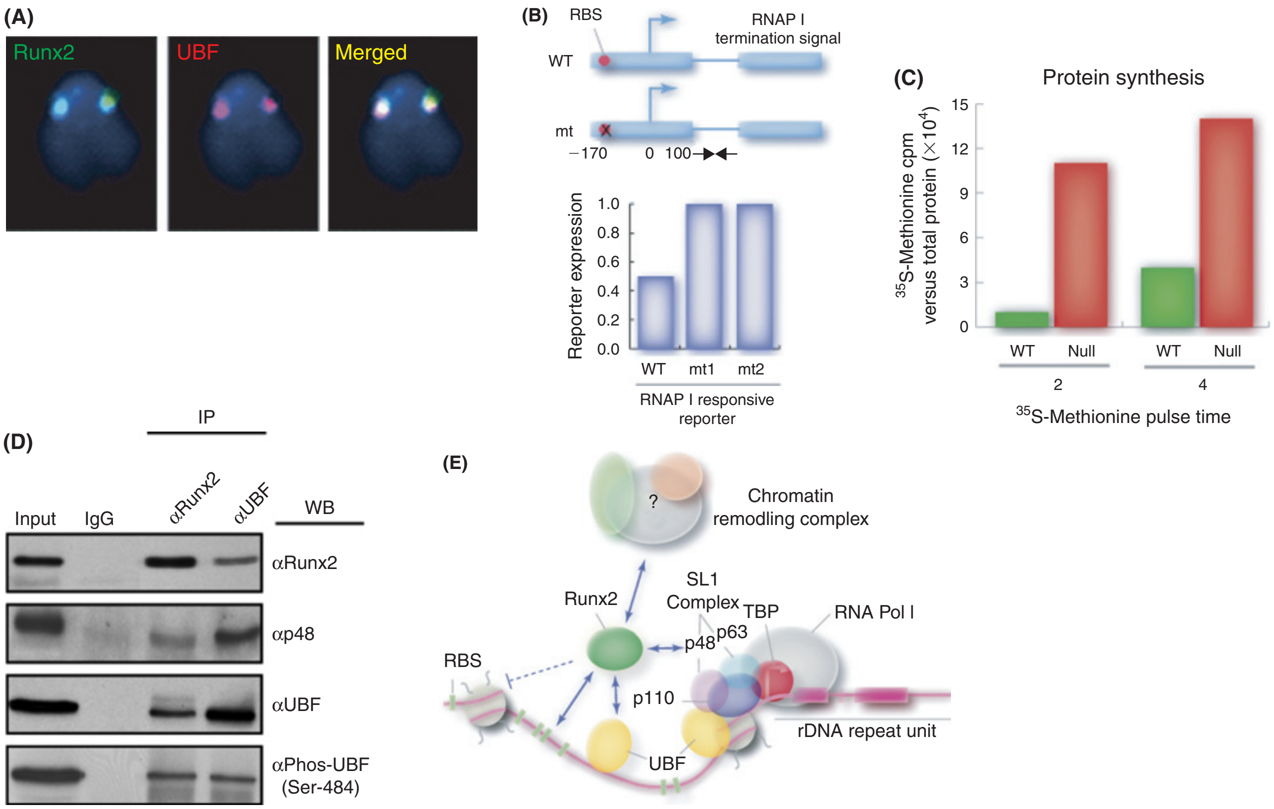


FIGURE 7 Runx2 regulates ribosomal RNA genes. (**A**) Immunofluorescence microscopy for Runx2 (green) and UBF1 (red) with DAPI staining (blue) and overlay for colocalization. Mitotic chromosome spreads from MCF-10A show that Runx2 colocalizes with nucleolar organizing regions (NORs). (**B**) Schematic of the rDNA-promoter reporters (wild-type [WT] and mutant [mt]) are shown with Runx binding site in red circle. RT-qPCR based reporter assay shows Runx2 inhibition of rDNA transcription. MC3T3 cells were transfected with hemagglutinin (HA) tagged *Runx2*. **C**, Primary calvarial cells from homozygous mouse embryos (17.5 days postcoitus) with WT or null Runx2 alleles. Total protein synthesis was measured by ^{3}H-methylmethionine incorporation at 0.5, 1, 2 and 4 hours using autoradiography. Incorporation of ^{35}S-methionine into proteins was measured by scintillation counting and normalized to total protein. **D**, Immunoprecipitates (IP) of endogenous Runx2 and UBF1 from osteoblastic cells react with antibodies against Runx2, UBF1, phospho-UBF1, or the p48 subunit of the SL1 complex in Western blots (WB). **E**, Diagram depicts lineage-specific regulation of rRNA synthesis. (See plate section)

mechanisms that control skeletal gene expression in tumors can provide insight into strategies for treatment. Our lab has demonstrated that transcription of skeletal genes in metastatic breast/prostate cancer cells is controlled by Runx2. We therefore determined the requirement for fidelity of Runx subnuclear localization for expression of bone proteins in breast/prostate cancers. The significance of intranuclear targeting is provided by single amino acid substitutions in the NMTS of lineage-specific Runx regulatory proteins. These mutations modify subnuclear targeting of Runx proteins and prevent osteolytic activity of metastatic breast cancer cells *in vivo* (Fig. 8). These findings suggest that therapeutic restoration of altered regulatory protein subnuclear targeting in leukemia and solid tumors may enhance capabilities for diagnosis and/or targeted treatment of cancer. The effectiveness of this strategy requires further understanding of the extent to which other regulatory proteins exhibit modified subnuclear targeting in metastatic cancers. Consistent with the subnuclear localization for tumor-related osteolysis or formation of osteoblastic lesions, perturbations in competency for subnuclear localization of Runx1 in myeloid progenitor cells leads to expression of a transformed phenotype (Barnes *et al.*, 2004; Javed *et al.*, 2005; Vradii *et al.*, 2005).

Runx2 Contributes to the Sequence of Metastatic Events through Regulation of Target Genes

One of the earliest indications of Runx2 involvement in the metastatic process was the characterization of the spectrum of matrix proteins, extracellular matrix signaling factors, and enzymes involved in bone turnover that are directly activated by Runx2 and are factors that have long been characterized as associated with stages of tumor progression and metastatic events. For example, Runx2 activates integrins in breast and prostate cancer cells (Pratap *et al.*, 2006). The vascular endothelial growth factor, essential for vascularization of the growth plate, has been shown to require Runx2 for activation during normal bone development (Zelzer *et al.*, 2001). Runx2 contributes to BSP and

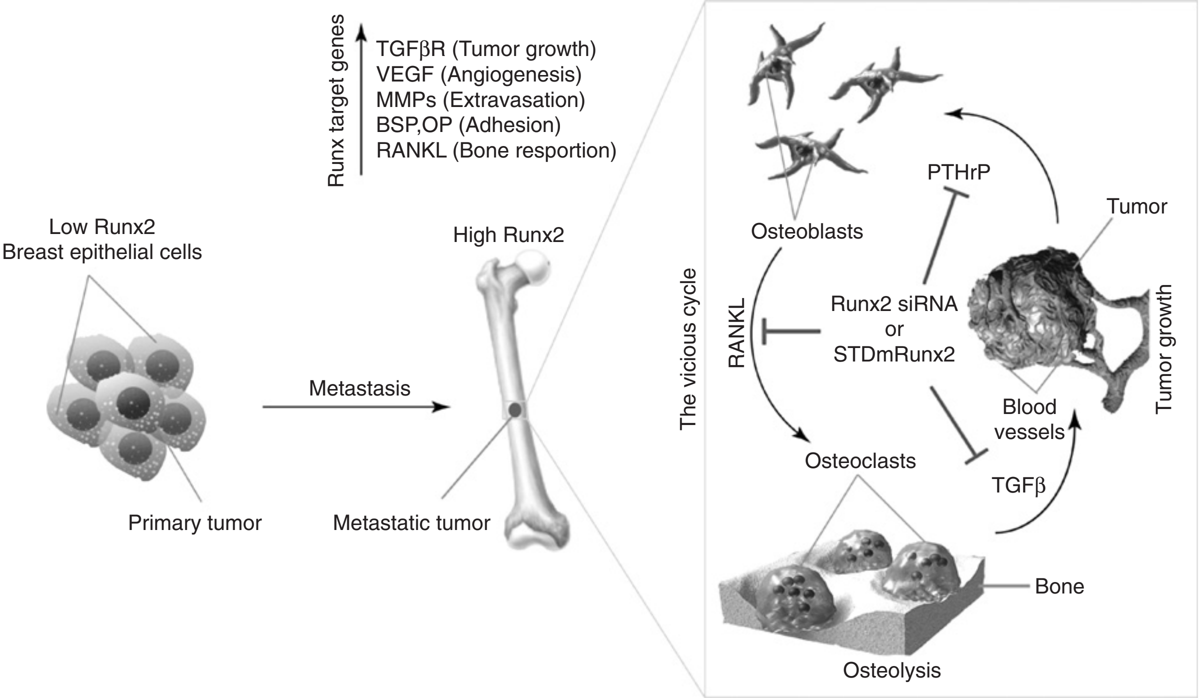


FIGURE 8 Vicious cycle of metastatic bone disease between breast cancer cells and bone. Once cancer cells arrive in bone, the four major participants in this vicious cycle include the cancer cells, osteoblasts, osteoclasts, and mineralized bone matrix, a major source of immobilized growth factors. Prostate cancer cells secrete factors that stimulate osteoblasts to proliferate, differentiate, and secrete growth factors. These factors are deposited into the bone matrix and also enrich the local microenvironment of the tumor cells. Tumor cells secrete osteolytic factors, most of which act via osteoblast production of the osteoclast differentiation factor RANKL. Growth factors released from the mineralized bone matrix as a consequence of osteoclastic bone resorption further enrich the local milieu. These interactions reinforce each other to accelerate cancer progression. Several of these factors are direct targets of Runx2 transcription factor, which is upregulated in highly aggressive prostate and breast cancer. When cancer cells expressing the mSTD Runx2 are introduced in mice, these cells lack the expression of cancer growth-promoting factors and do not form osteolytic lesions.

OPN, which have been observed in cancers with a high propensity for forming bone metastases, and these proteins are associated with poor survival (Bellahcene *et al.*, 1996; Bramwell *et al.*, 2006; Sharp *et al.*, 2004). Thus, high levels of Runx2 expression in the primary tumor may contribute to early events of tumor vascularization and modulate invasive properties of metastatic and nonmetastatic cancer cells (Pratap *et al.*, 2005).

Matrix metalloproteinases (MMPs) have long been implicated in tumor invasion and metastasis (Egeblad and Werb, 2002). Runx2 directly activates MMP genes, MMP9 and MMP13 (Pratap *et al.*, 2005; Selvamurugan *et al.*, 2004). We have reported that Runx2 siRNA knockdown decreases MMP9 expression in bone metastatic MDA-MB-231 breast cancer cells and reduces their invasive potential through matrigel (Pratap *et al.*, 2005). Furthermore, overexpression of Runx2 in poorly metastatic MCF-7 cells results in a significant increase in invasiveness in the matrigel invasion assay, concomitant with increased expression of several markers of metastasis (MMP2, MMP9, MMP13, and VEGF). Thus Runx2 represents a viable candidate for therapeutic targeting in primary tumors to block expression of many genes related to metastasis to bone.

Subnuclear Targeting Supports Metastasis and Osteolytic Bone Disease

Direct evidence for a functional role for Runx2 in metastatic cell lines was provided by inhibition of tumor growth, and osteolytic disease in the intratibial model of bone metastasis. The highly aggressive MDA-MB-231 cell lines were modified with stably integrated mutant Runx2 mutant proteins. MDA-MB-231 cells express high levels of Runx2 in striking contrast to MCF-7, the nonmetastatic breast cell line. Two Runx2 mutant proteins, either a dominant negative (Runx2 Δ230) or a point mutation protein that is defective in intranuclear targeting of Runx2 to transcriptionally active subnuclear domains (targeting deficient [TD] mRunx2 R398A/Y428A) when expressed in MDA-MB-231, decrease functional activity of the endogenous Runx2 in MDA-MB-231, breast cancer cells. As a result, *in vitro* invasive and *in vivo* bone osteolytic properties of the parental cells were blocked (Barnes *et al.*, 2004; Javed *et al.*, 2005). These findings suggest that fidelity of Runx2 intranuclear organization is obligatory for expression of target genes that mediate the osteolytic activity of metastatic breast cancer cells (Javed *et al.*, 2005).

Runx2 organization of regulatory complexes in the nuclear microenvironment of cancer cells can promote the end stage of metastasis to bone as a result of the formation of Runx2 protein–protein complexes. Of the many signaling proteins secreted by cancer cells, TGFβs and BMPs have long been implicated in early events of both tumorigenesis and metastasis as well as facilitating tumor growth and the osteolytic disease (Grimm and Rosen, 2006; Guise and Chirgwin, 2003; Yoneda *et al.*, 2001). TGFβ signals through both the Smad pathway and the mitogen-activated protein (MAP) kinase pathways and stimulates PTHrP secretion (Kakonen *et al.*, 2002). Runx2 protein is a downstream target of both pathways and plays a central role as a transcriptional mediator of Smad signaling (Chang *et al.*, 2002; Ji *et al.*, 2001; Kang *et al.*, 2005). This appears to be a critical component of the Runx2 activity in facilitating metastatic bone disease as tumor cells in the bone environment respond to TGFβ to continue the vicious cycle of tumor growth and osteolytic diseases (Chirgwin *et al.*, 2004; Feeley *et al.*, 2005; Fizazi *et al.*, 2003). PTHrP is stimulated by TGFβ (Kakonen *et al.*, 2002) and is now established as one of the principal factors responsible for activating the process of bone loss. Runx2 is also linked to RANKL production as well as inhibition of osteoprotegerin, an antagonist of osteoclast differentiation, thereby contributing to the osteolytic disease (Enomoto *et al.*, 2003). Thus identification of a master regulatory transcription factor Runx2, which mediates the TGFβ signaling pathway and controls the expression of genes directly linked to stages of tumor metastasis to bone and the accompanying bone disease, provides an opportunity to target an upstream regulator of many tumorigenic activities.

FUNCTIONAL INTERRELATIONSHIPS BETWEEN NUCLEAR STRUCTURE AND SKELETAL GENE EXPRESSION

The regulated and regulatory components that interrelate nuclear structure and function must be established experimentally. A formidable challenge is to define further the control of transcription factor targeting to acceptor sites associated with the nuclear matrix. It will be important to determine whether acceptor proteins are associated with a preexisting core filament structural lattice or whether a compositely organized scaffold of regulatory factors is assembled dynamically.

An inclusive model for all steps in the targeting of proteins to subnuclear sites cannot yet be proposed. However, this model must account for the apparent diversity of intranuclear targeting signals. It is also important to assess the extent to which regulatory discrimination is mediated by subnuclear domain-specific trafficking signals. Furthermore, the checkpoints that monitor the subnuclear distribution of regulatory factors and the sorting steps that ensure both structural and functional fidelity of nuclear domains in which replication and expression of genes occur must be defined biochemically and mechanistically.

There is emerging recognition that the placement of regulatory components of gene expression must be coordinated temporally and spatially to facilitate biological control. The consequences of breaches in nuclear structure–function relationships are observed in an expanding series of diseases that include cancer (McNeil *et al.*, 1999; Rogaia *et al.*, 1997; Rowley, 1998; Tao and Levine, 1999; Weis *et al.*, 1994; Yano *et al.*, 1997; Zeng *et al.*, 1998; Zhang *et al.*, 2000) and neurological disorders (Skinner *et al.*, 1997). Findings indicate the requirement for the fidelity of Runx/Cbfa/AML subnuclear localization to support regulatory activity for skeletogenesis *in vivo*. Although many of the human mutations in Runx2 associated with cleidocranial dysplasia occur in the DNA-binding domain, several mutations have been identified in the C terminus, which disrupt nuclear matrix association (Zhang *et al.*, 2000). As the repertoire of architecture-associated regulatory factors and cofactors expands, workers in the field are becoming increasingly confident that nuclear organization contributes significantly to the control of transcription. To gain increased appreciation for the complexities of subnuclear organization and gene regulation, we must continue to characterize mechanisms that direct regulatory proteins to specific transcription sites within the nucleus and sustain regulatory complex association with gene loci on mitotic chromosomes for epigenetic control of lineage and phenotype for these proteins to be in the right place at the right time.

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Chapter 15

Type I Collagen Structure, Synthesis, and Regulation

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INTRODUCTION

It has been almost 150 years since the term "collagen" was first adopted in the English language. This rope-like structure that yields gelatin upon boiling made its early appearance in evolution in primitive animals such as jellyfish, coral, and sea anemones (Bergeon, 1967). Today, the collagen family of proteins has grown to 28 different types and is used as a versatile biomaterial for delivery of drugs as well as for cosmetic purposes.

During those 150 years our understanding of collagen has evolved with advances in techniques and technology. The work of Nageotte in the early 1920s used acid solubilization to reveal the fibers that histologists had earlier described in sections of connective tissues (Nageotte, 1927); x-ray diffraction and then electron microscopy characterized those fibers that made up the collagen molecule. In addition to collagens involved in fibril formation, several other groups of nonfibrillar collagen have been discovered. Among these, some are involved in membrane formation that surrounds tissues such as basement and Descemet's membranes, cuticle of worms, and skeleton of sponges.

In this chapter, we are focusing on fibrillar collagens and, in particular, collagen type I, the most abundant extracellular protein, especially in bone, where it is essential for bone strength. We will discuss the structure and biosynthesis of type I collagen and associated proteins that maintain its homeostasis and recent results into the organization of regulatory elements in type I collagen genes, many of which are based on studies in transgenic mice. Then we will address how collagen synthesis is regulated by cytokines and growth factors.

The accepted definition of collagens is: "structural proteins of the extracellular matrix which contain one or more domains harboring the conformation of a collagen triple helix" (Myllyharju and Kivirikko, 2004; van der Rest and Garrone, 1991). The triple-helix motif is composed of three polypeptide chains whose amino acid sequence consists of Gly-X-Y repeats. Because of this particular peptide sequence, each chain is coiled in a left-handed helix, and the three chains assemble in a right-handed triple helix, where Gly residues are in the center of the triple helix and where the lateral chains of X and Y residues are on the surface of the helix (van der Rest and Garrone, 1991). In about one-third of the cases, X is a proline and Y is a hydroxyproline; the presence of hydroxyproline is essential to stabilize the triple helix and is a unique characteristic of collagen molecules. At the time of this review, 28 different types of collagens have been described, which are grouped in subfamilies depending on their structure and/or their function. The roman numerals denoting collagen types follow the order in which they were reported. For each collagen type, the alpha chains are identified with Arabic numerals (Myllyharju and Kivirikko, 2001). Although a standard nomenclature has been agreed on, the representation of the collagen names can be sometimes confusing. Throughout this chapter, we will address the unassembled collagen molecules as procollagens, the mouse gene as *Col1a1* or *Col1a2* (lower case) and the human in capital letters (*COL1A1* or *COL1A2*).

FIBRILLAR COLLAGENS

Types I, II, III, V, and XI and the newly described types XXIV and XXVII collagens (Boot-Handford *et al.*, 2003; Koch *et al.*, 2003) form the group of fibrillar collagens. The characteristic feature of fibrillar collagens is that they consist of a long continuous triple helix that self-assembles

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into highly organized fibrils. These fibrils have a very-high-tensile strength and play a key role in providing a structural framework for body structures such as skeleton, skin, blood vessels, intestines, or fibrous capsules of organs. Type I collagen, which is the most abundant protein in vertebrates, is present in many organs and is a major constituent of bone, tendons, ligaments, and skin. Type III collagen is less abundant than type I collagen, but its distribution essentially parallels that of type I collagen with the exception of bones and tendons, which contain virtually no type III collagen. Moreover, type III collagen is relatively more abundant in distensible tissues, such as blood vessels, than in nondistensible tissues. Type V collagen is present in tissues that also contain type I collagen. Type II collagen is a major constituent of cartilage and is also present in the vitreous body. Like type II, type XI and type XXVII collagens are present in cartilage. However, unlike other collagens, type XXVII appear to express in epithelial cells of cochlea, lung, gonad, and stomach (Boot-Handford *et al.*, 2003), suggesting that its function in these epithelial layers can not depend on the copolymerization with other collagens. Collagen type XXIV displays unique structural features of invertebrate fibrillar collagens and is expressed predominantly in bone tissue (Matsuo *et al.*, 2006).

Bone formation is a complex and tightly regulated genetic program that involves two distinct pathways at different anatomical locations (de Crombrugghe *et al.*, 2001; Karsenty and Wagner, 2002; Olsen *et al.*, 2000). In intramembranous ossification, mesenchymal cells condense and differentiate directly into mainly collagen type I producing osteoblasts, whereas in endochondral bone formation, a cartilage model that is initially rich in type II and type XI collagens, which are secreted by chondrocytes, is replaced by an osteum rich in collagen type I matrix. Cartilage formation in endochondral skeletal elements is initiated by the condensation of chondrogenic mesenchymal cells followed by the overt differentiation of cells in these condensations. After undergoing a unilateral form of proliferation, these cells gradually become hypertrophic. At the same time, cells around the condensations form the perichondrial layer that gives rise to the osteoblast-forming periosteum and ultimately to cortical bone. The process of cartilage replacement by a bone matrix involves invasion by preosteoblasts in the periosteum as well as blood vessels and hematopoietic cells of the zone of hypertrophic chondrocytes. Expression of the genes for collagen type I and those for collagen types II and XI follow distinct transcriptional codes that control osteoblastogenesis and chondrogenesis (Bridgewater *et al.*, 1998; de Crombrugghe *et al.*, 2001; Karsenty and Wagner, 2002; Lefebvre *et al.*, 2001; Lefebvre and de Crombrugghe, 1998). In addition to fibrillar collagens, collagen type X has been implicated in the morphogenic events of hypertrophic cartilage prior to their replacement by bone. Although knockout mice for collagen X showed no apparent phenotype (Rosati *et al.*, 1994), significant reduction in the amount and quality of bone minerals was evident (Paschalis *et al.*, 1996).

Collagen types XXIV and XXVII display mutually exclusive patterns of expression in the developing and adult mouse skeleton. Gene expression studies have shown that whereas *Col24a1* transcripts accumulate at ossification centers of the craniofacial, axial, and appendicular skeleton, *Col27a1* activity is instead confined to the cartilaginous anlagen of skeletal elements (Boot-Handford *et al.*, 2003; Koch *et al.*, 2003; Pace *et al.*, 2003). Additionally, structural considerations have suggested that collagens XXIV and XXVII are likely to form distinct homotrimers (Koch *et al.*, 2003). Together these observations have been interpreted to indicate that these newly discovered fibrillar collagens may participate in the control of important physiological processes in bone and cartilage, such as collagen fibrillogenesis and/or matrix calcification and mineralization (Boot-Handford *et al.*, 2003; Koch *et al.*, 2003; Pace *et al.*, 2003).

STRUCTURE, SYNTHESIS, AND ASSEMBLY OF TYPE I COLLAGEN

Structure

Fibril-forming collagens are synthesized in precursor form, procollagens. Each molecule of type I collagen is typically composed of two $\alpha1$ chains and one $\alpha2$ chain $[\alpha1(\mathrm{I})_2\text{–}\alpha2(\mathrm{I})]$ coiled around each other in a characteristic triple helix. Both the $\alpha1$ chain and the $\alpha2$ chain consist of a long helical domain preceded by a short N-terminal peptide and followed by a short C-terminal peptide (for reviews, see Myllyharju and Kivirikko, 2001, 2004, and van der Rest and Garrone, 1991).

The mechanism that controls the 2:1 stoichiometry of the collagen chains in type I collagen is not well understood. It is evident that a number of type I collagen molecules can be formed by three $\alpha1$ chains $[\alpha1(\mathrm{I})_3]$. The homotrimeric type I collagen isotype containing three pro $\alpha1(\mathrm{I})$ collagen chains $[\alpha1(\mathrm{I})_3]$ is a minor isotype, whose role is not well understood. Homotrimers are found embryonically (Jimenez *et al.*, 1977; Rupard *et al.*, 1988) in small amounts in skin (Uitto, 1979), in certain tumors and cultured cancer cell lines (Moro and Smith, 1977; Rupard *et al.*, 1988), and also during wound healing (Haralson *et al.*, 1987). Mesangial cells, which do not synthesize collagen type I *in vivo*, produce homotrimeric type I collagen in culture, further suggesting that homotrimers play a role in wound healing (Johnson *et al.*, 1992). The collagen I $\alpha2$-deficient mouse, otherwise known as the oim mouse (osteogenesis imperfecta model) is homozygous for a spontaneous nucleotide deletion in the *Col1a2* gene, resulting in a frameshift altering the carboxy-propeptide of the pro Col1a2 chain. Although the carboxy-propeptide is not present in mature type I collagen, it is responsible for association of the Col1a2 chain with the Col1a1 chains during assembly of the triple helix (Chipman *et al.*, 1993; Deak *et al.*, 1983; McBride *et al.*, 1997) (see sections on collagen diseases).

Type I collagen is secreted as a propeptide, but the N telopeptide and the C telopeptide are cleaved rapidly by specific proteases, ADAMTS 2 and BMP1, respectively, in order that shorter molecules assemble to form fibrils (Canty and Kadler, 2005). In fibrils, molecules of collagen are parallel to each other (Fig. 1); they overlap each other by multiples of 67 nm (distance D), with each molecule being 4.4 D (300 nm) long; there is a 40-nm (0.6 D) gap between the end of a molecule and the beginning of the other (see Fig. 1). This quarter-staggered assembly explains the banded aspect displayed by type I collagen fibrils in electron microscopy. In tissues, type I collagen fibrils can be parallel to each other and form bundles (or fibers), as in tendons, or they can be oriented randomly and form a complex network of interlaced fibrils, as in skin. In bone, hydroxyapatite crystals seem to lie in the gaps between collagen molecules.

Transcription

In humans the gene coding for the α1 chain of type I collagen is located on the long arm of chromosome 17 (17q21.3-q22), (chromosome 11 in mouse) and the gene coding for the α2 chain is located on the long arm of chromosome 7 (7q21.3-q22) (chromosome 6 in mouse). Both genes have a very similar structure (Chu *et al.*, 1984; D'Alessio *et al.*, 1988), and this structure is also very similar to that of genes coding for other fibrillar collagens (Vuorio and de Crombrugghe, 1990). The difference in size between the two genes (18 kb for the *Col1a1* gene and 38 kb for the *Col1a2* gene) is explained by differences in the size of the introns.

The triple helical domain of the α1 chain is coded by 41 exons, which code for Gly-X-Y repeats, and by two so-called joining exons. These joining exons code in part for the telopeptides and in part for Gly-X-Y repeats, which are part of the triple helical domain. The triple helical domain of the α2 chain is coded by 42 exons, plus two joining exons. Each of the corresponding exons coding for the triple helical domain of the α1 chain and for the triple helical domain of the α2 chain has a similar length (Table I). The only exception is that exons 34 and 35 in the *Col1a2*, which are 54 bp long each, correspond to a single 108-bp 34/35 exon in the α1 gene. Except for the two joining exons, each exon starts exactly with a G codon and ends precisely with a Y codon, and all the exons are 54, 108 (54 × 2), 162 (54 × 3), 45, or 99 bp long (see Table I). This organization suggests that exons coding for triple helical domains could have originated from the amplification of a DNA unit containing a 54-bp exon embedded in intron

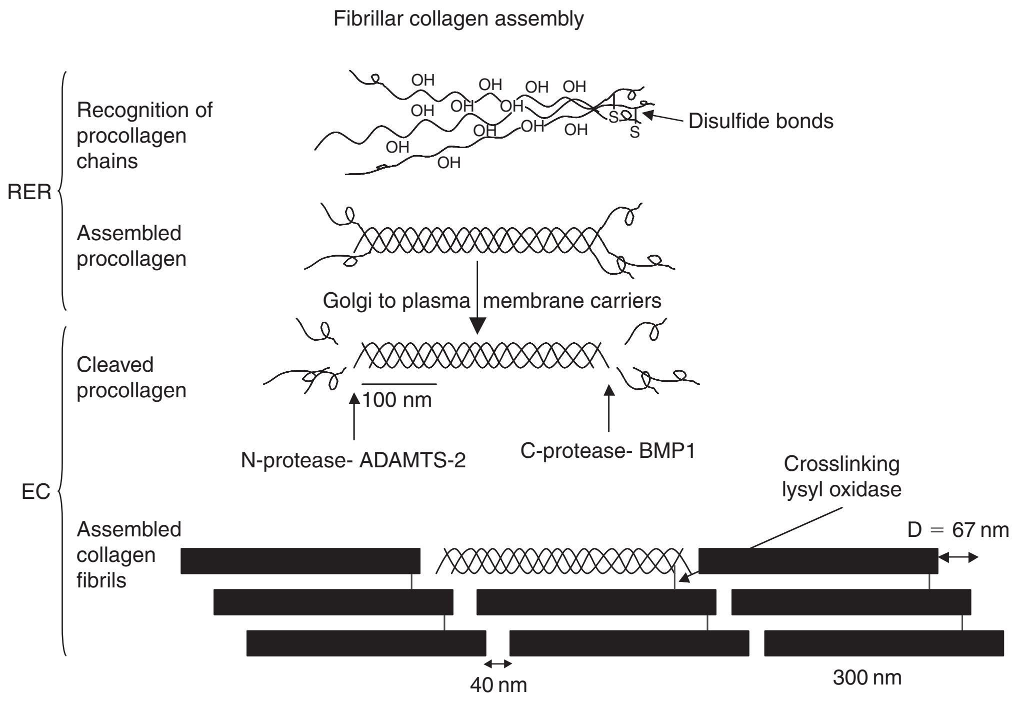

FIGURE 1 Schematic diagram of collagen assembly depicting the recognition of the alpha chains in the rough endoplasmic reticulum (RER) and the helix formation strengthened by the hydroxylated proline and lysine residues (OH). The collagen is then transported by Golgi to plasma membrane vesicles to extracellular space (EC) where the propeptides are removed by proteinases and the fibrils are assembled into collagen fibers that are crosslinked by lysyl oxidase.

TABLE I Size of Exons Coding for the Triple Helical Domain of Type I Collagen*

Exon	Size (bp)	Exon	Size (bp)	Exon	Size (bp)
7	45	21	108	35	54
8	54	22	54	36	54
9	54	23	99	37	108
10	54	24	54	38	54
11	54	25	99	39	54
12	54	26	54	40	162
13	45	27	54	41	108
14	54	28	54	42	108
15	45	29	54	43	54
16	54	30	45	44	108
17	99	31	99	45	54
18	45	32	108	46	108
19	99	33	54	47	54
20	54	34	54	48	108

**In the pro-α 1(I) collagen gene, exons 33 (54 bp) and 34 (54 bp) are replaced by a single 108-bp 33/34 exon. The two joining exons (exons 6 and 49) are not considered in this table (see text for details).*

sequences. One hundred and eight- and 162-bp exons would result from a loss of intervening introns. Forty-five- and 99-bp exons would result from recombinations between two 54-bp exons (Vuorio and de Crombrugghe, 1990).

For both the α1 chain and the α2 chain, the C propeptide plus the C telopeptide are coded by 4 exons (exons 48 to 51 of the *Col1a1* gene, exons 49 to 52 of the *Col1a2* gene). The first of these exons code for the end of the triple helical domain, the C-terminal telopeptide, and the beginning of the C-terminal propeptide. The three other exons code for the rest of the C-terminal propeptide. The C-terminal propeptide has a globular structure that is stabilized by two intrachain disulfide bonds (see Fig. 1). It contains three (α2 chain) and four (α1 chain) additional cysteine residues that form interchain disulfide bonds. The formation of disulfide bonds precedes the triple helix formation and plays an essential role in the intracellular assembly of the three a chains (see sections on translational and posttranslational modifications).

The signal peptide, the N propeptide, and the N telopeptide of the α1 chain, as well as of the α2 chain, are coded by the first six exons. The N propeptide of the α1 chain contains a cysteine-rich (10-cysteine residue) globular domain, a short triple helical domain, and a short globular domain, which harbors the N-terminal peptidase cleavage site (see Fig. 1). The N-terminal propeptide of the α2 chain does not contain a cysteine-rich domain but a short globular domain. The 3′-untranslated region of both the *Col1a1* gene and the *Col1a2* gene contains more than one polyadenylation site, which explains that mRNAs with different sizes will be generated. As in many other genes, the functional role of the different polyadenylation sites is still unknown.

Translation

After being transcribed, the pre-mRNA undergoes exon splicing, capping, and addition of a poly(A) tail, which gives rise to a mature mRNA. These mature mRNAs are then translated in polysomes, and the resulting proteins undergo extensive post-translational modifications before being assembled in a triple helix and released in the extracellular space (for reviews, see Lamande and Bateman, 1999; Myllyharju and Kivirikko, 2001).

Signal peptides are cleaved from the chains when their N-terminal end enters the cisternae of the rough endoplasmic reticulum. Both the pro-α1 chain and the pro-α2 chain undergo hydroxylation and glycosylation, and these modifications are essential for the assembly of type I collagen chains in a triple helix. About 100 proline residues in the Y position of the Gly-X-Y repeats, a few proline residues in the X position, and about 10 lysine residues in the Y position undergo hydroxylation, respectively, by a prolyl 4-hydroxylase, a prolyl 3-hydroxylase, and a lysyl hydroxylase. Hydroxylation of proline to hydroxyproline is critical to obtain a stable triple helix, and at 37°C, stable folding in a triple helical conformation cannot be obtained before at least 90 prolyl residues have been hydroxylated. These hydroxylases have different requirements to be active, and, in particular, they can act only when prolyl or lysyl residues occupy the correct position in the amino acid sequence of the a chain and when peptides are not in a triple helical configuration. Moreover, these enzymes require ferrous ions, molecular oxygen, α-ketoglutarate, and ascorbic acid to be active. This requirement for ascorbic acid could explain some of the consequences of scurvy on wound healing. When lysyl residues become hydroxylated, they serve as a substrate for a glycosyltransferase and for a galactosyltransferase, which add glucose and galactose, respectively, to the ε-OH group. As for hydroxylases, glycosylating enzymes are active only when the collagen chains are not in a triple helical conformation. Glycosylation interferes with the packaging of mature molecules into fibrils, and increased glycosylation tends to decrease the diameter of fibrils.

While hydroxylations and glycosylations described previously occur, after a mannose-rich oligosaccharide is added to the C propeptide of each pro α chain, C propeptides from two α1 chains and one α2 chain associate with the formation of intrachain and interchain disulfide bonds. After prolyl residues have been hydroxylated, and the three C propeptides have associated, a triple helix will form at the C-terminal end of the molecule and then extend toward the N-terminal end (see Fig. 1). This propagation of the triple helical configuration occurs in a "zipper-like fashion" (Prockop, 1990). If prolyl residues are not hydroxylated or if interchain disulfide bonds are not formed between the C propeptides, the a chain will not fold in a triple helix. Although the functions of the C-terminal sequences, which have been associated with initiation of triple helix formation, are thought to be well established, those of the N-terminal propeptide are poorly understood (Bornstein, 2002). The N propeptide of type I procollagen, as released physiologically by procollagen N-protease (ADAMTS 2), contains a globular domain largely encoded by exon 2 in the *Col1a1* gene, and a short triple helix that terminates in a non-triple-helical telopeptide sequence, which separates this helix from the major collagen helix. Bornstein and colleagues generated a mouse with a targeted deletion of exon 2 in the *Col1a1* gene, thus replicating the type IIB splice form of type II procollagen in type I procollagen (Bornstein *et al.*, 2002), surprisingly, homozygous mutant mice were essentially normal. In particular, none of the steps in collagen biogenesis thought to be dependent on the N propeptide were defective. However, there was a significant, but background-dependent, fetal mortality, which suggested a role for the type I collagen N propeptide in developmental processes.

Toman and colleagues have gone even further to demonstrate that propeptide may not be necessary for the selection and folding of procollagen. They engineered type I collagen genes that encode the N and C telopeptides with the entire triple helical domain and show that these sequences are sufficient for assembly of a triple helix in *Saccharomyces cerevisiae* (Olsen *et al.*, 2001). Other fibrillar collagens (types II, III, V, and XI) have a similar structure and thus would be expected to fold into triple helices without the propeptide regions in an analogous system.

Intracellular Transport

The newly formed triple-helical forms are then stabilized by Hsp47, a molecular chaperone of type I collagen molecules (Nagai *et al.*, 2000; Tasab *et al.*, 2000). This protein belongs to the serine protease inhibitor (serpin) superfamily containing a serpin signature sequence. Hsp47 resides in the endoplasmic reticulum (ER), as inferred from the presence of a carboxyl-terminal RDEL sequence similar to the ER retrieval signal, KDEL. Hsp47 binds to nascent procollagen chains in the ER of collagen-secreting cells and dissociates from them before reaching the *cis*-Golgi apparatus. Disrupting the *hsp47* gene in mice resulted in embryonic lethality in mice by 11.5 days postcoitus and caused a molecular abnormality in procollagens (Nagai *et al.*, 2000). Type I procollagen chains containing propeptides accumulated in the tissues, but the mature collagen chains normally processed were scarcely observed, suggesting that HSP47 is essential as a collagen-specific molecular chaperone for the proper processing of procollagen molecules, and the *hsp47* gene is needed for the normal development of mouse embryo (Nagai *et al.*, 2000). The Golgi complex with its associated Trans Golgi Network (TGN) consists of a complex network of anastomosing tubules. The TGN mediates the final modification of N-linked oligosaccharides to the complex and is involved in both the transport and sorting of membrane and secretory proteins (Griffiths and Simons, 1986). Previous works have shown that green fluorescent protein (GFP) fusion proteins are transported from the Golgi to the cell surface in tubular-saccular compartments that travel along microtubules (Hirschberg *et al.*, 1998; Polishchuk *et al.*, 2000; Puertollano *et al.*, 2003; Toomre *et al.*, 1999). These pleiomorphic Golgi to Plasma Membrane carriers (GPCs) can be 0.5 to 1.7 μm in length and have also been called transport containers (Toomre *et al.*, 1999) and post-Golgi carriers (Hirschberg *et al.*, 1998). A recent *in vitro* study has shown that exit from the TGN occurs by the formation of a tubular-reticular TGN domain that is a precursor structure to the release of tubular-saccular GPCs (Polishchuk *et al.*, 2003).

For the majority of cells, procollagen is secreted in the extracellular space, where a specific procollagen aminopeptidase (ADAMTS 2) and a specific procollagen carboxypeptidase (BMP1) cleave the propeptides, thereby triggering spontaneous self-assembly of collagen molecules into fibrils, giving rise to mature collagen molecules (Kadler, 2004; Kadler *et al.*, 1990). Cleavage of the propeptide decreases the solubility of collagen molecules dramatically. Thus a major extracellular function of C propeptides is thought to prevent fibril formation, while N propeptide influence fibril shape and diameter (Hulmes, 2002).

The free propeptides are believed to be involved in feedback regulation of collagen synthesis of types I and III collagens by fibroblasts in culture (Wiestner *et al.*, 1979). However, the mechanism of this inhibition remained elusive despite attempts by several groups to characterize it.

Fibrillogenesis

In the extracellular space, the molecules of mature collagen assemble spontaneously into quarter-staggered fibrils; this assembly is directed by the presence of clusters of hydrophobic and of charged amino acids on the surface of the molecules. Fibril formation has been compared with crystallization in that it follows the principle of "nucleated growth" (Prockop, 1990). Once a small number of molecules have formed a nucleus, it grows rapidly to form large fibrils. During fibrillogenesis, some lysyl and hydroxylysyl residues are deaminated by a lysine oxidase, which deaminates the ε-NH_2 group, giving rise to aldehyde derivatives. These aldehydes will associate spontaneously with ε-NH_2 groups from a lysyl or hydroxylysyl residue of adjacent molecules, forming interchain cross-links. These cross-links will increase the tensile strength of the fibrils considerably (see Fig. 1). *In vitro* studies have shown that procollagen molecules and their various structural domains have a remarkable capacity to control all stages of collagen assembly, from intracellular assembly to extracellular suprafibrillar assembly at a micrometer scale. The *in vivo* process is much more complex but we are beginning to understand some of this in particular cell types. Kadler and coworkers have recently shown that GPCs are indeed present *in vivo* in embryonic tendon fibroblasts and that some GPCs contain 28-nm-diameter collagen fibrils. Moreover, GPCs are targeted to novel plasma membrane protrusions, which they have termed "fibripositors" (fibril depositors). What was intriguing in this study is the fact that procollagen can be converted to collagen within the confines of the cell membrane, which is consistent with the observation of collagen fibrils in some GPCs and the known intracellular activation of BMP-1. In addition, fibripositors were shown to be always oriented along the tendon axis, which establishes a link between intracellular transport and the organization of the ECM (Canty *et al.*, 2004). Interestingly, fibripositor formation is not a constitutive process in procollagen-secreting cells. It is absent in postnatal development despite active procollagen synthesis, but occurs only during a narrow window of embryonic development when tissue architecture is being established. It is not known whether this phenomenon occurs in other types of specialized collagen-secreting cells (Canty and Kadler, 2005).

Assembly

The final assembly of fibrillar collagen involves the direct interaction of several molecules, which include other collagens, small leucine-rich proteoglycan (SLRP), and others. These interactions shape the diameter of the fibrils (Kuc and Scott, 1997; Vogel and Trotter, 1987) and patterning of the final matrix. SLRPs are a group of secreted proteins that includes decorin, biglycan, fibromodulin, lumican, and keratocan, among others, that play major roles in tissue development and assembly, especially in collagen fibrillogenesis (Iozzo, 1999). Biglycan and decorin are highly expressed in extracellular bone matrix and there is now substantial evidence to support an increasing role for biglycan and decorin in influencing bone cell differentiation and proliferative activity (Waddington *et al.*, 2003). The ability of decorin and biglycan to interact with collagen molecules and to facilitate fibril formation has implicated these macromolecules in important roles in the provision of a collagenous framework in bone, which eventually allows for mineral deposition. Initial mineral deposition is proposed to occur within or near the gap zones along the collagen fibers, and the structural architecture of the collagen fibers along with interacting noncollagenous proteins are likely to play a key role in directing placement of the mineral crystals (Dahl and Veis, 2003).

Molecular modeling techniques have lead to the proposal that decorin and biglycan adopt an open-horseshoe structure (Weber *et al.*, 1996) where the inner cavity interacts with a single triple helical molecule. The generation of mutated forms of decorin has demonstrated the importance of leucine-rich sequences 4 to 6 in mediating this interaction (Kresse *et al.*, 1997). In addition, reduction in the disulfide bridges at the C- and N-terminal of decorin also abolished interaction with type I collagen (Ramamurthy *et al.*, 1996) and this led to the proposal that the disulfide loop at the C terminal binds to adjacent collagen fibrils, thereby facilitating the lateral assembly and stabilization of the fibrils.

Interestingly, recent molecular analysis data have put forward the idea that decorin exists as a dimer in solution (Scott *et al.*, 2003), and if this is the case *in vivo,* then the nature of this interaction will be important when considering the mechanistic role of decorin in fibril assembly. The glycoseaminoglycan (GAG) moiety of decorin and biglycan have also been deemed to play an important role in collagen fibrillogenesis, where the interaction of glycosylated forms of these SLRPs with collagen appeared to be greater than nonglycosylated forms (Bittner *et al.*, 1996).

Further evidence for the role of decorin and biglycan in bone formation is provided by targeted deletion of the genes. The biglycan knockout (*Bgn* −/−) mouse (Xu *et al.*, 1998), unlike the *Dcn* −/− mouse (Danielson *et al.*, 1997), showed no gross skin abnormalities but rather a reduction in bone density. These mice were seen to develop an osteoporotic phenotype, failing to achieve peak bone mass owing to decreased bone formation with significantly shorter femurs (Ameye *et al.*, 2002). Within these animals lower osteoblast numbers and osteoblast activity were observed. *In vitro* experiments demonstrated that the number and responsiveness of bone marrow stromal cells to TGF-β, and hence osteogenic precursor cells, decreased dramatically with age, but apoptosis rates increased (Chen *et al.*, 2002a). The effects were not confined only to the skeletal tissues. Within the teeth the transition of predentin to dentin appeared to be impaired and the thickness of the enamel was dramatically increased (Goldberg *et al.*, 2002). Taken together these results would suggest that biglycan plays an important role in the formation of mineralized tissue. Furthermore, despite high sequence identity and somewhat similar patterns of localization, decorin and biglycan are not interchangeable in function and do not have the ability to rescue each other's knockout phenotypes. Notably, *Bgn* −/− and *Dcn* −/− double-knockout animals revealed that the effects of both gene deficiencies were additive in the dermis and synergistic in bone (Corsi *et al.*, 2002). The lack of both genes caused a phenotype with severe skin fragility and osteopenia, resembling a rare progeroid variant of Ehlers–Danlos syndrome.

Consequences of Genetic Mutations on Type I Collagen Formation

Osteogenesis imperfecta (also known as "brittle bone disease") is a genetic disease characterized by an extreme fragility of bones. Genetic studies have shown that it is due to a mutation in the coding sequence of either the pro *COL1A1* gene or the pro *COL1A2* gene, and more than 150 mutations have been identified (for review, see Byers, 2001) Most severe cases of osteogenesis imperfecta result from mutations that lead to the synthesis of normal amounts of an abnormal chain, which can have three consequences (Marini *et al.*, 2007). First, the structural abnormality can prevent the complete folding of the three chains in a triple helix, e.g., if a glycine is substituted by a bulkier amino acid that will not fit in the center of the triple helix. In this case, the incompletely folded triple helical molecules will be degraded intracellularly, resulting in a phenomenon known as "procollagen suicide." Second, some mutations appear not to prevent folding of the three chains in a triple helix, but presumably prevent proper fibril assembly. For example, Prockop's group has shown that a mutation of the *pro Col1a1* gene that changed the cysteine at position 748 to a glycine produced a kink in the triple helix (Kadler *et al.*, 1991). Finally, some mutations will not prevent triple helical formation or fibrillogenesis but might modify the structural characteristic of the fibrils slightly and thus affect their mechanical properties. In all these cases, the consequence on the mechanical properties of bone is probably similar. Mild forms of osteogenesis imperfecta most often result from a functionally null allele, which decreases the production of normal type I collagen. Null mutations are usually the result of the existence of a premature stop codon or of an abnormality in mRNA splicing. In these cases, the abnormal mRNA appears to be retained in the nucleus (Johnson *et al.*, 2000). A mouse model of osteogenesis imperfecta has been obtained by using a knockin strategy that introduced a $\mathrm{Gly}^{349} \rightarrow \mathrm{Cys}$ mutation in the pro *Col1a1* gene (Forlino *et al.*, 1999). This model faithfully reproduced the human disease. Another spontaneous mouse mutation the *oim/oim* mouse, analogous to human type III OI, carries a spontaneous deletion of G at nucleotide 3983 in the $\alpha2$ chain of collagen type I, which alters the reading frame to result in the final 48 amino acids of the COOH-terminal propeptide generating a new stop codon, with addition of an extra amino acid (Chipman *et al.*, 1993). In these mice, collagen type I is made of $\mathrm{a(I)}_3$ homotrimers in place of the normal $\alpha1(\mathrm{I})_2\ \alpha2(\mathrm{I})_1$ heterotrimers.(Chipman *et al.*, 1993; Kuznetsova *et al.*, 2004; Miles *et al.*, 2002) which results in marked skeletal fragility, with thinning of the long bones and reduced mechanical strength.(Chipman *et al.*, 1993; Pereira *et al.*, 1995) An additional organ pathology has been recently described by Phillips and coworkers, namely a glomerulopathy characterized by abnormal renal collagen deposition (Brodeur *et al.*, 2007; Phillips *et al.*, 2002).

Ehlers–Danlos syndrome type VIIA and VIIB are two rare dominant genetic diseases characterized mainly by an extreme joint laxity. They result from mutations in the pro *COL1A1* gene (Ehlers–Danlos syndrome type VIIA) or in the pro *COL1A2* gene (Ehlers–Danlos syndrome type VIIB) that interfere with the normal splicing of exon 6, and a little less than 20 mutations have been described. These mutations can affect the splice donor site of intron 7 or the splice acceptor site of intron 5; in the latter case, there is efficient recognition of a cryptic site in exon 6 (Byers *et al.*, 1997). Thus, these mutations induce a partial or complete excision of exon 6. They do not appear to affect the secretion of the abnormal procollagen molecules, but they are responsible for the disappearance of the cleavage site of the N-terminal propeptide and thus for the presence of partially processed collagen molecules in fibrils that fail to provide normal tensile strength to tissues (Byers, 2001). Nevertheless, these mutations seem to affect the rate of cleavage of the N-terminal propeptide rather than to completely prevent it, which explains that the phenotype is less severe than for patients who do not have a functional N-proteinase (Ehlers–Danlos syndrome type VIIC).

Collagen Type I and Osteoporosis

Because type I collagen is the most abundant protein in bone and because mutations in the *COL1A1* gene are a major cause of osteogenic imperfecta, this gene has been considered a strong candidate for susceptibility to osteoporosis (Ralston and de Crombrugghe, 2006). Indeed polymorphisms in both the promoter (Garcia-Giralt *et al.*, 2002) and in the first intron of *COL1A1* (Grant *et al.*, 1996) have been associated with changes in bone mineral density (BMD). The polymorphism of intron 1 is located in a binding site for the transcription factor Sp1 and has been associated with various osteoporosis-related symptoms such as bone density and fractures (Grant *et al.*, 1996; Uitterlinden *et al.*, 1998), postmenopausal bone loss (Harris *et al.*, 2000; MacDonald *et al.*, 2001), bone geometry (Qureshi *et al.*, 2001), bone quality (Mann *et al.*, 2001), and bone mineralization (Stewart *et al.*, 2005). The osteoporosis-associated Sp1 polymorphism causes increased Sp1 binding, enhanced transcription, and abnormally high production of *COLIA1* mRNA and protein (Mann *et al.*, 2001), which result in an imbalance between the *COLIA1* and *COLIA2* chains. This is thought to lead to impairment of bone strength and reduced bone mass in carriers of the Sp1 polymorphism (Stewart *et al.*, 2005). Retrospective meta-analyses of previous studies have indicated that the Sp1 polymorphic allele is associated with reduced BMD and with vertebral fractures (Efstathiadou *et al.*, 2001; Mann and Ralston, 2003). In a recent large prospective meta-analysis of more than 20,000 participants from several European countries of the GENOMOS study, homozygotes for the Sp1 polymorphism were found to be associated with lower BMD at the lumbar spine and femoral neck and a predisposition to vertebral fractures (Ralston *et al.*, 2006). In this study, however, the BMD association was only observed for homozygotes of the Sp1 polymorphism, in contrast to previous studies where heterozygotes also showed a reduction in BMD (Mann and Ralston, 2003). It should be noted that the association between *COLIA1* alleles and vertebral fracture reported in GENOMOS and other studies was not fully accounted for by the reduced bone density, suggesting that the Sp1 allele may also be a measure of bone quality. Furthermore, the existence of an extended haplotype defined by the Sp1 polymorphism and other promoter polymorphisms has been proposed to exert stronger effects on BMD than the individual polymorphisms (Garcia-Giralt *et al.*, 2002; Stewart *et al.*, 2006). Evidence has been presented that suggests that the promoter polymorphism at position −1663 interacts with the transcription factor NMP4, which plays a role in osteoblast differentiation by interacting with Smads (Garcia-Giralt *et al.*, 2005).

Transcriptional Regulation of Type I Collagen Genes

Expression of the *pro Col1a1* gene and the *pro Col1a2* gene is coordinately regulated in a variety of physiological and pathological situations. In many of these instances it is likely that the control of expression of these two genes is mainly exerted at the level of transcription, suggesting that similar transcription factors control the transcription of both genes.

This section considers successively the proximal promoter elements of these genes and then the nature of cell-specific enhancers located in other areas of these genes including intronic sequences. Information about the various DNA elements has come from transient expression experiments in tissue culture cells, *in vitro* transcription experiments, and experiments in transgenic mice. *In vitro* transcription experiments and, in large measure, transient expression experiments identify DNA elements that have the potential of activating or inhibiting promoter activity. These DNA elements can be used as probes to detect DNA-binding proteins. However, transient expression and *in vitro* transcription experiments do not take into account the role of the chromatin structure in the control of gene expression. Transgenic mice are clearly the most physiological system to identify tissue-specific elements; the DNAs that are tested are integrated into the mouse genome and their activities are presumably also influenced by their chromatin environment. In transgenic mice experiments, reporter genes such as green fluorescent protein (GFP, luciferase or the *Escherichia coli* β-galactosidase offer the advantage that their activity is indicative of promoter activation and location of the expression. All three transgenes can be detected *in vivo* without having to kill the mouse. In addition, X-Gal histochemical stain for β-galactosidase can identify the cell types in which the transgene is active by histology.

Transient transfection experiments using various sequences upstream of the transcription start site of either the pro α1(I) or the pro α2(I) promoter, cloned upstream of a reporter gene, and introduced in fibroblasts, have delineated positive *cis*-acting regulatory segments in these two sequences that were designated as minimal proximal promoters. Footprint experiments and gel-shift assays performed using these regulatory elements as DNA templates have also characterized sequences that interact with DNA-binding proteins to modulate the expression of the two genes.

Proximal Promoters of Type I Collagen Genes

Transcription Factors Binding to the pro α1(I) Proximal Promoter

In the pro α1(1) collagen gene, a close homology exists between human and mouse promoters. Brenner and colleagues demonstrated by progressively deleting the mouse *pro-Col1a1* promoter that sequences downstream of −181 bp are needed for high-level transient transfection (Brenner *et al.*, 1989). Therefore, the sequence between −220 and +110 has been used as the proximal promoter (Fig. 2) and contains binding sites for DNA-binding factors that also bind to the proximal *pro Col1a2* promoter (Ghosh, 2002). These DNA elements in the mouse pro *Col1a1* proximal promoter include a binding site for CBF between −90 and −115 (Karsenty and de Crombrugghe, 1990). A second CCAAT box located slightly more upstream is, however, unable to bind CBF, suggesting that sequences surrounding the CCAAT motif also have a role in CBF binding. Similar results were observed in the human *COL1A1* promoter (Saitta *et al.*, 2000). DNA transfection experiments with

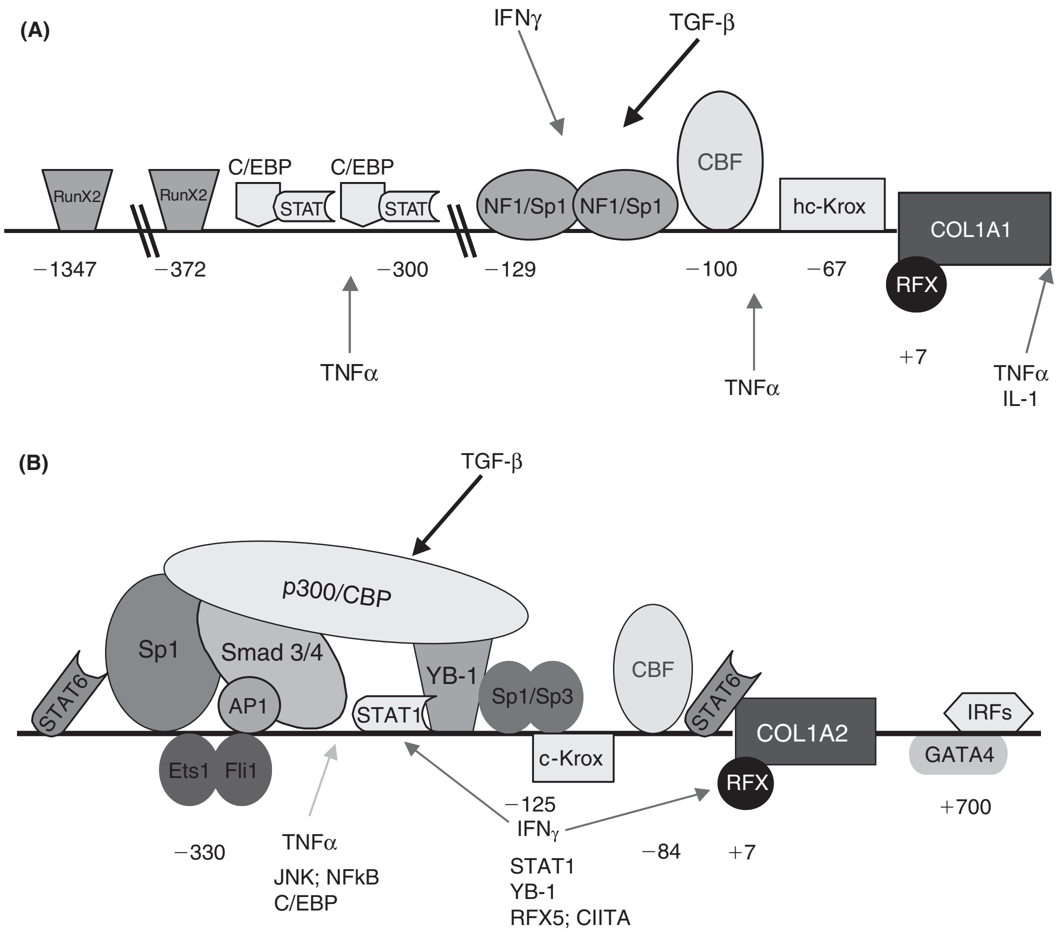

FIGURE 2 Schematic diagram illustrating the known transcription factors that bind to the proximal promoter of the human and murine *Col1a1* (**A**) and *Col1a2* (**B**) promoters. Also shown are the cytokines that modulate the expression of these genes. The data are accumulated from several papers as cited in the text.

the *pro Col1a1* promoter showed that point mutations in the CBF-binding site decreased promoter activity (Karsenty and de Crombrugghe, 1990). The CBF-binding site is flanked by two identical 12-bp repeat sequences that are binding sites for Sp1 and probably other GC-rich binding proteins (Nehls *et al.*, 1991). In transient transfection experiments, a mutation in the binding site that prevents the binding of Sp1 surprisingly increased the activity of the promoter, and overexpression of Sp1 decreased the activity of the promoter (Nehls *et al.*, 1991). It is possible that several transcription factors with different activating potentials bind to overlapping binding sites and compete with each other for binding to these sites; the overall activity of the promoter could then depend on the relative occupancy of the different factors on the promoter DNA. Substitution mutations in two apparently redundant sites between −190 and −170 and between −160 and −130 that abolished DNA binding resulted in an increase in transcription (Karsenty and de Crombrugghe, 1990). Formation of a DNA–protein complex with these two redundant elements in the pro *Col1a1* promoter was also shown to be completed by the sequence of the *pro Col1a2* promoter between −173 and −143, suggesting that both type I promoters contained binding sites for a similar (Karsenty and de Crombrugghe, 1991). A member of the Krox family, designated c-Krox, binds to these two sites in the pro *Col1a1* promoter (Galera *et al.*, 1994). In addition, c-Krox binds to a site located near the CCAAT box in the pro *Col1a1* promoter and to three GC-rich sequences in the pro *Col1a2* proximal promoter, located between −277 and −264 bp, between −175 and −143 bp, and near the CCAAT box, respectively (Galera *et al.*, 1996). More recently, c-Krox has been shown to exert its action by synergistic association with SP1 (Kypriotou *et al.*, 2007).

The *Col1a1* promoter region spanning base pairs −9 to −56 bound purified recombinant YY1 and the corresponding binding activity in nuclear extracts was supershifted using a YY1-specific antibody. Mutation of the TATA box to TgTA enhanced YY1 complex formation. YY1 functions as a positive regulator of constitutive activity in fibroblasts. Although YY1 is not sufficient for transcriptional initiation, it is a required component of the transcription machinery in this promoter (Riquet *et al.*, 2001).

A *RunX2/Cbfa1*-binding element is present in the *Col1a1* promoter of mouse, rat, and human at approximately position −372 (Kern *et al.*, 2001). This site binds *RunX2/Cbfa1*only weakly and does not act as a *cis*-acting activator of transcription when tested in DNA transfection experiments. These may interact with the upstream osteoblast-specific element (OSF2) site at −1347 (Kern *et al.*, 2001) as discussed in the section on "upstream element in the pro *Col1a1*" later.

It is likely that the transcriptional function of various transcription factors and eventually their DNA-binding properties offer opportunities for regulation by intracellular signaling pathways. These pathways are triggered by a variety of cytokines such as TNFα, which exerts its inhibitory action on *Col1a1* expression through NF-κB (Rippe *et al.*, 1999).

Factors Binding to the pro-COL1A2 *Proximal Promoter*

Several functional *cis*-acting elements have been identified in the approximately 400-bp proximal promoter of the mouse pro *Col1a2* gene (+54 to −350 bp) and the human minimal sequence between +52 and −378bp. The first transcription factor found to bind to this promoter was the ubiquitous CCAAT-binding protein, CBF. This transcription factor is formed by three separate subunits, named A, B, and C, which have all been cloned and sequenced (Maity and de Crombrugghe, 1998). All three subunits are needed for CBF to bind to the sequence containing the CCAAT box located between −84 and −80 and activate transcription in both human and mouse genes (see Fig. 2B). *In vitro* data suggest that the A and C subunits first associate to form an A–C complex and that this complex then forms a heteromeric molecule with the B subunit (Sinha *et al.*, 1995). Mutations in the CCAAT box that prevent the binding of CBF decrease the transcriptional activity of the pro *Col1a2* proximal promoter three to five times in transient transfection experiments of fibroblastic cell lines (Coustry *et al.*, 1995). Purified CBF as well as CBF composed of its three recombinant subunits also activate the pro Col1a2 promoter in cell-free nuclear extracts previously depleted of CBF (Coustry *et al.*, 1995). Two of the three subunits of CBF contain transcriptional activation domains. More recently, a single substitution of T to an A *in vivo* in the presence of an upstream enhancer of the human sequence suggested that CBF is involved in patterning of the collagen type I expression in the dorso-ventral as well as the rostro-caudal axis of mouse skin fibroblasts (Tanaka *et al.*, 2004).

In addition to the binding site for CBF, footprinting experiments and gel-shift studies identified other binding sites in the first 350 bp of the mouse *pro Col1a2* promoter. Three GC-rich sequences, located at about −160 bp (between −176 and −152 bp) and −120 bp (between −131 and −114 bp) have been shown to interact with DNA-binding proteins by footprint experiments and gel-shift assays (Hasegawa *et al.*, 1996). A deletion in the mouse promoter encompassing these three foot-printed sequences completely abolished the transcriptional activity of the *pro Col1a2* proximal promoter in transient transfection experiments using fibroblastic cell lines. The corresponding regions in the human *pro COL1A2* promoter were also protected in *in vivo* and *in vitro* footprinting experiments (Ihn *et al.*, 1996) and bound transcriptional activators. However, gel-shift assays performed using the human *pro COL1A2* promoter have suggested that a *cis*-acting element located at −160 bp represents a repressor element (Ihn *et al.*, 1996), implying that interactions between proteins interacting with the activator elements at −300, −125, and −80 bp and proteins binding to a repressor element at −160 bp regulate this gene. Spl and Sp3 have been shown to bind the TCCTCC motif located between −123 and −128 bp; both transcription factors activate this promoter (Ihn *et al.*, 2001) (see Fig. 2B). Furthermore, proteins that bind to the two proximal segments also bind to the most upstream GC-rich segment, at −300 bp, with the exception of CBF, suggesting a redundancy among functionally active DNA segments of the pro *COL1A2* proximal promoter.

TGF-β mediates its action in the human promoter through the combination of the ubiquitous transcription factor Sp1, the Smad3/4 complex, and the coactivators p300/CBP in what is termed a TGF-β-responsive element (TbRE) (Zhang *et al.*, 2000). This segment contains three GC-rich motifs between −330 and −255, capable of binding Sp1, C/EBP, AP1, and Smad complexes (Chen *et al.*, 1999, 2000a; Kanamaru *et al.*, 2003; Tamaki *et al.*, 1995; Zhang *et al.*, 2000). Interaction among these nuclear factors is synergistic and requires binding of Sp1 and Smad3/4 to the GC-rich elements and the downstream CAGAC Smad site, respectively (Ghosh *et al.*, 2000; Poncelet and Schnaper, 2001; Zhang *et al.*, 2000).

Tumor necrosis-alpha (TNFα) and interferon-γ (IFNγ) suppress matrix production. In contrast to the single DNA element that mediates TGF-β stimulation of the COL1A2 proximal promoter, both TNFα and IFN-γ have been shown to inhibit COL1A2 transcription by interfering with the formation of the TGF-β-induced complex, and by stimulating the interaction of negative factors with responsive DNA elements located 5′ and 3′ of it. This so called: "cytokine responsive element" plays an important role in maintaining homeostasis. Involvement of AP1 and NF-κB in transducing the inhibitory action of TNFα on *COL1A2* gene expression has been shown using immortalized fibroblasts from mice that lack either the AP1 activator JNK1 or the NF-κB essential modulator NEMO. Specifically, the loss of JNK1 prevented the TNFα antagonism of TGF-β, but preserves TNFα inhibition of constitutive *COL1A2* expression. TGF-β antagonism by TNFα involves JNK1 phosphorylation of c-jun leading to off-DNA competition of the latter molecule for Smad3 binding to the cognate DNA site and/or for interaction with the p300/CBP coactivators (Kouba *et al.*, 1999; Verrecchia *et al.*, 2001, 2002).

Binding of INF-γ to its receptors leads to tyrosine phosphorylation of JAK tyrosine kinases and this in turn results in STAT1 phosphorylation. In *COL1A2*, STAT1 activation results in competition with Smad3 for interaction with p300/CBP (Inagaki *et al.*, 2003). In addition, JAK1 can also activate transcription factor YB-1; this activation results in both inhibition of constitutive promoter activity through YB-1 binding the −125 TC-box, and antagonism of TGF-β signals through YB-1 competition with Smad3 and/or p300/CBP (Ghosh *et al.*, 2001; Higashi *et al.*, 2003). For more details, see "Growth Factors" and "Cytokines."

Est1/Fli1, have also been shown to bind the same sequence with an opposite effects on collagen type I transcription. A functional Ets transcription factor was identified in *COL1A2* in close proximity of Sp1 sites. Ets1 stimulated, whereas Fli1 inhibited promoter activity. Sp1 binding was essential for inhibition of Fli1. Moreover, overexpression of Fli1 in dermal fibroblasts led to decrease in *COL1A2* mRNA and protein levels (Czuwara-Ladykowska *et al.*, 2001). Furthermore, TGF-β treatment of dermal fibroblasts leads to dissociation of Ets1 from the CBP/p300 complexes and alters their responses to TGF-β in favor of matrix degradation (Czuwara-Ladykowska *et al.*, 2002).

Furthermore a CpG motif at +7 has been shown to be preferentially methylated and bound by RFX proteins in cells acquiring a collagen I-negative state. Cell transfection experiments in conjunction with DNA-binding assays have assigned positive or negative properties to each of the proteins binding to the proximal promoter of *pro COL1A2* (Sengupta *et al.*, 2002). The degree of methylation at the +7 CpG site, on the other hand, has been shown to modulate the binding affinity of RFX proteins and, consequently, their ability to downregulate promoter activity by recruiting associated proteins that interfere with the assembly of positively acting transcriptional complexes (Xu *et al.*, 2003, 2004).

Organization of Upstream Segments of Type I Collagen Genes

Upstream Elements in the pro Col1a1 Gene

In complete contrast with its high-level activity in transient expression and *in vitro* transcription experiments, the 220-bp *pro Col1a1* proximal promoter is almost completely inactive in stable transfection experiments and in transgenic mice (Rossert *et al.*, 1996). By increasing the length of this promoter, evidence from different groups showed that the 2.3-kb human promoter linked to different transgenes resulted in a high degree of tissue-specific expression of the reporter gene in bone, tail, and skin. However, this expression of the transgene was not identical to that of the endogenous gene (Slack *et al.*, 1991). Indeed, in latter experiments using embryos from the same mice, *in situ* hybridization assays showed no expression of the transgene in perichondrium and in skeletal muscle, implying that additional sequences might be needed to obtain expression of the gene in all type I-collagen producing cells (Liska *et al.*, 1994), In newborn mice carrying a 3.6-kb rat *Col1a1* promoter linked to a CAT reporter gene, high levels of transgene expression were found in extracts of bone, tooth, and tendon (Bogdanovic *et al.*, 1994; Pavlin *et al.*, 1992). Similarly, the mouse 3.6-kb promoter fused to β-galactosidase transgene resulted in a similar pattern, but localized to three distinct *cis*-acting sequences that directed expression in the skin, bone, and tendon (Rossert *et al.*, 1995). The skin element was between −220 bp and −900 bp. The bone element was further delineated to 117 bp between −1656 bp and −1540 bp, and the tendon to a sequence between −2300 bp and −3600 bp (Rossert *et al.*, 1996) (Fig. 3), These experiments suggested a modular arrangement of separate *cis*-acting elements that activate the pro *Col1a1* gene in different type I collagen-producing cells. The direct consequence of such a modular arrangement is that it should be possible to selectively modulate the activation of type I collagen genes in well-defined subpopulations of type I collagen-producing cells. Perhaps the best illustration of *cis*-acting element responsible for the activation of the mouse pro *Col1a1* gene is the osteoblast element. This 117-bp segment is very well conserved amongst species. In the mouse promoter, located between −1656 and −1540 bp, is a minimal sequence able to induce high levels of expression of the reporter gene exclusively in osteoblasts. In these mice the transgene becomes active at the same time during embryonic development when osteoblasts first appear in the different ossification centers. This so-called "osteoblast-specific element" can be divided into three subsegments that have different functions. The 29-bp A segment, which is located most 5′ (−1656 to −1628 bp), is required to activate the gene in osteoblasts. A deletion of the A element or a 4-bp mutation in the TAAT sequence of this segment completely abolished the expression of the reporter gene in osteoblasts of transgenic mice. The C segment, which is located at the 3′ end of the 117-bp sequence (−1575 to −1540 bp), is required to obtain consistent high-level expression of the reporter gene in transgenic mice (Rossert *et al.*, 1996). When this C segment was deleted, the *lacZ* gene was expressed at very low levels and only in a small proportion of transgenic mice. The function of the intermediary segment (B segment) is still poorly understood, but it could be to prevent a promiscuous expression of the gene and, in particular, expression of the gene in the nervous system.

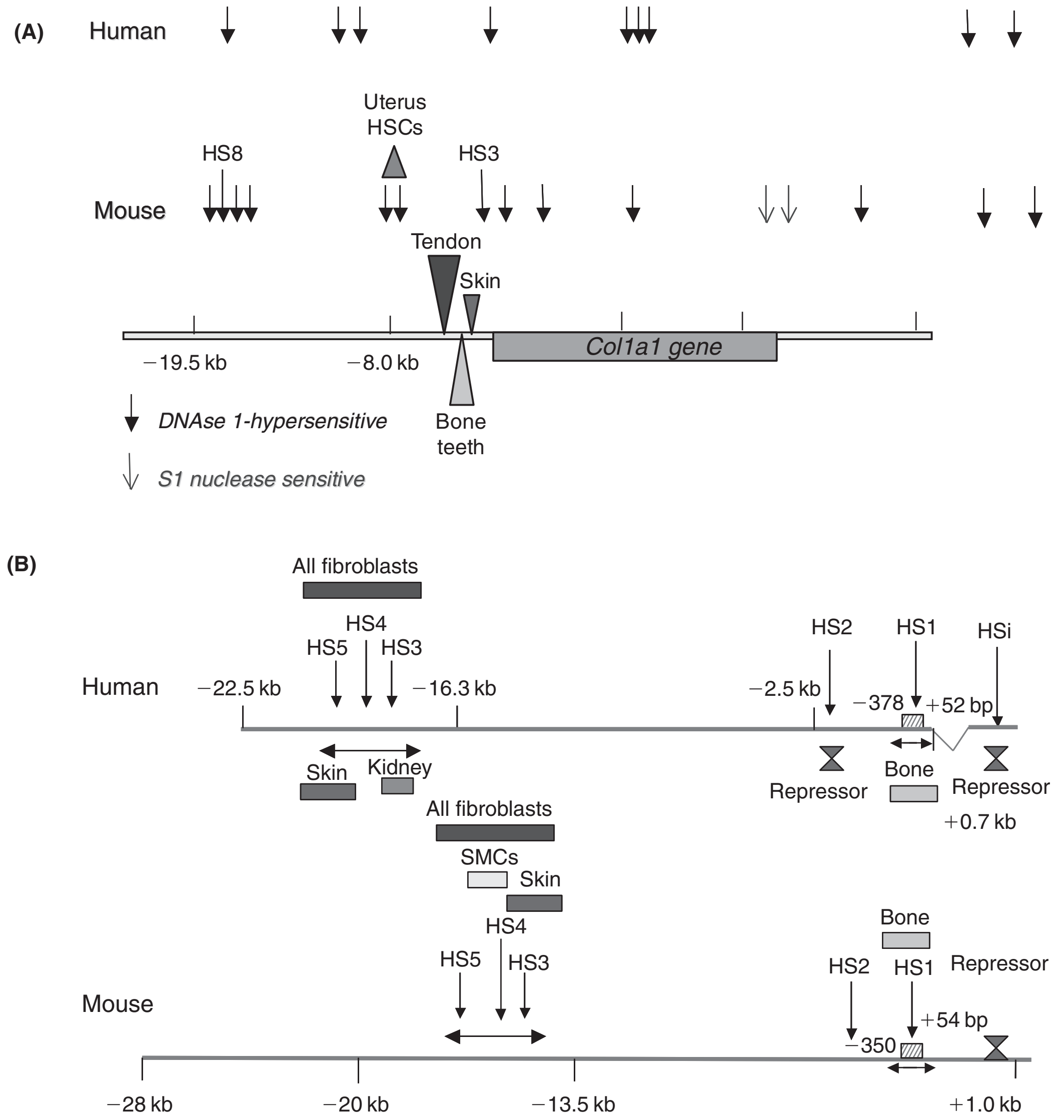
(A)
Human
Uterus
HSCs
HS8
HS3
Mouse
Tendon
Skin
Col1a1 gene
−19.5 kb
−8.0 kb
DNAse 1-hypersensitive
Bone
teeth
S1 nuclease sensitive
(B)
All fibroblasts
HS4
HS5
HS3
HS2
HS1
HSi
Human
−22.5 kb
−16.3 kb
−2.5 kb
−378
+52 bp
Skin
Kidney
Repressor
Bone
Repressor
+0.7 kb
All fibroblasts
SMCs
Skin
HS4
HS5
HS3
Bone
HS2
HS1
Repressor
Mouse
+54 bp
−350
−28 kb
−20 kb
−13.5 kb
+1.0 kb

FIGURE 3 DNaseI hypersensitive sites (HS) of the human and murine *Col1a1* (**A**) and *Col1a2* (**B**) genes depicted by vertical arrows, which indicate the position within the gene where the sequence may be involved in transcriptional regulation. Areas of high homology in the promoter (>85%) are indicated by horizontal arrows. Also indicated are the regions in which *cis*-acting sequences have been shown to express in transgenic mice and the tissues or cells in which they are expressed and called "elements."

In addition to the 117-bp region, a consensus Cbfa1-binding site, termed OSE2, is present at approximately −1347 bp of the rat, mouse, and human genes (Kern *et al.*, 2001). *RunX2/Cbfa1* can bind to this site, as demonstrated by electrophoretic mobility shift assay and supershift experiments using an anti-Cbfa1 antibody. Mutagenesis of the *Col1a1* OSE2 at −1347 bp reduces the activity of a *Col1a1* promoter fragment 2- to 3-fold. Moreover, multimers of this OSE2 at −1347 bp confer osteoblast-specific activity to a minimum *Col1a1* collagen promoter fragment in DNA transfection experiments. However, this region did not confer expression in transgenic mice, suggesting that other specific factors contribute to collagen expression of collagen type I in osteoblasts. Indeed osterix, a zinc finger transcription factor, was found to be essential for bone formation and to act downstream of *RunX2/Cbfa1*. It was shown to bind to segment C of the osteoblast element. The tendon element was found to be a combination of sequences between −3.2 and −3.6 kb termed TSE1 and TSE2 (Terraz *et al.*, 2002). Gel-shift experiments of this region showed that scleraxis, which is a basic helix-loop-helix transcription factor that is expressed selectively in tendon fibroblasts, binds TSE2, whereas TSE1 binds NFATc transcription factors (Lejard *et al.*, 2007).

In pursuit of the controlling regions of this gene, a chromatin structure analysis of the human *COL1A2* gene has revealed the presence of several proximal and distal 5′ and 3′ DNase I hypersensitive sites (HS) (Barsh *et al.*, 1984).

Breindl's team used the same technique and mapped a 55-kb sequence spanning the mouse gene, including 24 kb of 5′- and 13 kb of 3′-flanking sequences (Salimi-Tari *et al.*, 1997). Nine HS sites were found, seven of which were in the promoter sequence (HS3–HS9) up to −20 kb (see Fig. 3A). All of these hypersensitive sites were present in collagen type I-producing cells except for HS8. When these sequences were fused to a reporter gene in transgenic animals, they revealed an additional *cis*-acting sequence in uterine smooth muscle cells between −7 to −8 kb (Krempen *et al.*, 1999) and liver stellate cells (Yata *et al.*, 2003).

Upstream Elements of the Mouse pro Col1a2 *gene*

The activity of the mouse 350-bp *pro Col1a2* proximal promoter in transgenic mice is very low compared with that of the corresponding endogenous gene. Although this low-level activity appears to be present selectively in fibroblasts and mesenchymal cells (Niederreither *et al.*, 1992), the precise sequences and the factors responsible for this tissue specificity have not yet been identified. An element located 13.5 to 17 kb upstream of the start site of transcription, and named "far-upstream enhancer," increased the levels of expression of the *lacZ* and luciferase reporter genes considerably when it was cloned upstream of the 350-bp mouse *pro Col1a2* proximal promoter. Moreover, this element by itself contributed to the tissue-specific expression of a reporter gene. Indeed, when it was cloned upstream of a minimal promoter that has no tissue-specific expression by itself, i.e., first 220 bp of the *pro Col1a1* proximal promoter, it conferred a tissue-specific expression to the *lacZ* reporter gene in transgenic mice (Bou-Gharios *et al.*, 1996).

Interestingly, in transgenic mice harboring the *lacZ* reporter gene cloned downstream of a *pro Col1a2* promoter segment containing the far-upstream enhancer, fibroblastic cells expressed the *lacZ* reporter gene at very high levels, but only a subset of osteoblastic cells expressed this reporter gene, whereas odontoblasts and fully differentiated tendon fibroblasts did not (Bou-Gharios *et al.*, 1996), suggesting that a *cis*-acting element active in all osteoblasts is missing from this promoter/enhancer sequence. Besides this heterogenous expression of the *lacZ* reporter gene in bone, the lack of expression of the *lacZ* gene in odontoblasts suggests that other elements exist that control expression in these cells and strongly supports a modular organization of different regulatory domains in the mouse *pro Col1a2* promoter, as described for the *pro Col1a1* promoter.

In order to verify the transgene-staining results, both luciferase and β-galactosidase activity in tissue homogenate were measured because the transgenic line used harbored both β-galactosidase and luciferase transgenes. Although β-galactosidase staining was no longer present in many tissues by the time transgenic mice are weaned, luciferase activity of the same tissues remained significantly higher than in controls. The endogenous mRNA level followed the overall trend of the transgene and very little transgene mRNA was detected by three months of age. The skin showed the highest level of endogenous mRNA during development, which was matched by transgene expression (Ponticos *et al.*, 2004a), and although mRNA level drops by day 10 after birth, the transgene remains significantly high until three weeks of age, suggesting perhaps a specific role of this enhancer in the dermis.

The role of the enhancer in the regulation of the collagen type I gene was also revealed by measuring activation of the transgenes after injury to tissues either by injection of fibrogenic cytokines such as TGFβ-1 or by physical injury. Such challenge appears to stimulate a significant increase in transcription of both the endogenous collagen gene and the transgenes. Although mRNA levels of collagen has been shown to diminish in many tissues after birth and throughout adulthood (Goldberg *et al.*, 1992), acute injury reactivates the *lacZ* transgene to a level that is visually apparent (Ponticos *et al.*, 2004a). This finding makes such transgenic animals an important tool and provides a model to address questions about the nature of cells that are active in fibrosis. For example, in muscular dystrophy where fibrosis bears the hallmark of the disease (Morrison *et al.*, 2000), this model can be used to identify those cells that are active in producing collagen type I as shown with glomerulosclerosis (Chatziantoniou *et al.*, 1998) and the Tsk mouse model bred with these transgenic mice (Denton *et al.*, 2001). More importantly, this will allow us to test the direct effects of various antifibrotic agents on the cells that are actively synthesizing collagen type I.

In the human *COL1A2* gene, six distinct DNase I-hypersensitive sites were mapped within 22 kb upstream and around the start site of transcription. Their spatial arrangement, cell type specificity, and relative availability to DNase I digestion are comparable to those of the mouse gene (Antoniv *et al.*, 2001). The more striking finding of the chromatin survey was the identification of three strong cell-type-specific hypersensitive sites (HS3–5), at a location comparable to those of the mouse far-upstream enhancer and residing within nearly identical sequences (see Fig. 3B). DNase I footprinting correlated areas of sequence identity with twelve distinct binding sites of nuclear proteins, the majority of which are likely not to be tissue-specific. Using transgenic analysis, it was found that the proximal −378 promoter contains elements that drive tissue-specific transcription in subsets of fibroblastic and osteoblastic cells, and this activity is significantly augmented when the proximal promoter is linked to the far-upstream enhancer. The results of the human transgenes thus indicate that the predominant function of the upstream element is to broaden and intensify tissue-specific transcription from the proximal promoter. Unlike the mouse enhancer that was only active in a subset of osteoblasts, the human sequence was active in all osteoblasts and it is most likely that the osteoblast element resides in the proximal promoter (Antoniv *et al.*, 2001).

Mode of Action of Tissue-Specific Elements

The mode of action of the different lineage-specific transcription elements and their postulated cognate-binding proteins is still unknown, but a study of hypersensitive sites (Antoniv *et al.*, 2001; Bou-Gharios *et al.*, 1996) and *in vivo* footprinting experiments (Chen *et al.*, 1997) strongly suggest that the chromatin structure of discrete areas in the regulatory regions of type I collagen genes is different in cells when these genes are being transcribed actively compared with cells in which they are silent. These experiments suggest that, in intact cells, transcription factors such as CBF bind to the proximal promoters of type I collagen genes only in cells in which the genes are transcribed actively (Coustry *et al.*, 2001). Although *in vivo* footprint experiments show a protection of the CCAAT box in different fibroblastic cell lines, such a protection does not exist in cell lines that do not produce type I collagen. Similarly, hypersensitive sites corresponding to the far-upstream enhancer of the *pro Col1a2* promoter can be detected only in cells that express type I collagen. The importance of chromatin structure is also highlighted by comparison of transient and stable transfection experiments. When a chimeric construct harboring the *pro Col1a1* osteoblast-specific element cloned upstream of a minimal promoter and of the *lacZ* reporter gene was transfected stably in different cell lines, it was expressed in the ROS17/2 osteoblastic cell line, but not in two fibroblastic cell lines or in a cell line that does not produce type I collagen. In contrast, in transient transfection experiments, the same chimeric construction was expressed at high levels by all cell lines. These results suggest a model where the binding of a lineage-specific transcription factor to specific enhancer segments of type I collagen genes would result in opening the chromatin around the promoter and allow ubiquitous transcription factors to bind to the proximal promoter and to activate transcription of the genes.

The discovery of the far-upstream enhancer raised the question of whether the enhancer could confer tissue specificity on its own, and if individual elements of the proximal promoter contribute differently to *COL1A2* transcription *in vivo*. A series of mutations introduced in each of the transcription factors binding sites within the proximal promoter such as the three Sp1 sites, GC-rich Sp1/Sp3-binding sites, and the CBF-binding site reduced transgene activity suggesting strong evidence that these protein interactions operate *in vivo* as well, and in concert with the enhancer-bound complex (Tanaka *et al.*, 2004). The unique expression pattern of transgenic mice harboring mutations in the binding site of transcription factor CBF/NFY lead to loss of *LacZ* expression in the ventral and head regions of the dermis, as well as in the muscles of the forelimbs. The patterning of specific cell lineages implies that CBF/NFY may be essential for *COL1A2* activity in those cells that do not express the transgene. These transgenic data therefore raise the intriguing possibility that CBF/NFY may be implicated in patterning COL1A2 expression in some mesenchyme cell lineages through a yet-to-be-defined mechanism. The results suggested cooperativity between the far-upstream enhancer and proximal promoter in assembling tissue-specific protein complexes. They confirmed *in vitro* observations indicating that interactions among proximal promoter elements are required for optimal transcription. They also indicated that transcription factors binding to individual promoter elements are responsible for distinct properties of *COL1A2* expression *in vivo* (Ramirez *et al.*, 2006).

In the enhancer sequence of the mouse *Col1a2* gene, the first tissue-specific element to be characterized was a vascular smooth muscle cells (vSMC) element; a 100-bp sequence at about −16.6 kb upstream of the transcription start site regulates collagen expression exclusively in vSMC (Ponticos *et al.*, 2004b). This expression was shown to be activated through the binding of the homeodomain protein Nkx2.5, and further potentiated by the presence of GATA6. In contrast, this element was repressed by the binding of the zinc finger protein delta-EF1/ZEB1. A model of regulation where the activating transcription factor, Nkx2.5, and the repressor,, delta-EF1/ZEB1 compete for an overlapping DNA-binding site. This element is important in understanding the molecular mechanisms of vessel remodeling and is a potential a target for intervention in vascular diseases where there is excessive deposition of collagen in the vessel wall.

First Intron Elements

First Intron of the pro COL1A1 *Gene*

Different negative or positive regulatory segments have been identified within the first intron, but most of the transcription factors binding to these regulatory segments are still unknown. A sequence of the first intron of the human *COL1A2* gene located about +600 bp downstream of the transcription start site binds AP-1, and a mutation that abolished this binding diminished the expression of a reporter gene in transient transfection experiments (Liska *et al.*, 1990) This AP-1 was also found to mediate the repressive effect of ras in fibroblasts (Slack *et al.*, 1995). Another segment of the first intron of the human gene, which extends from 820 to 1093 bp, has been shown to inhibit the activity of a reporter gene in transient transfection experiments (Liska *et al.*, 1992). This sequence contains two binding sites for an Sp1-like transcription factor, and mutations in these two Sp1-binding sites tended to increase the activity of the reporter gene (Liska *et al.*, 1992). An Sp1-binding site is also located at about 1240 bp, in the human gene, and a frequent G→T polymorphism in this Sp1-binding site (G1242T) has been linked with low bone mineral density and increased risk of osteoporotic vertebrate fracture (Grant *et al.*, 1996), which suggests that it may be important for normal levels of type I collagen synthesis by osteoblasts.

The phenotype of Mov 13 mice suggested that the first intron of the pro α1(I) collagen gene could play a role in the expression of this gene. These mice, which harbor a retrovirus in the first intron of the *pro Col1a1* gene (Harbers *et al.*, 1984), express this gene in osteoblasts and odontoblasts, but not in fibroblastic cells (Schwarz *et al.*, 1990). Nevertheless, the presence of tissue-specific regulatory elements in the first intron of the *pro Col1a1* gene has long been controversial. Two groups have reported that, in transgenic mice harboring the proximal promoter of either the human or the rat *pro Col1a1* gene, the pattern of expression of the reporter gene was the same whether or not these mice harbored the first intron of the *pro Col1a1* collagen gene (Sokolov *et al.*, 1993). In contrast, data obtained by *in situ* hybridization in transgenic mice harboring 2.3 kb of the human *pro Col1a1* proximal promoter suggested that the first intron of this gene was necessary to obtain high-level expressions of the transgene in the dermis of skin (Liska *et al.*, 1994). Only mice harboring the first intron, in addition to the 2.3-kb proximal promoter segment, expressed the human growth hormone reporter gene at high levels in skin. In order to clarify this issue, Bornstein's group generated knockin mice with a targeted deletion of most of the first intron (Hormuzdi *et al.*, 1998). Mice homozygous for the mutated allele developed normally and showed no apparent abnormalities. Nevertheless, in heterozygous mice, the mutated allele was expressed at normal levels in skin, but at lower levels in lung and muscle, and its levels of expression decreased with age in these two tissues more than in wild type mice. Thus, the first intron does not play a role in the tissue-specific expression of the *pro Col1a1* gene, but it seems to be important for maintaining normal transcriptional levels of this gene in certain tissues (Hormuzdi *et al.*, 1999).

First Intron of the pro COL1A2 *Gene*

The first intron of the mouse *pro Col1a2* gene has also been shown to contain a tissue-specific enhancer in transient transfection experiments (Rossi and de Crombrugghe, 1987). In transgenic mice, however, the presence of this tissue-specific enhancer apparently had no effect on the pattern of expression of a CAT reporter gene (Goldberg *et al.*, 1992). However, recent transgenic analysis of the human gene suggested that despite the homologies between the two genes, species-specific differences have been reported regarding the function of individual *cis*-acting elements, such as the first intron sequence (Antoniv *et al.*, 2005). *In vitro* DNase I footprinting of the sequence corresponding to the open chromatin site identified a cluster of three distinct areas of nuclease protection that span from nucleotides +647 to +759 of the *COL1A2* gene. These areas contain consensus sequences for GATA and IRF transcription factors. Gel mobility shift and chromatin immunoprecipitation assays corroborated this finding by documenting binding of GATA-4 and IRFs 1and 2 to the first intron sequence (Antoniv *et al.*, 2005). Moreover, a short sequence encompassing the three footprints was found to inhibit expression of transgenic constructs containing the *COL1A2* proximal promoter and far-upstream enhancer in a position-independent manner. Mutations inserted into each of the footprints restored transgenic expression to different extents. These results therefore indicated that the intronic sequence corresponds to a repressor element, the activity of which seems to be mediated by the concerted action of GATA and IRF proteins (see Fig. 2B). Furthermore, GATA4 was found to bind the promoter sequence of HS2 at −2.3 kb in the COL1A2 gene. Overexpression of this transcription factor inhibited the expression of the transgene as well as endogenous gene in fibroblasts (Wang *et al.*, 2005). The presence of a common repressor transcription factor in both HS2 and HS intron led to the suggestion that the "switch-off" of collagen in adults may occur by cooperation of the intronic sequence and HS2 to block the enhancer activity from exerting its effect through its cooperation with the proximal promoter in (HS1) (Ramirez *et al.*, 2006).

Post-transcriptional Regulation of Type I Collagen

Even if the control of expression of type I collagen genes appears to be mainly exerted at the level of transcription, type I collagen production is also be regulated at a post-transcriptional level. For example, TGF-β and IFN-γ modulate not only the levels of transcription of type I collagen genes, but also the stability of the corresponding mRNAs. Similarly, activation of hepatic stellate cells is associated with a dramatic increase in the stability of the *pro Col1a1* mRNA. Run-on experiments have shown that the half-life of this mRNA was increased about 15-fold in activated rat hepatic stellate cells when compared with quiescent ones (Stefanovic *et al.*, 1997). Several lines of evidence indicate that expression of the genes coding for the two collagens I and III is controlled predominantly post-transcriptionally at the mRNA level, as the 3′-UTR of the collagen family mRNA has been shown to be a target for mRNA-binding protein. hnRNP A1, E1, and K substantially participate in the coordinative upregulation of collagen I and III synthesis, which is involved in mRNA stabilization (Lindquist *et al.*, 2000). Indeed, all three *COL1A1*, *1A2*, and *3A1* 3′-UTRs, contain hnRNP K-like consensus motifs and do actually bind it. *COL1A1* and *COL1A2* contain a classical CU-rich hnRNP E1-binding site and it is also able to bind hnRNP E1, although not *COL3A1*, which lacks a more extended

CU-rich region. Furthermore, these mRNA-binding proteins have been shown to increase in fibrotic conditions and are suggested to coordinate expression of type I and III collagens by common post-transcriptional mechanisms targeted at the mRNA 3′-UTR level (Thiele *et al.*, 2004).

The sequence of the mouse pro *Col1a1* mRNA surrounding the start codon could also play a role in regulating mRNA stability. This sequence has been shown to form a stem-loop structure, and mutations that prevent its formation decreased the stability of the mRNA dramatically (Stefanovic *et al.*, 1999). Indeed, the 5′-UTR of *COL1A1*, *COL1A2*, and *COL3A1* mRNAs are involved in such translational control. These 5′ stem loops, together with their cognate binding proteins, should help coordinate translation and couple the translation apparatus to the rest of the collagen biosynthetic pathway (Stefanovic and Brenner, 2003).

FACTORS INVOLVED IN TYPE I COLLAGEN GENE REGULATION

Different cytokines, hormones, vitamins, and growth factors can modify type I collagen synthesis by osteoblasts and/or fibroblasts. The effects of these molecules have been studied mainly *in vitro* by using either bone organ cultures or cell cultures. However, in some instances the *in vivo* effects of these factors on type I collagen synthesis have also been studied. A degree of complexity is due to the fact that some factors can act on type I collagen synthesis but can also act indirectly by modifying the secretion of other factors, which will themselves affect type I collagen synthesis. For example, TNF-α will inhibit type I collagen production through the NF-κB pathway, but it will also induce the secretion of prostaglandin E_2 (PGE_2) and of IL-1, which themselves affect type I collagen production. Furthermore, PGE_2 will induce the production of IGF-1, which also modifies the rate of type I collagen synthesis.

Transforming Growth Factor β

In mammals, the TGF-β family consists of three members (TGF-β1, TGF-β2, and TGF-β3), which have similar biological effects but different spatial and temporal patterns of expression. These three molecules are part of a large family, the TGF-β superfamily, which also contains proteins such as bone morphogenetic proteins (BMPs), activins/inhibins, growth and differentiation factors (GDFs), as well as Nodal and its related proteins (Chang *et al.*, 2002; Massague and Chen, 2000). Members of the TGF-β family are multifunctional proteins that regulate cell proliferation and differentiation, extracellular matrix modification, angiogenesis, apoptosis, and immunosuppression (Feng and Derynck, 2005; Jones *et al.*, 2006; Schier and Shen, 2000). A TGF-β protein exerts its function by binding to and bringing together on the cell surface type I and II receptors to form a ligand–receptor complex (Leask and Abraham, 2004). Five members of type II and seven members of type I receptors [Activin receptor-like kinase (ALK) 1–7] have been characterized in mammals (Graham and Peng, 2006). Upon phosphorylation by the type II receptor, the type I receptor phosphorylates and activates Smads, which are intracellular signaling molecules for members of the TGF-β superfamily. Phosphorylated Smads are released from the receptors and form oligomeric complexes with a common-partner Smad, Smad4, and translocate into the nucleus where they bind to specific DNA sequences called Smad-binding elements, and act as transcription factors to regulate the transcription of target genes. Smad2 and Smad3 respond to TGF-βs, Activins, Nodal and Lefty, whereas Smad1, Smad5, and Smad8 mediate BMP signaling (Miyazawa *et al.*, 2002). Several transcription factors and transcriptional coactivators have been shown to cooperate with Smad complexes, such as Sp1, AP-1, PEBP2/CBF, TFE-3, ATF-2, or CBP/p300 (reviewed in Attisano and Wrana, 2000).

TGF-βs are secreted by many cell types, including monocytes/macrophages, lymphocytes, platelets, fibroblasts, osteoblasts, and osteoclasts (Leask and Abraham, 2004). Synthesis by bone cells is quantitatively important because, *in vivo*, the highest levels of TGF-β are found in platelets and bone (Seyedin *et al.*, 1985). Nearly all cells, including osteoblasts and fibroblasts, have TGF-β receptors (Verrecchia and Mauviel, 2007).

The three mammalian TGF-β isoforms are synthesized as homodimeric proproteins (proTGF-β) that have a mass of 75 kDa. The dimeric propeptides are cleaved from the mature TGF-β 24-kDa dimer in the *trans*-Golgi by furin-type enzymes. Mature TGF-β1 then associates noncovalently with a dimer of its N-terminal propeptide (also called LAP, for latency-associated peptide). This complex is referred to as the small latent complex (Annes *et al.*, 2003). Early in the assembly of TGF-β and LAP, disulfide linkages are formed between cysteine residues of LAP and specific cysteine residues in another protein, the latent-TGF-β binding protein (LTBP) to form the Large Latent Complex (Annes *et al.*, 2003). The LTBP is removed extracellularly by either proteolytic cleavage by various proteases such as plasmin, thrombin, plasma transglutaminase, or endoglycosylases, or by physical interactions of the LAP with other proteins, such as *thrombospondin-1*, which is able to transform latent TGF-β1 into active TGF-β1 *in vitro*. Analysis of mice harboring a targeted disruption of the thrombospondin-1 gene suggests that it likely plays an important role *in vivo* (Crawford *et al.*, 1998). In particular, the phenotype of thrombospondin-1-null mice was relatively similar to the one of TGF-β1-null mice, and fibroblasts isolated from the former mice had a decreased ability to activate TGF-β1. Nevertheless, thrombospondin-1 is probably not the only molecule that activates TGF-β *in vivo* (Abdelouahed *et al.*, 2000).

The four latent TGF-β binding proteins (LTBP1–4) belong to the LTBP/fibrillin family of large extracellular glycoproteins (Todorovic *et al.*, 2005). LTBP1, LTBP3, and LTBP4 form a subgroup within the family, because they covalently interact with latent TGF-β and have an important role as regulators of TGF-β function: LTBPs facilitate the secretion of latent TGF-β, direct its localization in the ECM, and regulate the activation of the cytokine. In mice ablation of the *Ltbp3* gene and attenuation of *Ltbp4* expression both result in developmental defects associated with reduced TGF-β activity (Dabovic *et al.*, 2002; Sterner-Kock *et al.*, 2002), further demonstrating that *Ltbp3* and *Ltbp4* modulate extracellular TGF-β levels in a specific and nonredundant manner.

The role of TGF-β on type I collagen synthesis has been demonstrated both *in vivo* and *in vitro*. *In vivo*, subcutaneous injections of platelet-derived TGF-β in newborn mice increased type I collagen synthesis by dermal fibroblasts with formation of granulation tissue (Roberts *et al.*, 1986). Injections of platelet-derived TGF-β onto the periostea of parietal bone of newborn rats stimulated bone formation, and thus accumulation of extracellular matrix (Noda and Camilliere, 1989). Transgenic mice that overexpressed mature TGF-β1developed hepatic fibrosis and renal fibrosis (Sanderson *et al.*, 1995). Increased expression of TGF-β2 in osteoblasts in transgenic mice resulted in an osteoporosis-like phenotype with progressive bone loss. The bone loss was associated with an increase in osteoblastic matrix deposition and osteoclastic bone resorption (Erlebacher and Derynck, 1996). Conversely, expression of a dominant-negative TGF-β receptor mutant in osteoblasts led to decreased bone remodeling and increased trabecular bone mass (Filvaroff *et al.*, 1999). In humans, mutations in the latency-associated peptide of TGF-β1 (LAP) causes Camurati–Engelmann disease, a rare sclerosing bone dysplasia inherited in a autosomal-dominant matter. It is unclear whether these mutations impair the ability of the LAP to inhibit TGF-β activity or whether the mutations cause accelerated degradation of TGF-β (Janssens *et al.*, 2000; Kinoshita *et al.*, 2000).

More recently, transgenic mice expressing a kinase-deficient type II TGF receptor (T-RIIΔk) in fibroblasts demonstrated unexpected skin and lung fibrosis (Denton *et al.*, 2003). Moreover, the fibrotic phenotype of explanted dermal fibroblasts from the T-RIIDk-deficient transgenic mice showed that expression of the mutant receptor leads to multilevel activation of the TGF-β ligand–receptor axis and that activation depends on endogenous T-RI receptor kinase activity (Denton *et al.*, 2005). This paradoxical increased acivity of the TGF-β-ligand–receptor axis in these transgenic mice occurs despite previous findings that the kinase-deficient TGF-β receptors are dominant negative inhibitors of signaling in several experimental systems, when expressed at high expression levels compared with wild-type receptors. Further evidence that TGF-β and its signaling pathways significantly influence collagen gene regulation is manifested in Smad3 −/− mice exposed to a single dose of 30 to 50 Gy of gamma-irradiation; these mice showed significantly less epidermal acanthosis and dermal influx of mast cells, macrophages, and neutrophils and decreased expression of TGF-β than skin from wild-type littermates suggesting that inhibition of Smad3 could decrease tissue damage and reduce fibrosis after exposure to ionizing radiations (Flanders *et al.*, 2002). Conversely, C57BL/6 mice with bleomycin-induced lung fibrosis receiving an intratracheal injection of a recombinant adenovirus expressing Smad7 demonstrated suppression of Type 1 procollagen mRNA, reduced hydroxyproline content, and no morphological fibrotic responses in the lungs, indicating that gene transfer of Smad7 prevents bleomycin-induced lung fibrosis (Nakao *et al.*, 1999). More recently, using mice with a targeted deletion of Smad3, Roberts *et al.* (2006) demonstrated that lack of Smad3 prevents the epithelial-to-mesenchymal transition of lens epithelial cells following injury, and attenuates the development of fibrotic sequelae. Together, these various experimental approaches demonstrate the direct implication of Smad3 and TGF-β in fibrotic diseases.

In vitro, TGF-β stimulates the synthesis of most of the structural components of the extracellular matrix by fibroblasts and osteoblasts, including type I collagen (for a review, see Leask and Abraham, 2004). It also decreases extracellular matrix degradation by repressing the synthesis of collagenases and stromelysins and by increasing the synthesis of tissue inhibitors of metalloproteinases (TIMPs). It increases lysyl-oxidase activity, which may favor interchain cross-linking in collagen fibrils (Feres-Filho *et al.*, 1995). Finally, it stimulates the proliferation of both fibroblasts and osteoblasts, in contrast to its inhibitory effect on the proliferation of epithelial cells. Moreover, TGF-β may have a role in controlling the lineage-specific expression of type I collagen genes during embryonic development, because there is an excellent temporal and spatial correlation between activation of type I collagen genes and the presence of immunoreactive TGF-β in the extracellular environment (Niederreither *et al.*, 1992).

One way in which TGF-β exerts its action is at a pretranslational level, by increasing mRNA levels of the *pro Col1a1* and *pro Col1a2* transcripts. This increase in type I collagen mRNA levels can be caused by an increase in the transcription rate of type I collagen genes and/or by an increase in procollagen mRNA stability, with the relative contribution of these two mechanisms depending on cell types and on culture conditions. The second effect is through Smad proteins as indicated later: The effect of TGF-β on transcription of the human pro *COL1A2* gene involves Smad complexes reviewed by Ramirez *et al.* (2006). This effect is mediated through a sequence of the promoter located between −378 and −183 bp upstream of the start site of transcription and is called TGF-β-responsive element (TβRE), as demonstrated by transfection experiments

(Inagaki *et al.*, 1994). Footprinting experiments performed with this sequence revealed two distinct segments interacting with DNA-binding proteins (Inagaki *et al.*, 1994). It contains two binding sites for Sp1, as well as a binding site for C/EBP (Greenwel *et al.*, 1997; Inagaki *et al.*, 1994). The DNA sequence between −271 and −250 contains a CAGA box that binds Smad 3/Smad 4 complexes (Chen *et al.*, 1999; Zhang *et al.*, 2000), as well as a binding site for AP-1 (Chung *et al.*, 1996). Data have shown that Smad 3/Smad 4 complexes can bind to CAGA box and mediate TGF-β induced stimulation of the *pro COL1A2* gene, in cooperation with Sp1 proteins (Zhang *et al.*, 2000). Other studies have suggested a role of AP-1 in mediating the effects of TGF-β (Chung *et al.*, 1996). This Smad/AP1 interaction was later found to take place "off DNA" (Verrecchia *et al.*, 2001). The transcriptional coactivator CBP/p300, which can bind to Smad complexes, also plays an important role in mediating the effects of TGF-β on the transcriptional activity of the *pro COL1A2* gene (Ghosh *et al.*, 2000). Thus, TGF-β probably activates the transcription of the *pro COL1A2* gene through the binding of a multimeric complex, which includes Smad 3/Smad 4, Sp1, CBP/p300, and possibly AP-1. More recently, using Affymetrix microarrays to detect cellular genes, whose expression is regulated by TGF-β through Smad3, identified the gene for an early growth response factor-1 (EGR-1) as a Smad3-inducible gene (Chen *et al.*, 2006a). It was also found that TGF-β enhanced endogenous Egr-1 interaction with a consensus Egr-1-binding site element and with GC-rich DNA sequences of the human *COL1A2* promoter *in vitro* and *in vivo*. Furthermore, forced expression of Egr-1 by itself caused dose-dependent upregulation of *COL1A2* promoter activity and further enhanced the stimulation induced by TGF-β (Chen *et al.*, 2006a). Other transcriptional coactivators such as the steroid receptor coactivator-1 may also participate in TGF-β effects (Dennler *et al.*, 2005).

In the rat *pro Col1a1* gene, a TGF-β response element has been described at about 1.6 kb upstream of the start site of transcription (Ritzenthaler *et al.*, 1993) and between −174 and −84 bp in the human pro α1 collagen gene (Jimenez *et al.*, 1994). The latter sequence contains an Sp1-like binding site, but none of these two sequences seems to contain a potential Smad-binding site. In addition to direct action of TGF-β, part of the profibrotic properties may be indirect, mediated by an increased production of a cysteine-rich protein called connective tissue growth factor (Leask and Abraham, 2003).

Connective tissue growth factor (CTGF, CCN2)

CTGF (also called CCN2), a member of the CCN family of matricellular proteins, has long been known to promote differentiation and proliferation of chondrocytes and osteoblasts (for a review, see Takigawa *et al.*, 2003). CCN2 promotes fibroblast proliferation, matrix production, and granulation tissue formation, as well as cell adhesion and migration. Experiments using recombinant CTGF and neutralizing antibodies targeting CTGF have suggested that CTGF mediates at least some of the effects of TGF-β on fibroblast proliferation, adhesion, and ECM production, including collagen and fibronectin (Crean *et al.*, 2002; Weston *et al.*, 2003). Moreover, an expression vector encoding CTGF transfected into fibroblasts can activate a reporter driven by the type I collagen promoter, suggesting that a CTGF response element exists in the promoter of type I collagen (Shi-wen *et al.*, 2000). Perhaps the most significant recent insights into the specific physiological roles of the CCN2 have come from the generation of mutant mice lacking CCN2 (Ivkovic *et al.*, 2003). *Ccn2* −/− mice display severely malformed ribcages and die soon after birth owing to a failure to breathe. These mice exhibit impaired chondrocyte proliferation and proteoglycan production within the hypertrophic zone. Excessive chondrocytic hypertrophy and a concomitant reduction in endochondral ossification are also observed. Further support for the idea that CCN2 regulates bone formation in development comes from studies of transgenic mice that overproduce CCN2 under the control of the mouse type XI collagen promoter. These mice develop normally but show dwarfism within a few months of birth owing to a reduced bone density (Nakanishi *et al.*, 2001). The molecular basis for this deformity has not yet been explored; however, a possible explanation is that CCN2 overexpression results in abnormally premature ossification, before proper chondrocyte maturation.

CCN2 is constitutively expressed in fibrotic and embryonic fibroblasts independently of TGF-β (Chen *et al.*, 2006b; Holmes *et al.*, 2001, 2003). Experiments using *Ccn2* −/− MEFs have shown that loss of CCN2 results in an inability of TGF-β to induce expression of approximately one-third of those mRNAs induced in *Ccn2* +/+ MEFs (Shi-wen *et al.*, 2006). Consistent with the fact that CCN2 is required only for a subset of TGF responses, *Ccn2* −/− MEFs show no impairment of the generic Smad pathway, emphasizing the relative selectivity of CCN2-dependent action. In contrast to the lack of effect of loss of CCN2 expression on basal type I collagen and alpha-smooth muscle actin expression, the ability of TGF-β to induce these proteins is impaired in *Ccn2* −/− MEFs (Shi-wen *et al.*, 2006).

Expression of the *CTGF* gene in fibroblasts is strongly induced by TGF-β but not by other growth factors, and intradermal injections of TGF-β in neonatal mice induced an overexpression of *CTGF* in skin fibroblasts (Frazier *et al.*, 1996; Igarashi *et al.*, 1993). The ability of TGF-β to induce CCN2 also requires protein kinase C and the Ras/MEK/ERK MAP kinase cascade (Chen *et al.*, 2002b; Stratton *et al.*, 2002). As in the case of other TGF-β-responsive promoters that do not require the transcription factor AP-1, the induction of CCN2 by TGF-β is antagonized by hyperactive AP-1 or Jun N-terminal kinase (JNK) (Leask *et al.*, 2003) because of the ability of active Jun to bind to Smads off DNA and inhibit Smads from interacting with the target DNA sequences (Verrecchia *et al.*, 2001).

Although a specific CTGF receptor has yet to be identified, CTGF appears to perform many of its functions through integrins, heparin sulfate-containing proteoglycans, and the low-density lipoprotein receptor-related protein (Gao and Brigstock, 2003; Segarini *et al.*, 2001; Weston *et al.*, 2003).

Growth Factors

Fibroblast Growth Factor

FGFs comprise a family of 23 genes encoding structurally related proteins divided into six subfamilies of FGFs (Ornitz and Marie, 2002). The most recent member is FGF23, which is mainly produced by osteocytes in bone and acts as a hormone primarily to inhibit phosphate reabsorption in the proximal tubules of the kidney (Liu *et al.*, 2007; Razzaque and Lanske, 2007). Most members of the FGF family bind to four distinct FGF receptor tyrosine kinase molecules (FGFR) and activate the receptors. Historically, FGF2 (basic fibroblast growth factor) was the first FGF ligand to be isolated from growth plate chondrocytes (Sullivan and Klagsbrun, 1985). Subsequently, *Fgf2* gene expression has also been observed in periosteal cells and in osteoblasts (Hurley *et al.*, 1999; Sabbieti *et al.*, 1999). FGF signaling affects the expression and activity of several transcription factors that are required for calvarial osteogenesis. In rat or mouse calvarial cells, FGF2 activates osteocalcin transcription. This activity is inhibited by the transcription factor MSX2 and is activated by DLX5 (Newberry *et al.*, 1997, 1999). FGF2 can up-regulate *Twist* expression in mouse calvarial mesenchyme (Rice *et al.* 2000). *Twist* heterozygous mice show altered FGFR protein expression (Rice *et al.*, 2000), suggesting that Twist acts upstream of FGF signaling pathways. Additionally, Twist could be a potential transcriptional regulator that mediates the negative effect of FGF2 on type I collagen expression in calvarial cells (Fang *et al.*, 2001). Thus, FGF/FGFR, MSX, and Twist functionally interact to control cranial suture development in a coordinated manner. FGF_2 production by calvarial osteoblasts is upregulated by FGF2 itself, and by parathyroid hormone, PGE_2, and TGF-β. Thus, the balance between high and low levels of endogenous FGF2 may constitute a mechanism to control proliferation and ensure normal cranial vault development (Moore *et al.*, 2002).

Bone resorption by osteoclasts is required to maintain the shape of craniofacial bones during development. It is therefore significant that FGF2 can increase the formation of osteoclast-like cells and activate mature osteoclasts through FGFR1 (Chikazu *et al.*, 2000). In addition, FGF2 increases the expression of metalloproteinases, collagenases 1 and 3 (Newberry *et al.*, 1997; Tang *et al.*, 1996; Varghese *et al.*, 2000) tissue inhibitors of metalloproteinases (TIMP) 1 and 3 (Varghese *et al.*, 1995) and stromelysin-3, which regulates collagenase activity in calvarial cells (Delany and Canalis, 1998). These mechanisms may control bone matrix degradation and remodeling by FGFs during calvarial expansion. Targeted deletion of FGF2 causes a relatively subtle defect in osteoblastogenesis, leading to decreased bone growth and bone density. However, no defects in chondrogenesis were observed (Montero *et al.*, 2000).

Insulin-like Growth factor

Insulin-like growth factor I (IGF-I) is synthesized by many cells, including cells of the osteoblastic lineage, as well as chondroblasts and osteoclasts (Rajaram *et al.*, 1997). IGF-I plays an important role in regulating peak bone mineral density and bone size (Mohan and Baylink, 2005; Mohan *et al.*, 2003). The IGFs interact with specific cell surface receptors, designated type I and type II IGF receptors (IGF-IR and IGF-IIR). Most of the actions of IGF-I and IGF-II are mediated by the IGF-IR, which is a transmembrane receptor with tyrosine kinase activity.

The IGFs in serum and other extracellular environments are bound to specific IGF-binding proteins (IGFBPs), which represent a family of six secreted proteins with a common domain organization. Each IGFBP has unique properties and exhibits specific functions. IGFBPs 2 to 5 are present in bone; IGFBP-4 and -5 are expressed at the highest levels. Unlike IGFBP-4, which has an inhibiting effect on IGF actions, IGFBP-5 has a potentiating effect, both *in vivo* and *in vitro*, probably by binding directly to sites that are independent of the IGF receptor (Govoni *et al.*, 2005).

IGF-I and IGF-II can stimulate osteoblast and fibroblast proliferation and increase type I collagen production by these cells. Their effect on type I collagen production by osteoblasts has been demonstrated by using both fetal rat calvariae and osteoblastic cells, and it is related to an increase in corresponding mRNA transcripts (McCarthy *et al.*, 1989; Thiebaud *et al.*, 1994; Woitge and Kream, 2000).

In vivo, administration of IGF-I to hypophysectomized rats increased mRNA transcripts for pro *Col1a1* and pro *Col1a2* genes in parietal bones (Schmid *et al.*, 1989). Furthermore, in calvaria of mice, in which the *IgfI* gene was disrupted, there was a reduced rate of collagen synthesis. Using the *Cre-LoxP* model to disrupt IGF-I in all *Col1a2*-expressing cells demonstrated that locally produced IGF-I plays a critical role in embryonic and postnatal growth. Local IGF-I from *Col1a2*-producing cells is required for bone matrix mineralization during embryonic development and postnatal growth (Govoni *et al.*, 2007). Moreover, to determine the effects of locally expressed IGF-I on bone remodeling, a transgene was produced in which murine IGF-I cDNA was cloned downstream of a gene fragment comprising 3.6 kb of 5′ upstream regulatory sequence and most of the first intron of the rat *Col1a1* gene. Transgenic calvaria showed an increase in osteoclast numbers per bone surface, as well as increased collagen synthesis and cell proliferation. Femur length, cortical width, and cross-sectional area were also increased in transgenic femurs, whereas femoral trabecular bone volume displayed little change. Thus, broad overexpression of IGF-I in cells of the osteoblast lineage increased indices of bone formation and resorption (Jiang *et al.*, 2006). Furthermore, in calvaria of mice, in which the IGF-1 gene is disrupted, there was a reduced rate of collagen synthesis (Woitge and Kream, 2000).

Cytokines

Tumor Necrosis Factor alpha

Tumor necrosis factor α (TNF-α) is a cytokine secreted mainly by monocytes/macrophages, but osteoblasts seem to be able to produce TNF-α under certain conditions (Gowen *et al.*, 1990). After being cleaved from its propeptide, TNF-α undergoes trimerization and binds to type I receptors, which transduce most of the effects of TNF-α, or to type II receptors,which activate them and transmit signals to the nucleus via different transcription factors.

TNF-α levels are elevated in various bone disorders such as rheumatoid arthritis and osteoporosis (Beutler and Cerami, 1988; Pacifici, 1996). In bone tissue, TNF-α inhibits osteoblast function and increases osteoclastogenesis, thus favoring net matrix destruction (Centrella *et al.*, 1988; Chou *et al.*, 1996; Panagakos *et al.*, 1996). Similarly, TNF-α stimulates fibroblast and osteoblast proliferation, inhibits the production of extracellular matrix components, including type I collagen and its modifying enzyme, lysyl oxidase (Kouba *et al.*, 1999; Pischon *et al.*, 2004; Verrecchia *et al.*, 2000), as well as that of the transcription factor Sox9 (Murakami *et al.*, 2000), and increases collagenase production and thus extracellular matrix degradation (Iraburu *et al.*, 2000).

In vivo, inoculation of nude mice with TNF-α-producing cells decreased type I collagen production in skin and liver, impaired wound healing, and decreased TGF-β1 synthesis in skin (Houglum *et al.*, 1998). TNF-α also increases PGE_2 and interleukin 1 production by osteoblasts and fibroblasts, which themselves modulate type I collagen synthesis.

In dermal fibroblasts, inhibition of collagen synthesis in fibroblasts by TNF-α is associated with a decrease in mRNA levels for the *pro Col1a1* and *pro Col1a2* transcripts and in the transcription of type I collagen genes (Solis-Herruzo *et al.*, 1988). Inagaki *et al* (1995) showed that TNF-α increases binding of a protein complex that recognizes the negative *cis*-acting element located immediately next to the TβRE, and postulated that TNF-α counteracts the TGF-β elicited stimulation of collagen gene expression through overlapping nuclear signaling pathways. Kouba *et al.* (1999) characterized this specific TNF-α response element and termed it (TaRE) between nucleotides −271 and −235 relative to the transcription initiation site. Electrophoretic mobility supershift assays identified the NF-κB family members NF-κB1 and RelA as transcription factors binding the TaRE and mediating TNF-α repression of *COL1a2* promoter activity. By using a gene knockout approach, the same group showed that, in primary fibroblasts from NF-κB essential modulator (NEMO) knockout mice, lack of NF-κB activation prevented repression of basal *COL1a2* gene expression by TNF-α. Similar regulatory mechanisms take place in dermal fibroblasts, transfected with dominant negative forms of IKK-a, a critical kinase upstream of NF-κB (Verrecchia *et al.*, 2002). More interestingly, the antagonist activities of TGF-β and TNF-α in *COL1a2* may be the result of steric interactions between transcription factors binding to TβRE and TaRE, respectively (Greenwel *et al.*, 2000; Verrecchia *et al.*, 2001).

The inhibitory effects of TNF-α on the rat *pro Col1a1* proximal promoter was shown to be mediated by a sequence located between −378 and −345 bp through the binding of proteins of the C/EBP family, such as C/EBP delta and p20C/EBP beta (Iraburu *et al.*, 2000). Other TNF-α response elements have been identified within the *pro Col1a1* gene between −101 and −38 bp and between 68 and 86 bp, in dermal fibroblasts and hepatic stellate cells, respectively (Hernandez *et al.*, 2000; Mori *et al.*, 1996). The latter *cis*-acting element binds proteins of the Sp1 family, whereas the proteins binding to the former one have not been identified (Hernandez *et al.*, 2000; Mori *et al.*, 1996). Using two lines of transgenic mice harboring a growth hormone reporter gene under the control of either, at 2.3 kb, the human *pro COL1A1* proximal promoter plus the first intron, or at 440 bp of this promoter plus the first intron, Chojkier's group reported that different *cis*-acting elements mediate the inhibitory effects of TNF-α, depending on the tissue (Houglum *et al.*, 1998). In skin, the inhibitory effect of TNF-α on the activity of the reporter gene was mediated through a *cis*-acting element located between −2.3 kb and −440 bp (Buck *et al.*, 1996). In contrast, this inhibitory effect of TNF-α was mediated in liver through an element located between −440 and +1607 bp (Houglum *et al.*, 1998).

Interferon γ

Interferon γ (IFN-γ) is a cytokine produced both by monocytes/macrophages and by type I helper T cells. IFN-γ binds to the IFN-γ receptor complex (IFNGR1 and IFNGR2), followed by the activation of receptor-associated janus kinase (JAK), which in turn phosphorylates and activates the signal transducer and activator of transcription (STAT1). Once phosphorylated, STAT1 molecules dimerize and translocate to the nucleus where they modulate target gene transcription either by direct interaction with specific sequences or through protein–protein interactions (Darnell *et al.*, 1994; Horvai *et al.*, 1997).

In vitro, IFN-γ decreases osteoblast and fibroblast proliferation and type I collagen synthesis by these cells. This latter effect seems to be caused both by a decrease in type I collagen mRNA stability (Czaja *et al.*, 1987; Kahari *et al.*, 1990) and by a decrease in the transcription rate of the pro *Col1a1* gene (Diaz and Jimenez, 1997; Yuan *et al.*, 1999). Transfection studies using different segments of the human pro *COL1A1* proximal promoter have shown the existence of an IFN-γ response element between −129 and −107 bp that can bind transcription factors NF1 and members of the Sp1 family (Yuan *et al.*, 1999). However, mutations in NF1 and Sp1 sites, which abrogate the binding of these factors, repress the basal *COL1A1* promoter activity but are unable to abrogate the IFN-γ-mediated inhibition, suggesting that NF1 and Sp1 are not involved in this inhibitory action of IFN-γ (Yuan *et al.*, 1999).

In the human *COL1A2* gene, an IFN-γ response element has been identified between −161 and 125 bp by using transfection experiments in dermal fibroblasts (Higashi *et al.*, 1998). Simlarly to the action of TNF-α, Ulloa *et al.* (1999) reported that IFN-γ abrogates the TGF-β stimulation of TGF-β responsive element containing reporter constructs in fibrosarcoma cells by inducing the level of Smad7 via the JAK-STAT1 signaling pathway. To account for the antagonistic action of IFN-γ and TGF-β on expression of the *COL1a2* gene, however, evidence suggests another mechanism involving p300/CBP. This evidence indicates that IFN-γ activated STAT1 sequesters the endogenous p300 and reduces its interaction with Smad3, thus inhibiting the TGF-β/Smad-induced collagen gene transcription (Ghosh *et al.*, 2001). Moreover, Higashi *et al.* (2003) showed that Y-box binding factor (YB-1) binds to the IFN-γ responsive element at −165 to −150 and mediates transcriptional repression of *COL1A2*. The use of YB-1 as a therapeutic target was successfully used to downregulate collagen type I, as a strategy to treat liver fibrosis *in vivo*. This effect was potentiated by the addition of exogenous IFN-γ (Inagaki *et al.*, 2005).

Other Cytokines

Interleukin 1

Interleukin 1 (IL-1) is a cytokine secreted mainly by monocytes/macrophages, but also by other cells, including fibroblasts, osteoblasts, synoviocytes, and chondrocytes. Two forms of IL-1 have been described, IL-1 a and IL-1β, which have little primary structure homology but bind to the same receptor and have similar biological activities (Stylianou and Saklatvala, 1998).

IL-1 modulates the type I collagen synthesis at different levels, and the variations may be owing to cell type, species, and age differences but, at present, the molecular mechanisms by which IL-1 modulates the type I collagen gene transcription are not clear. *In vitro*, IL-1 has an inhibitory effect on type I collagen production by osteoblasts, which is owing to an inhibition of type I collagen gene transcription (Harrison *et al.*, 1990). Nevertheless, it can be masked when low doses of IL-1 are used, as this cytokine stimulates the production of PGE$_2$, which in turn can modulate type I collagen synthesis. Slack *et al.* (1995) have suggested that the inhibitory effects of IL-1 on the transcription of the human pro *COL1A1* collagen gene by osteoblasts could be mediated through the binding of AP-1 to the first intron of the gene (Slack *et al.*, 1995), but this has not been confirmed. *In vitro*, IL-1β inhibits the expression of the *COL1A2* in human lung fibroblasts at the transcriptional level by a PGE$_2$-independent effect, as well as through the effect of endogenous fibroblast PGE$_2$ released under the stimulus of the cytokine (Diaz *et al.*, 1993). More recently, in SSc fibroblasts, pre-IL-1 a was shown to form a complex with IL-1α-binding proteins that are translocated into the nuclei of fibroblasts via HAX-1 (HS1-associated protein X-1) and IL-1 receptor type II in order to increase production of collagen and IL-6 (Kawaguchi *et al.*, 2006). In contrast, the biological impact of IL-1β on tendon fibroblasts showed that the presence of IL-1β significantly decreased the level of collagen type I mRNA. These effects were found to be mediated by selective upregulation of EP(4) receptor, which is a member of G-protein-coupled receptor that transduces the PGE$_2$ signal via the p38 MAPK pathway (Thampatty *et al.*, 2007).

Interleukin 13

Interleukin 13 (IL-13) is a major inducer of fibrosis. Indeed, IL-13 induces expression of TGF − β1 in macrophages. The increase in TGF-β expression requires both TNF-α and signaling through IL-13 receptor α2 to activate an AP-1 variant, which stimulates the TGF-β promoter. Prevention of IL-13Ra2 expression, IL-13Ra2 gene silencing, or blockade of IL-13Ra2 signaling lead to marked downregulation of TGF-β1 production and collagen deposition in bleomycin-induced lung fibrosis (Fichtner-Feigl *et al.*, 2006). Thus, IL-13R a2 signaling during prolonged inflammation could be an important therapeutic target for the prevention of TGF-β1-mediated fibrosis.

Interleukin 4

Interleukin-4 (IL-4) is secreted by type 2 helper T cells and by mastocytes. *In vitro*, IL-4 increases type I collagen production by human fibroblasts by increasing both transcriptional levels of type I collagen genes and stability of the corresponding mRNAs (Serpier *et al.*, 1997). In bronchial fibroblasts, IL-4 positively regulates *pro COL1A1* transcription by direct promoter activation and increases the TIMP-2/MMP-2 ratio, thereby supporting the profibrotic effect of this cytokine. Furthermore, a combined action of SP1, NF-κB, and STAT6 contribute to the IL-4 mediated *COL1A2* gene activation. An AP2 site adjacent to a STAT6 consensus motif TTC N(3/4) GCT is located within 205 bases from the transcription start site and seems to support the moderate IL-4-induction of COL1A1 gene expression (Bergeron *et al.*, 2003; Buttner *et al.*, 2004).

Other Interleukins

Interleukin 6 (IL-6) has been shown to induce collagen type I and is elevated in patients with systemic sclerosis (Kawaguchi *et al.*, 1999) and in cultured hepatic stellate cells (Nieto, 2006).

Interleukin 10 (IL-10) is secreted mainly by monocytes/macrophages, inhibits type I collagen genes transcription and type I collagen production by skin fibroblasts (Reitamo *et al.*, 1994).

Oncostatin M is produced mainly by activated T cells and monocytes/macrophages and belongs to the hematopoietic cytokine family. It is mitogenic for fibroblasts and stimulates type I collagen production by fibroblasts by increasing transcriptional levels of type I collagen genes (Ihn *et al.*, 1997). Transfection studies performed using different segments of the human pro α2(I) collagen gene have shown that a 12-bp segment located between −131 and −120 bp, and that contains a TCCTCC motif, mediated the stimulatory effects of oncostatin M (Ihn *et al.*, 1997).

Arachidonic Acid Derivatives

PGE_2, a product of the cyclooxygenase pathway, is synthesized by various cell types, including endothelial cells, monocytes/macrophages, osteoblasts, and fibroblasts. Its production by the fibroblasts is increased by IL-1 and TNF-α. PGE_2 has a biphasic effect on type I collagen synthesis by bone organ cultures and by osteoblastic cells (Ono *et al.*, 2005). At low concentration, it increases type I collagen synthesis, whereas at higher concentrations it decreases type I collagen synthesis. PGE_2 induces the production of IGF-I by osteoblastic cells, and part of the stimulatory effect of low doses of PGE_2 on type I collagen production seems to be indirect, mediated by a stimulation of IGF-I production (Raisz *et al.*, 1993b). Nevertheless, part of this stimulatory effect is independent of IGF-I production and persists after blocking the effects of IGF-I (Raisz *et al.*, 1993a). It is notable that when PGE_2 is added to fibroblasts in culture, it inhibits type I collagen synthesis and decreases the levels of the corresponding mRNAs. Most of the effects of PGE_2 are mediated through an increase in cAMP levels (Sakuma *et al.*, 2004) and the activation of collagen synthesis by low doses of PGE_2 could be caused by such a mechanism, because cAMP analogues can also increase collagen synthesis in bone (Fall *et al.*, 1994). In contrast, the inhibitory effect of PGE_2, which has been shown to be caused by an inhibition of transcription of type I collagen genes, is not mediated through a cAMP-dependent pathway but through a pathway involving the activation of protein kinase C (Sakuma *et al.*, 2004). A study using an osteoblastic cell line transfected stably with various segments of the rat pro Col1a1 promoter cloned upstream of a CAT reporter gene has shown that PGE_2 acts through an element located more than 2.3 kb upstream of the start site of transcription (Raisz *et al.*, 1993b). A more recent study using fibroblasts transfected transiently with a construct containing 220 bp of the mouse *pro Col1a1* proximal promoter has shown that PGE_2 can also act through a *cis*-acting element located within this promoter segment (Riquet *et al.*, 2000).

Hormones and Vitamins

Corticosteroids

It has been known for many years that the administration of corticosteroids to patients results in osteoporosis and growth retardation. In mice, corticosteroids have also been shown to decrease collagen production in calvariae (Advani *et al.*, 1997). *In vitro*, incubation of fetal rat calvariae with high doses of corticosteroids, or with lower doses but for a prolonged period of time, decreased the synthesis of type I collagen (Canalis, 1983; Dietrich *et al.*, 1979); this inhibitory effect could also be observed with osteoblastic cell lines (Hodge and Kream, 1988). Moreover, cortisol increases interstitial collagenase transcript levels by post-transcriptional mechanisms in osteoblastic cells (Delany *et al.*, 1995b). Nuclear run-off experiments performed using osteoblasts derived from fetal rat calvariae showed that glucocorticoids downregulate transcriptional levels of the *pro Col1a1* gene, as well as stability of the corresponding mRNA (Delany *et al.*, 1995a). Because corticosteroids inhibit the secretion of IGF-I, part of their inhibitory effect on type I collagen synthesis could be indirect, but calvariae from IGF-I-null mice maintain their responsiveness to glucocorticoids (Woitge and Kream, 2000).

When added to fibroblasts in culture, corticosteroids usually decrease type I collagen synthesis by acting at a pretranslational level, which is in agreement with their *in vivo* effect on wound healing (Cockayne *et al.*, 1986). Stable transfection experiments using the mouse pro *Col1a2* proximal promoter fused to a CAT reporter gene and transfected into fibroblasts have shown that sequences located between −2048 and −981 bp and between −506 and −351 bp were important for the corticosteroid-mediated inhibition of transcription (Perez *et al.*, 1992). However, the *cis*-acting element(s) responsible was not sufficient to block the effect alone (Meisler *et al.*, 1995), and further experiments from the same group indicated that glucocorticoids coordinately regulate procollagen gene expression through TGF-β elements. Depression of procollagengene expression by glucocorticoids through the TGF-β element is mediated by decreased TGF-β secretion, possibly involving a secondary effect on regulatory proteins encoded by noncollagenous protein genes (Cutroneo and Sterling, 2004).

Bone mineral density (BMD) decreases by 2% to 4.5% after just 6 months of glucocorticoid administration to healthy men, but subsequently the rate of bone loss declines (LoCascio *et al.*, 1990). Considerable evidence indicates that glucocorticoid-induced bone loss occurs in two phases in both humans and mice: an early, rapid phase in which bone mass is lost because of excessive bone resorption and a slower, later phase in which bone is lost because of inadequate bone formation (LoCascio *et al.*, 1990; Weinstein *et al.*, 1998). Transgenic mice overexpressing 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) using the osteoclast-specific murine tartrate-resistant acid phosphatase (TRAP) promoter (Reddy *et al.*, 1995) exhibited decreased cancellous osteoclasts after glucocorticoid administration and were protected from the glucocorticoid-induced early, rapid loss of BMD (Jia *et al.*, 2006), suggesting that direct effects of glucocorticoids on osteoclasts are more important than these mediators in the early, rapid loss of bone mass that follows glucocorticoid administration. Similarly, when an osteoblast-specific 2.3-kb *Col1a1* promoter drives (11β-HSD2) expression in mature osteoblasts, 11β-HSD2 should metabolically inactivate endogenous glucocorticoids in the targeted cells, thereby reducing glucocorticoid signaling; collagen synthesis rates were lower in calvarial organ cultures of transgenic mice than in wild type. Furthermore, the inhibitory effect of 300 n*M* hydrocortisone on collagen synthesis was blunted in transgenic calvariae. Trabecular bone parameters measured by microcomputed tomography were also reduced in L3 vertebrae, but not femurs, of 7- and 24-week-old transgenic females. This effect was not seen in male mice, suggesting that endogenous glucocorticoid signaling is required for normal vertebral trabecular bone volume and architecture in female mice (Sher *et al.*, 2004).

Thyroid Hormones

Thyroid hormones have been shown to inhibit type I collagen production by cardiac fibroblasts, and this effect was associated with a decrease in the levels of pro *Col1a1* mRNA (Chen *et al.*, 2000b). Transfection studies have shown that thyroid hormones modulate transcriptional levels of the *pro Col1a1* gene through a *cis*-acting element located between −224 and 115 bp (Chen *et al.*, 2000b). In addition, thyroid hormone (T3) regulates the FGFR1 promoter in osteblasts through a thyroid receptor-binding site at position −279/−264 (O'Shea P *et al.*, 2007).

Parathyroid Hormone

Parathyroid hormone (PTH) binds to specific receptors in osteoblasts and upregulates RANKL expression, a protein essential for osteoclast development and survival. PTH signaling occurs via a PTH receptor /1/cAMP/ protein kinase A/CREB cascade. Runx2 may contribute to the osteoblast specificity of PTH signaling (Fu *et al.*, 2006) by downregulating OPG expression (Boyle *et al.*, 2003). *In vitro*, parathyroid hormone inhibits type I collagen synthesis by osteoblastic cell lines as well as by bone organotypic cultures (Kream *et al.*, 1986). This inhibitory effect is associated with a decrease in the levels of procollagen mRNAs (Kream *et al.*, 1986). When calvariae of transgenic mice harboring a 1.7-, 2.3-, or 3.6-kb segment of the rat *pro Col1a1* proximal promoter were cultured in the presence of parathyroid hormone, there was a parallel decrease in the incorporation of [^{3}H]proline and in the activity of the reporter gene, suggesting that the *pro Col1a1* promoter contains a *cis*-acting element located downstream of −1.7 kb, which mediates the inhibition of the *pro Col1a1* gene expression induced by parathyroid hormone (Bogdanovic *et al.*, 2000; Kream *et al.*, 1993). Furthermore, the effect of parathyroid hormone on the levels of expression of the reporter gene were mimicked by cAMP and potentiated by a phosphodiesterase inhibitor, suggesting that the inhibitory effects of parathyroid hormone are mediated mainly by a cAMP-signaling pathway (Bogdanovic *et al.*, 2000).

Vitamin D

The classic role of the vitamin D endocrine system is to stimulate calcium absorption in the intestine, thus maintaining normocalcemia and indirectly regulating bone mineralization (van Driel *et al.*, 2004). The actions of vitamin D are mediated through the vitamin D receptor (VDR), which acts as a ligand-activated transcription factor to regulate the expression of target genes. The VDR heterodimerizes with retinoid X receptor (RXR) and associates with the transcriptional complex on promoters of target genes. *In vitro*, the active metabolite of vitamin D_3, 1,25$(OH)_2D_3$, has been shown to inhibit type I collagen synthesis by bone organ cultures and by osteoblastic cells, and this inhibitory effect is caused by an inhibition of the transcription of type I collagen genes (Bedalov *et al.*, 1998; Harrison *et al.*, 1989). Transfection studies performed with the rat pro *Col1a1* proximal promoter led to the identification of a vitamin D responsive element between −2.3 and −1.6 kb (Pavlin *et al.*, 1994). Nevertheless, when transgenic mice, harboring a 1.7-kb segment of the rat pro *Col1a1* promoter cloned upstream of a CAT reporter gene, were treated with 1,25$(OH)_2D_3$, the levels of expression of the CAT reporter gene decreased, which suggests that a vitamin D response element is located downstream of −1.7 kb (Bedalov *et al.*, 1998). Similarly, when calvariae from these transgenic mice were cultured in the presence of 1,25$(OH)_2D_3$, it inhibited reporter gene expression (Bedalov *et al.*, 1998). It is of note that part of the effects of vitamin D on type I collagen could be mediated through an inhibition of the production of IGF-I, because vitamin D has been shown to inhibit IGF-I production and increases the concentrations of inhibitory IGFBP-4 (Scharla *et al.*, 1991). Indeed, effects of 1,25-$(OH)_2D_3$ on release of the IGFs were independent of bone resorption and support the conclusion that 1,25-$(OH)_2D_3$ modulated the production and secretion of IGF-I and IGF-II in calvarial cells (Linkhart and Keffer, 1991). The results of these studies and similar studies on PTH in calvarial cells suggest that PTH, TGF-β, and 1,25-$(OH)_2D_3$ differentially regulate mouse calvarial cell IGF-I and IGF-II production. Furthermore, IGFBP-5 has been shown to reduce the effects of 1,25$(OH)_2D_3$ by blocking cell cycle progression at G_0/G_1 in osteoblasts and by decreasing the expression of cyclin D1. Moreover, IGFBP-5 can interact with VDR to prevent RXR:VDR heterodimerization and IGFBP-5 may attenuate the 1,25$(OH)_2D_3$-induced expression of bone differentiation markers (Schedlich *et al.*, 2007).

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Chapter 16

Collagen Cross-Linking and Metabolism

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INTRODUCTION

In constituting about 90% of the matrix protein of bone, collagen clearly plays an important role in determining the characteristic of the tissue. Much of the research on collagen has focused on the extensive post-ribosomal modifications that occur during biosynthesis of the molecule, as these intracellular changes have major influences on the assembly, cross-linking, mineralization, and degradation of collagen fibrils. The aim of this chapter is to bring together current knowledge on the mechanisms of collagen cross-linking and how these are influenced by specific post-ribosomal modifications. These changes are also viewed in the context of collagen metabolism, with particular reference to the utilization of certain collagen metabolites as markers of bone metabolism.

Although 27 genetically distinct collagen types are known (Myllyharju and Kivirikko, 2004), bone contains predominantly the principal fibrillar form, collagen type I, but with small amounts of collagens V and III. Collagen V interacts with type I fibrils (Birk *et al.*, 1988; Chanut-Delalande *et al.*, 2001) and may have some regulatory role on fibril diameter and orientation, as has been shown for cornea. Collagen III in bone is generally limited to anatomically distinct regions, such as tendon insertion sites (Keene *et al.*, 1991). Thus, for the purposes of this chapter, the properties of collagen type I will be considered, as these dominate the primarily structural function of collagen in bone.

CROSS-LINK FORMATION

Collagen type I fibrils form spontaneously within the extracellular space once the N- and C-terminal propeptides of procollagen have been removed by specific proteases. During fibrillogenesis, the final enzymatic modification of collagen occurs: conversion of lysine or hydroxylysine residues within both N- and C-terminal telopeptides to aldehydes by lysyl oxidase. Subsequently, all collagen cross-linking steps occur spontaneously by virtue of the specific alignment of molecules within the fibrils.

As indicated in an overview of the cross-linking process (Fig. 1), the hydroxylation state of telopeptide lysine residues is crucial in determining the pathway of collagen cross-linking; this step is determined by an intracellular modification during collagen biosynthesis.

Hydroxylation of telopeptide lysine residues is known to be accomplished by a separate enzyme to the one that act on lysines in the central chain portion destined to become the helix, and further research in this area has expanded our knowledge of the tissue-specific control of collagen cross-linking. Following preliminary evidence for a telopeptide lysyl hydroxylase from studies *in vitro* (Royce and Barnes, 1985; Uzawa *et al.*, 1999), and from a rare form of osteogenesis imperfecta, Bruck syndrome (Bank *et al.*, 1999), the long form of lysyl hydroxylase-2, a splice variant of the enzyme also known as procollagen-lysine, 2-oxoglutarate, 5-dioxygenase-2 (PLOD-2), was shown to accomplish hydroxylation of telopeptide lysine residues (Mercer *et al.*, 2003; van der Slot *et al.*, 2003). Initially, PLOD-2, which has been localized to chromosome 3, was thought to be an unlikely candidate for the defect in Bruck syndrome, known to be located on chromosome 17 (Bank *et al.*, 1999), but subsequent studies demonstrated the heterogeneity of this disorder, with some variants being derived from PLOD-2 deficiency (Ha-Vinh *et al.*, 2004). Further studies have confirmed that, of the three known PLOD enzymes, the long splice variant of lysyl hydroxylase 2 (LH2b) directs the pathways of collagen cross-linking in MC3T3-E1 cells (Pornprasertsuk *et al.*, 2004), and that there appears to be tissue-specific control of this (Walker *et al.*, 2005) and other lysyl hydroxylase enzymes (Eyre *et al.*, 2002). Some studies of telopeptide hydroxylation have associated increased

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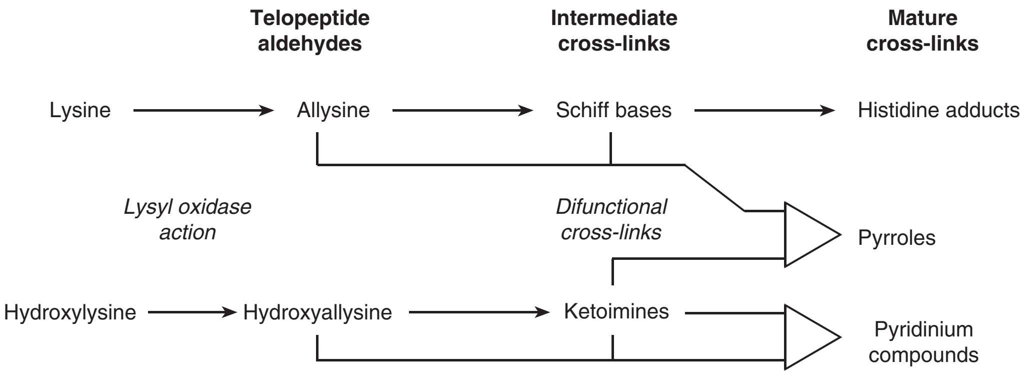

FIGURE 1 Formation of collagen cross-links from lysine- or hydroxylysine-derived telopeptide aldehydes giving rise to Schiff base or ketoimine difunctional bonds, respectively. On maturation, the Schiff bases are converted primarily to nonreducible, histidine adducts, whereas the ketoimines react with hydroxylysine aldehyde or a second ketoimine to give pyridinium cross-links. Pyrrole cross-link formation requires the presence of both lysine- and hydroxylysine-derived products.

expression of LH2b generally with fibrosis, particularly in skin (van der Slot *et al.*, 2003; van der Slot *et al.*, 2004), leading to attempts at enzyme inhibition as a therapeutic target (Zuurmond *et al.*, 2005). The hypothesis prompting this approach, that the presence of hydroxylysine-derived cross-links results in intractable collagen, has yet to be verified experimentally, and other data now indicate that the association of LH2 with fibrosis may, at least in part, result directly from elevated collagen synthesis in response to overexpression of LH2 (Wu *et al.*, 2006).

All of the three known lysyl hydroxylase isoenzymes were shown to hydroxylate collagenous sequences *in vitro* but LH2 exhibited no activity against 23- or 29-residue synthetic peptide substrates corresponding to the C- or N-terminal telopeptide domains, respectively; this enzyme appeared to require a full length α-chain as substrate (Takaluoma *et al.*, 2007). LH3 is a multifunctional enzyme that, in addition to lysyl hydroxylation, has galactosyltransferase and glucosyltransferase activities (Heikkinen *et al.*, 2000); the latter appears to be the predominant function of LH3 (Ruotsalainen *et al.*, 2006; Salo *et al.*, 2006) providing intriguing new insights into possible functions of O-glycosylation of collagen hydroxylysine (Sipila *et al.*, 2007).

CROSS-LINK STRUCTURE

Intermediate Cross-Links

The preponderance of hydroxylysine aldehydes in bone collagen telopeptides ensures that most of the difunctional cross-links initially formed are relatively stable bonds. Thus, in contrast to tissues such as skin, where the telopeptide lysine aldehydes interact with adjacent molecules to give Schiff base (–N = CH–) cross-links, the presence of the hydroxyl group allows an Amadori rearrangement to a more stable, ketoimine form. Both the Schiff base and the ketoimine forms of cross-link are reducible by borohydride, a technique that enabled the Schiff base compounds to be stabilized for identification (Bailey *et al.*, 1974). Although the ketoimine bonds are sufficiently stable to allow isolation of peptides containing these bonds, the cross-links are quantified after reduction with borohydride to the well-characterized compounds dihydroxylysinonorleucine (DHLNL) and hydroxylysinonorleucine (HLNL).

The reducible, bifunctional cross-links are referred to as intermediates because of their conversion during maturation of the tissue to nonreducible compounds, which are generally trivalent. Such a process can therefore be considered to provide additional stability to the fibrillar network, although, because of some ambiguities in the mechanisms involved, this has not been demonstrated directly.

Pyridinium Cross-Links

One of the first maturation products of the intermediate cross-links to be identified was pyridinoline (PYD) or hydroxylysyl pyridinoline (HP), a trifunctional 3-hydroxy-pyridinium compound (Fujimoto *et al.*, 1978). An analogue, deoxypyridinoline (DPD) or lysyl pyridinoline (LP), has also been identified in bone (Ogawa *et al.*, 1982). Both of these compounds (Fig. 2) are derived from intermediate ketoimines by reaction either with another difunctional cross-link (Eyre and Oguchi, 1980) or with a free hydroxylysine aldehyde group (Robins and Duncan, 1983). The chemistry of these two proposed mechanisms is very similar, but there are implications in terms of structural function of the cross-links. The involvement of two difunctional compounds results in a cross-link among three collagen molecules, whereas the alternative mechanism seems more likely to link only two molecules (as shown in Fig. 2). The difunctional, reducible cross-links necessarily link adjacent molecules within a pentafilament microfibril

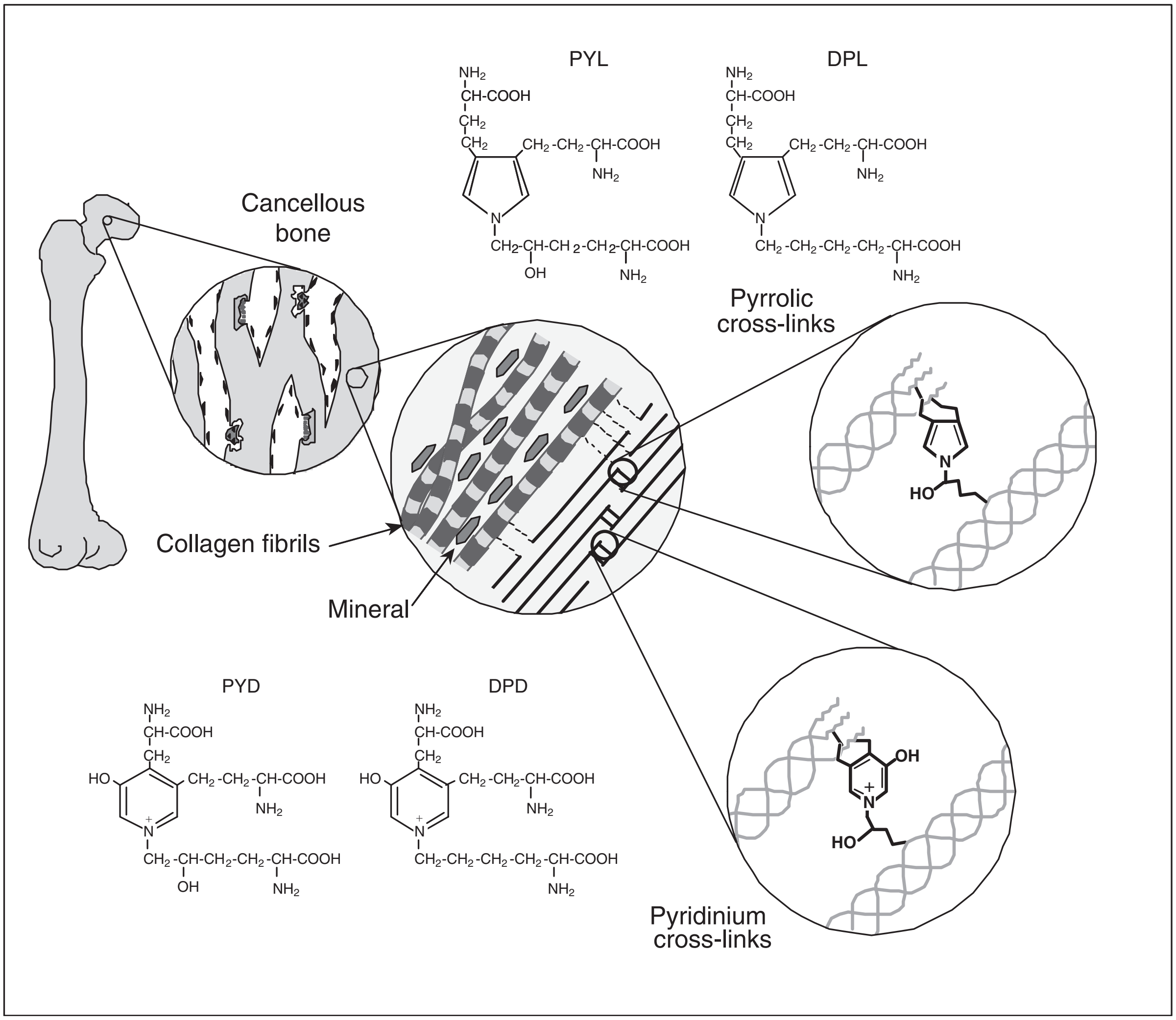

FIGURE 2 Stabilization of bone matrix by pyridinium and pyrrolic cross-links. Slightly higher concentrations of the mature cross-links are present in cortical compared with remodeling cancellous bone (inset) showing schematically mineralized collagen fibrils having a banded appearance arising from the precise alignment of collagen molecules in a quarter-staggered array. The overlap is stabilized by cross-links at both N- and C-terminal ends. (Insets) Pyridinium and pyrrole compounds linking N-terminal telopeptides to an adjoining helix: cross-linking may also involve telopeptides from two different molecules in register. Pyridinium cross-links are present at both N- and C-terminal sites, but pyrroles are located predominantly at the N terminus. Depending on the degree of hydroxylation of the helical lysine residue, two analogues of both the pyridinium and the pyrrolic cross-links are formed.

(Orgel *et al.*, 2006), whereas modeling studies suggest that conversion to mature, trifunctional bonds is accompanied by the formation of a transverse network of inter-microfibrillar bonds (Malone *et al.*, 2004; Robins, 2006).

Pyrroles

Based on the observation that tissues solubilized by enzyme treatment gave a characteristic pink color with *p*-dimethylaminobenzaldehyde, Scott and colleagues (Scott *et al.*, 1981) suggested that collagen contained pyrrolic cross-links. These compounds were termed Ehrlich chromogens (EC) and, in later experiments, diazo-affinity columns were used to bind covalently the pyrrole-containing peptides from enzyme digests of bone (Scott *et al.*, 1983) and skin (Kemp and Scott, 1988); these were partially characterized by amino acid analysis. A similar affinity chromatography approach was used to demonstrate that Ehrlich chromogen cross-links were present at the same loci as the pyridinium cross-links in bovine tendon (Kuypers *et al.*, 1992). This work culminated in a proposed structure and mechanism of formation for pyrroles analogous to that for pyridinium cross-link formation: this mechanism involves reaction of a difunctional, ketoimine cross-link with a lysyl aldehyde-derived component rather than hydroxylysyl aldehyde-derived component (Kuypers *et al.*, 1992), where the latter may be a second difunctional cross-link (Hanson and Eyre, 1996).

Isolation and characterization of the pyrrolic cross-link(s) were hampered by the instability of the pyrrole to acid or alkali hydrolysis. The use of repeated enzyme digestion of decalcified bone matrix to isolate pyrrole-containing peptides was not possible because, as these peptides were reduced in size and enriched, the pyrrole tended to oxidize or polymerize. By synthesizing new Ehrlich reagents, however, it was possible to both stabilize the pyrrolic cross-links and facilitate their isolation and characterization by mass spectrometry (Brady and Robins, 2001). Both predicted analogues of the pyrrole (Fig. 2) were identified as the derivatized cross-link. Consistent with previous nomenclature, the trivial names pyrrololine (PYL) and deoxypyrrololine (DPL) have been proposed for the underivatized cross-links, and a convergent total chemical synthesis of DPL has been described (Adamczyk *et al.*, 2001).

Location of Cross-Links

Within the quarter-staggered, fibrillar array of collagen molecules, almost all cross-links have been shown to be located at the 4D overlap position (see Fig. 2). Thus, N-telopeptide-derived cross-links are linked to the C-terminal part of the helix [residue 930 in the α1(I) chain], whereas C-telopeptide-derived cross-links are adjacent to the N-terminal end of the helix at residue 87. Because there is no oxidizable lysine in the C-telopeptide of the α2(I) chain, a more restricted number of cross-links is possible at this site compared to the N-terminal end. It has been established that the pyridinium and pyrrole cross-links are both located at these sites but that there are differences in their relative amounts. Thus, in bone collagen, the pyrrolic cross-links involve predominantly the N-terminal telopeptide (Hanson and Eyre, 1996), although there is some evidence for their location at the C-terminal end (Brady and Robins, 2001). Pyridinium cross-links are present at both ends of the molecule (Hanson and Eyre, 1996; Robins and Duncan, 1987) but, in human tissue, there is more DPD relative to PYD at the N terminus compared with the C-telopeptide-derived cross-linking region (Hanson and Eyre, 1996). The helical Hyl residue toward the N-terminal end (residue 87) is much more likely to be glycosylated than its C-terminal counterpart so that glycosylated pyridinium cross-links are relatively common, whereas glycosylated pyrrolic cross-links have not yet been detected.

Spectrum of Cross-Linking

The variations in telopeptide lysine hydroxylation give rise to a spectrum of different cross-linking patterns (Fig. 3). Bone collagen occupies a central position in this spectrum by virtue of the partial hydroxylation within the telopeptides, resulting in the formation of both pyridinium and pyrrolic cross-links. In cartilage, where telopeptide hydroxylation is essentially complete, only pyridinium cross-links are present with no pyrrolic forms. At the opposite end of the spectrum, skin also has no pyrrolic cross-links because of the absence of any telopeptide lysyl hydroxylase activity in this tissue. Helical lysyl hydroxylase activity, predominantly accomplished by the LH1 enzyme, has a much less profound effect on cross-link composition (Fig. 3) but does control the relative proportions of PYD:DPD and PYL:DPL. These cross-link changes are exemplified by the kyphoscoliotic type of Ehlers-Danlos syndrome (EDS VIA) or Nevo syndrome in which LH1 deficiency results in a change of over twofold in the PYD:DPD ratio in urine, reflecting mainly that in bone (Giunta *et al.*, 2005).

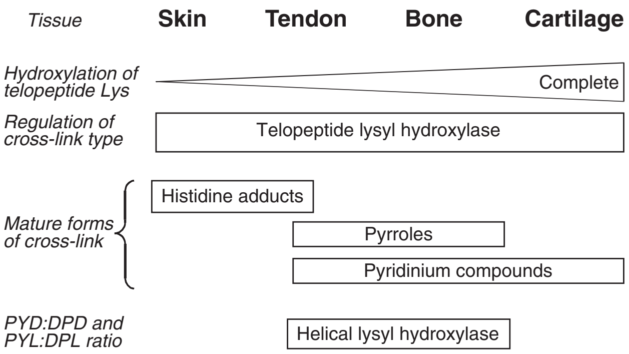
Tissue
Skin
Tendon
Bone
Cartilage
Hydroxylation of telopeptide Lys
Complete
Regulation of cross-link type
Telopeptide lysyl hydroxylase
Mature forms of cross-link
Histidine adducts
Pyrroles
Pyridinium compounds
PYD:DPD and PYL:DPL ratio
Helical lysyl hydroxylase

FIGURE 3 Spectrum of tissue-specific cross-linking resulting from the activity of telopeptide lysyl hydroxylase to give complete hydroxylation in cartilage but no significant hydroxylation of telopeptide lysine in skin collagen. The action of this intracellular enzyme regulator leads to the differences shown in mature cross-links. Pyrrolic cross-links are absent at the extremes of activity but are major components of bone and some tendons. The action of the intracellular enzyme, helical lysyl hydroxylase, regulates the relative proportions of mature pyridinium and pyrrolic cross-links in bone and tendon.

Age-Related Changes in Lysyl Oxidase-Derived Cross-Links

The conversion of the intermediate, bifunctional cross-links to pyridinium compounds is well documented, but the stoichiometry is less clear. Studies of the aging *in vitro* of bone indicated a 2:1 molar ratio of the ketoimine precursor to the pyridinium cross-link (Eyre, 1981), an observation that is consistent with the proposed mechanism of formation of the trivalent cross-link (Eyre and Oguchi, 1980). The maturation of bifunctional cross-links to the pyrrolic cross-links is extremely difficult to follow owing to instability of the pyrrole during isolation. In addition, as a relatively reactive species, pyrrolic cross-links have the potential to undergo further interactions within the fibril during the maturation process, although there appears to be little change in total bone pyrrole concentrations during aging (Bailey *et al.*, 1999).

In most soft tissues, the content of intermediate, reducible bonds is very low after the cessation of growth (Robins *et al.*, 1973), but bone is unusual in retaining a relatively large proportion of reducible bonds. One possible reason for this is the continual turnover through the remodeling of bone, resulting in a higher proportion of recently formed fibrils compared with other tissues. In support of this view, low bone turnover in osteopetrotic rats was found to be associated with high concentrations of pyridinium cross-links in cancellous and compact bone, which were partially normalized by the restoration of osteoclast formation with colony-stimulating factor 1 treatment (Wojtowicz *et al.*, 1997). The ultimate concentrations of pyridinium cross-links attained in these experiment were, however, not markedly different and it is unclear whether the observed differences in kinetics play an important part *in vivo*. Studies from samples obtained *in vivo* show that, although the overall collagen content of bone decreases, there appears to be no dramatic change in mature enzymatic cross-link concentrations with age (Bailey *et al.*, 1999; Nyman *et al.*, 2006). The situation may be confounded by subtle differences between areas that are undergoing active remodeling and sites containing older bone (Hernandez *et al.*, 2005; Nyman *et al.*, 2006) but again the relative differences in cross-link content are small.

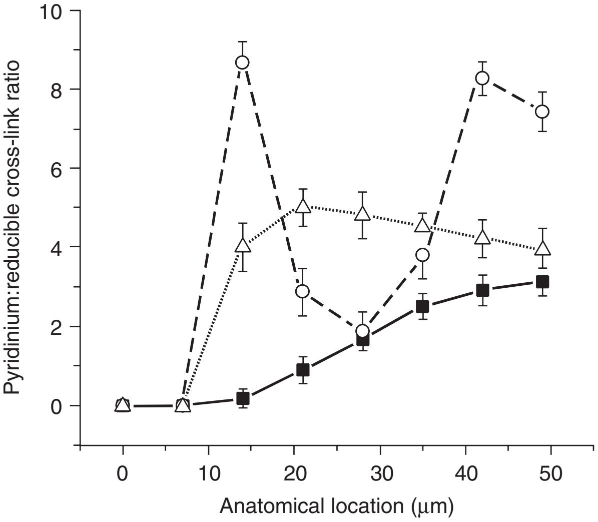

FIGURE 4 The spatial variation in pyridinium: reducible collagen cross-link ratio at bone-forming trabecular bone surfaces as a function of the anatomical distance from the outer edge of the osteoid surface (Paschalis *et al.*, 2004). The data depict measurements in iliac crest biopsy samples taken from patients with high-turnover osteoporosis (open circles) and low-turnover osteoporosis (open triangles) in comparison with healthy controls of similar age (solid squares), at equivalent anatomical locations (Paschalis *et al.*, 2004). Adapted with permission of the American Society for Bone and Mineral Research from Paschalis *et al.* (2004).

Direct Measurement of Cross-link Maturation in Tissues

The rates of conversion of intermediate to mature cross-links in tissues have generally been assessed by rather time-consuming and laborious analysis of individual cross-links in borohydride-reduced tissues. Such analyses do not, of course, give any indication of the localization of the different cross-links within the tissue. By using a newly developed application of Fourier transform infrared (FTIR) spectroscopy combined with imaging techniques, it has been possible to visualize within bone tissue sections the relative abundance of intermediate and mature cross-links (Paschalis *et al.*, 2001).

Bands in the amide I spectral region had been used previously for FTIR analysis of collagen, but by analyzing the spectra of isolated bone peptides containing either PYD or DHLNL, Paschalis *et al.* (2001) were able to assign a band at $\sim 1660\,\text{cm}^{-1}$ primarily to the mature, trifunctional cross-link and another at $\sim 1690\,\text{cm}^{-1}$ to intermediate cross-links characterized by DHLNL. Thus, by measuring the 1660:1690 band ratio, the relative proportions of mature and immature cross-link could be measured in a spatially resolved manner in thin sections of undecalcified bone. In bone trabeculae, the mature:intermediate cross-link ratio was much higher in the geometric center compared with lower ratios at the periphery and in osteoid-containing regions, consistent with the growth patterns in bone (Paschalis *et al.*, 2003). This technique does not measure the cross-links directly but assesses their presence from characteristic perturbations in the amide I part of the spectrum: it is likely that little distinction can be made with these methods between any of the trifunctional, mature cross-links, or between any of the intermediate, difunctional compounds. The method is, however, extremely valuable in facilitating imaging of the spatial distribution of collagen cross-link maturation, as evidenced by the differences in maturation patterns (Fig. 4) in iliac crest biopsies between normal postmenopausal women and those prone to fracture (Paschalis *et al.*, 2004).

Age-Related Changes in Sugar-Derived Cross-links

Although there are numerous sugar-derived modifications known to occur in collagens from all tissues, in bone pentosidine is the only well-characterized cross-link studied *in vivo* mainly owing to its stability to acid hydrolysis. Pentosidine is the glucose-mediated cross-link formed between a modified lysine residue and arginine (Sell and Monnier, 1989) and recent studies have revealed some intriguing correlations between the age-related changes in concentration of this cross-link and the physical properties of the bone, both *in vitro* and *in vivo*. Many studies have shown an increase in pentosidine concentrations with age (Odetti *et al.*, 2005; Nyman *et al.*, 2006; and a concomitant decrease in mechanical bone quality (Wang *et al.*, 2002). Using postmortem vertebrae, regression analysis showed significant negative correlations between pentosidine concentrations and vertebral failure load and work to fracture after accounting for bone mineral density (Viguet-Carrin *et al.*, 2006) suggesting an association of these cross-links with increased fragility. In an attempt to assess the potential effect on fracture, femoral necks from a group of fracture patients were compared to postmortem age-matched controls in compartments with high and low degrees of mineralization. The results showed that pentosidine content was significantly higher in fracture patients in the areas of low mineral density and elevated in the high-density areas (Saito *et al.*, 2006). Incubation *in vitro* of fetal bovine cortical bone at 37°C in phosphate buffered saline induced marked increases in enzymatic cross-links, but to levels much lower than those found *in vivo* (Garnero *et al.*, 2006). How an increase in intermolecular cross-linking might have a deleterious effect on quality is less clear with the only evidence being associative; in this study, there was a large increase (55-fold) in pentosidine but only to very low concentrations of cross-link (10 mmol/mol). In fact, in all of these studies the relative quantities of measured pentosidine is in the order of 20–40 mmol/mol of collagen, at least an order of magnitude lower than the combined lysyl oxidase-mediated cross-links, which can approach the mol/mol level in bone. It is difficult therefore, to envisage how such small amounts could affect bone collagen strength directly. One possibility, however, is that other sugar-derived lysine-arginine cross-links, which are not stable to acid hydrolysis, may be more important, but would be expected to correlate to the more readily detectable pentosidine. The difficulty in measuring these cross-links is that they need to be isolated by enzymatic digestion, which is extremely difficult for bone. Concentrations of these acid unstable cross-links have been measured in soft collagenous tissues and can reach levels that may conceivably affect collagen stiffness and strength; glucosepane measured in the skin of diabetic subjects reached concentrations of about 1 mol/mol collagen by the ninth decade of life (Sell *et al.*, 2005).

Other Age-Related Changes

Changes in protein structure owing to age-related modifications such as progressive deamidation, racemization, or, as discussed in the previous section, nonenzymatic glycosylation of specific amino acid residues are well recognized. These changes have profound effects on the functional properties of the matrix and may alter interactions with cells and other matrix constituents, thus affecting the metabolism of the protein. Although a detailed discussion of the many protein modifications that occur during aging is beyond the scope of this chapter, specific changes because of isomerization and racemization of aspartyl residues in collagen telopeptides are discussed because of their implications for the measurement of collagen metabolites as bone resorption markers.

Isomerization and Racemization of Asp in Telopeptides

The racemization of amino acids in proteins has long been used as a means of assessing the “age” of proteins (Helfman and Bada, 1975). Different amino acids racemize at different rates, but aspartyl (or asparaginyl) residues racemize particularly rapidly because of the association with isomerization events. Conversion to a D-aspartyl or -asparaginyl residue occurs more readily when this residue is adjacent to a glycine, thus allowing the formation of a succinimide intermediate, which leads to L- and D-isomers of both α and β forms. The presence of isomeric forms of -Asp-Gly- bonds in collagen telopeptides was recognized by Fledelius and colleagues (Fledelius *et al.*, 1997), who showed that the proportion of β-aspartyl residues within the C-terminal telopeptide of $\alpha1(I)$ increased with age in human and animal tissues. Measurements in urine reflected similar age changes, with higher α/β ratios detected in children compared with adults (Fledelius *et al.*, 1997). Later studies of the Asp-Gly bond in the N-telopeptide of the $\alpha2(I)$ chain revealed that isomerization also occurs at this end of the molecule (Brady *et al.*, 1999), although there were some differences between N- and C-terminal telopeptides in the relative α/β ratios in bone and urine.

The isomerization and racemization of aspartyl residues in telopeptides potentially have applications in monitoring the relative rates of metabolism of different pools of bone. A systematic study of the kinetics of isomerization and racemization of C-telopeptide aspartyl residues using synthetic peptides aged *in vitro* indicated that the ratio most discriminatory in terms of indicating biological age was $\alpha L/\beta D$ (Cloos and Fledelius, 2000). Analysis of these ratios in bone samples using immunoassays specific for each form of CTX indicated that children and patients with Paget’s had a turnover time of 2–3 months, whereas those from healthy adults and patients with osteoporosis had longer half-lives. In an extension to these studies, analyses of the relative rates of turnover of a wide range of human tissues using the specific CTX immunoassays (Gineyts *et al.*, 2000) suggested that collagen turnover in most of the soft tissues examined, including arteries, heart, lung, and skeletal muscle, was much higher than that in bone. These rather surprising results probably arise because of the limited solubilization of the tissue with trypsin, although the amounts solubilized were not reported (Gineyts *et al.*, 2000). Without heat denaturation before trypsinization, however, only younger, less cross-linked tissue will be extracted, leading to an overestimate of the turnover rate.

There are now several available assays to assess serum, urine, and cell culture α and β C-terminal peptides (see Table I). Other assays differentiating among the αL, αD

TABLE I Various forms of the C-terminal telopeptide of collagen type I detected by the commercially available assays

Assay	Telopeptide fragment detected
αCTX RIA	αCTX; αCTX-x-αCTX; αCTX-x-βCTX
Urinary CTX	βCTX; βCTX-x-αCTX; βCTX-x-βCTX
Serum CTX (CrossLaps; Roche Elecsys)	βCTX-x-βCTX
ααCTX ELISA	αCTX-x-αCTX

βL, and βD also have been used but discriminating among the three altered forms added little more value than comparing alpha and any isomerized or racemized fragment when used as a direct estimate of resorption (Cloos *et al.*, 2003b). Comparison of the αL-CTX isoform to the kinetically slowest form, αD-CTX may represent useful "extremes" of the young-old profile when assessing metastases (Cloos *et al.*, 2003a).

The β telopeptide serum assays have been widely used to indicate increased bone resorption in various conditions expected to be associated with increased resorption of mature bone (Cloos *et al.*, 2003b), including menopause and fracture (Kawana *et al.*, 2002) and bone loss in hemodialysis patients (Okuno *et al.*, 2005). The β peptides also appear to reflect attenuation of resorption in therapies such as hormone replacement (HRT), bisphosphonates (Byrjalsen *et al.*, 2007; Okabe *et al.*, 2004), and calcitonin (Zikan and Stepan, 2002).

The α-peptide assays, or preferably α/β ratios generated from measuring both forms of the peptide, can provide additional information on metabolism rates. The rate of turnover would be expected to have a direct effect on the maturity of collagen in bone, as slowing down resorption should allow more time for alpha to beta maturation; therefore, the collagen fragments detected would be expected to show a more mature profile. An example of this is a study using both urinary α-α and β-β peptide assays to monitor the effects of antiresorptive therapies (Byrjalsen *et al.*, 2007). Although all of the treatments (alendronate, ibandronate, HRT, and raloxifene) significantly reduced both α-α peptides and β-β peptides compared to placebo, only the bisphosphonates significantly changed the ratio, demonstrating an increased maturation of bone allowed by the decreased turnover.

Measuring α fragments may also provide an improved index of bone loss from areas of intense remodeling of immature bone that has had insufficient time to isomerize. As discussed previously, in Paget's disease there is a significant increase in α/β ratio (Garnero *et al.*, 1997; Alexandersen *et al.*, 2005) but the α/β ratio also responds extremely sensitively to antiresorptive therapy and may indicate an increase in bone maturation with treatment (Alexandersen *et al.*, 2005). Metastatic bone cancer is another condition where rapid remodeling may result in non-isomerized peptide fragments being released into the serum and urine through resorption of more immature areas of bone. Measuring α-α alone or as an α-α/β-β ratio provided significant discrimination between breast cancer patients with or without bone metastases (Cloos *et al.*, 2004), as was comparison of the aging extremes of the isomerization process, αL/αD, although α-α alone was still the best at differentiating those subjects with metastases (Cloos *et al.*, 2003a). The number of bone metastases also correlated with urinary α-αCTX measurements, as demonstrated in another study of patients with primary breast cancer (Leeming *et al.*, 2006).

In addition to representing an opportunity to estimate the age of bone being resorbed at any given time, the presence of isomerized aspartyl residues may be postulated as a mechanism *in vivo* for targeting older bone for local remodeling. This nonstochastic or targeted remodeling is suggested to occur primarily at load-bearing areas in response to micro-damage (Han *et al.*, 1997). In an *in vitro* study using both young and aged bone, it was shown that osteoclastogenesis and total resorption was accelerated in older bone (Henriksen *et al.*, 2007). Although the α/β ratio of the peptides released was used as a marker to differentiate between the two samples and to monitor relative resorption of old versus new bone, it is tempting to speculate that such modifications may be used *in vivo* to recognize mature bone that is fit for remodeling.

DEGRADATIVE PATHWAYS

Action of Osteoclastic Cells

The major role of osteoclasts in bone resorption has been discussed extensively in this volume and will not be described here in detail. Of the many proteases expressed by osteoclasts, current evidence indicates that the cysteine proteinase, cathepsin K, plays a major role in degrading bone matrix. Initially cloned from rabbit osteoclasts (Tezuka *et al.*, 1994), cathepsin K was subsequently shown to be expressed predominantly in this cell type for other mammalian species. Studies of human osteoclastic cells showed that cathepsin K inhibition with antisense probes markedly decreased the resorptive activity measured by pit assay (Avnet *et al.*, 2006). Unlike other cathepsins, recombinant cathepsin K was shown to be capable of solubilizing demineralized, fibrillar bone collagen (Garnero *et al.*, 1998a). The importance of cathepsin K for bone metabolism *in vivo* was demonstrated by the discovery that pycnodysostosis, an autosomal recessive skeletal dysplasia, resulted from mutations in this enzyme (Gelb *et al.*, 1996). The lack of cathepsin K resulted in decreased bone turnover measured by histomorphometry as well as poor bone quality and structural characteristics revealed by quantitative backscattered electron imaging and small angle X-ray scattering (Fratzl-Zelman *et al.*, 2004). This disorder was also associated with the accumulation of phagocytosed collagen fragments in lysosomal vacuoles (Everts *et al.*, 2003). Changes in the patterns of urinary bone markers occurred in pycnodysostosis (Nishi *et al.*, 1999), and studies *in vitro* confirmed that cathepsin K activity influenced the fragments produced from both N-terminal (Atley *et al.*, 2000) and C-terminal (Sassi *et al.*, 2000) sites of collagen type I.

Although cathepsin K clearly has an important function in osteoclastic bone resorption, there are many other enzymes that may play a role. Matrix metalloproteinases (MMPs), which are abundant in bone (Knott *et al.*, 1997), include collagenases with the ability to cleave native collagen fibrils, although less effectively than cathepsin K (Garnero *et al.*, 1998), and gelatinases able to degrade further the denatured chain fragments produced. Some debate continues on the relative roles of cathepsin K and MMPs in osteoclastic bone resorption. Although osteoclast activity assays *in vitro* suggested that MMPs contribute little resorptive activity (Fuller *et al.*, 2007), these enzymes may have more indirect roles through, for example, modifying osteoclast migration and recruitment (Delaisse *et al.*, 2003). As the major bone-resorbing enzyme, however, cathepsin K constitutes a primary therapeutic target for attenuating bone resorption (Kumar *et al.*, 2007).

EXTRASKELETAL PROCESSING OF COLLAGEN FRAGMENTS

Hepatic and Renal Influences

There is currently little evidence on whether the liver plays a significant role in the further processing of collagen fragments. Early studies showed that ^{125}I-labeled monomeric $\alpha 1$(I) chains injected into rats were taken up rapidly by liver endothelial and Kupffer cells (Smedsrod *et al.*, 1985). For the endothelial cells at least, this process was receptor mediated and was accompanied by lysosomal degradation of the denatured collagen chains. Whether the relatively small fragments of collagen that emanate from bone will be similarly sequestered and metabolized by the liver is unknown.

In contrast, there is good evidence that the kidney has an important role in controlling the patterns of collagen degradation products from bone and other tissues. Initially, evidence was again obtained from animal experiments in which immunostaining of rat kidney sections with antibodies recognizing only denatured collagen showed large accumulations of collagen fragments in proximal renal tubules (Rucklidge *et al.*, 1986). Subsequent studies following the fate of injected ^{3}H-labeled collagen fragments by autoradiography showed rapid uptake by proximal tubule epithelial cells and vacuolar transport to lysosomes (Rucklidge *et al.*, 1988) where antibody reactivity was lost, presumably through degradation of the peptides.

Analyses of serum and urinary concentrations of pyridinium cross-link components in children provided evidence that free pyridinium cross-links were in part produced in the kidney (Colwell and Eastell, 1996). This study showed that the proportion of free DPD in serum was about half that in urine. Analysis of free DPD in serum for older children revealed a negative correlation with the total cross-link output (Colwell and Eastell, 1996) and a similar correlation was noted in urine for a group of pre- and postmenopausal women (Garnero *et al.*, 1995). These data led to the hypothesis that the renal processing of collagen fragments was a saturable process whereby increased collagen turnover resulted in a progressive decrease in the proportion of free cross-links and a corresponding increase in their peptide forms (Colwell and Eastell, 1996; Garnero *et al.*, 1995; Randall *et al.*, 1996). This hypothesis is probably an oversimplification, however, as an analysis of the results for a wide range of healthy individuals and patients with metabolic bone diseases indicated only a weak correlation between the proportion of free DPD and total cross-link output (Robins, 1998). Comparisons of serum and urinary immunoassays for telopeptide markers indicated a greater degree of renal processing of the N-terminal relative to C-terminal components (Fall *et al.*, 2000), although it is unclear whether this is related to the increased protease resistance of C-telopeptides imparted by the presence of isoaspartyl residues. Several studies have established that the patterns of collagen cross-link-containing components can be affected by various treatments for disease (Garnero *et al.*, 1995; Robins, 1995; Kamel *et al.*, 1995), and the effects of amino-bisphosphonates have received most attention in this respect.

Effects of Bisphosphonates

Much interest in this aspect was created by a report that measurements of free and peptide-bound cross-links in patients receiving acute, intravenous treatment with pamidronate for three days showed essentially no changes in free pyridinium cross-link concentrations, whereas there were large decreases in telopeptide-based assays and, to a lesser extent, in HPLC measurements of total cross-links (Garnero *et al.*, 1995). Although these finding appeared to overestimate the bisphosphonate effects compared with another similar study (Delmas, 1993), subsequent investigations of the effects of longer term bisphosphonate treatment have confirmed that there are changes in the patterns of collagen degradation components. Treatment of postmenopausal women with the amino-bisphosphonate, neridronate, over a four-week period resulted in a significant increase in the proportion of free DPD, with an apparently greater response to therapy in the peptide-bound fraction (Tobias *et al.*, 1996). This study also showed that there were no changes in the proportion of free PYD, leading to an increased PYD/DPD ratio in the peptide fraction. The changes in cross-link ratio were initially thought to represent altered tissue contributions to the cross-links, but, as DPD is more prevalent at the N-terminal portion of collagen (Hanson and Eyre, 1996), these changes probably indicate differential effects on proteolytic degradation of the N- and C-telopeptide cross-linked regions.

Bisphosphonates appear to inhibit bone resorption through several mechanisms involving direct effects on osteoclasts and their precursors (Flanagan and Chambers, 1991; Murakami *et al.*, 1995; Hughes *et al.*, 1995) or indirectly through effects on osteoblasts (Sahni *et al.*, 1993). Two classes of these compounds may be distinguished pharmacologically; the more potent, nitrogen-containing bisphosphonates act by inhibiting farnesyl diphosphate synthase, an enzyme of the mevalonate pathway, thereby preventing prenylation of small GTPase-signaling proteins required for normal cellular osteoclast function (Coxon *et al.*, 2006). Amino-bisphosphonates have also been shown to activate caspase-3-like enzymes, the cysteine proteinases that act as the main executioner enzymes during apoptosis (Benford *et al.*, 2001). It is conceivable, therefore, that these compounds may also affect the activity of enzymes involved in the degradation of collagen fragments. Whether this occurs in bone, which seems likely in view of the accumulation of bisphosphonates in this tissue, or in other organs involved in peptide processing is at present unknown. Thus, in addition to inhibiting bone resorption, bisphosphonates may also alter the patterns of collagen degradation products, a fact that is crucial in interpreting biochemical monitoring of these processes (see Disturbances of Degradative Metabolism).

Gastrointestinal Effects

Studies have shown that bone resorption is influenced by nutrient intake (Bjarnason *et al.*, 2002; Clowes *et al.*, 2002a), and some of these effects may have been interpreted previously as circadian or diurnal variations. Insulin clamp studies indicated that postprandial hyperinsulinemia was unlikely to explain the suppression of bone turnover with feeding, and suggested that the hypoglycemia-induced decrease in bone resorption may be related to changes in PTH or other hormones (Clowes *et al.*, 2002b).

Attempts to explain the postprandial decrease in bone resorption subsequently focused on the gastrointestinal hormones, glucose-dependent insulinotropic peptide (GIP), glucagon-like peptide-1 (GLP-1), and GLP-2. Of these, only GLP-2 appeared to play a direct role in bone remodeling, as evidenced by measurements of biochemical markers after intravenous or subcutaneous injections of these peptides (Henriksen *et al.*, 2003). There was an acute, dose-dependent decrease in bone resorption after GLP-2 administration, suggesting that part of the circadian variation in resorption may be owing to the nutrient-induced release of this hormone during the nonfasting period of the day. GLP-2 injections in postmenopausal women given at 10 PM reduced the nocturnal rise in bone resorption and increased bone formation markers (Henriksen *et al.*, 2004), and an extended study confirmed these findings (Henriksen *et al.*, 2007). The gastric hormone, grehlin, is increased during fasting, paralleling the changes in bone resorption, suggesting that this peptide might also mediate the effect. An observed normal postprandial decrease in bone resorption in patients with gastrectomy (Holst *et al.*, 2007) argues against this suggestion, however, and other studies have confirmed the lack of acute effects (Huda *et al.*, 2007).

RELEASE OF CROSS-LINKED COMPONENTS FROM BONE *IN VITRO*

The use of osteoclastic cells cultured on dentine slices or with bone particles has given information on the mechanisms and extent of collagen degradation in bone. Cross-linked N-telopeptide fragments (NTX) were shown to be released into medium from human bone, whereas no free pyridinium cross-links could be detected by HPLC (Apone *et al.*, 1997). Confocal microscopy of labeled bone surfaces has revealed the intracellular pathway of proteins, including degraded collagen type I, through osteoclasts (Nesbitt and Horton, 1997), and other studies indicate that cathepsin K co-localized with the degrading collagen (Nesbitt and Horton, 2003). These types of study, combining immunolocalization of collagen fragments with the response to enzyme inhibitors, provide a powerful technique to address the cellular mechanism of bone collagen resorption. Cathepsin K was shown to solubilize demineralized bone *in vitro* through cleavage at sites in both the telopeptides and within the collagen helix (Garnero *et al.*, 1998a).

Collagen Metabolites as Markers of Bone Metabolism

N- and C-Terminal Propeptides as Formation Markers

Procollagen type I, the initially synthesized product, is about 50% larger than the collagen molecule in fibrils, having large extension peptides at both N- and C-terminal ends. These propeptides are removed *en bloc* by separate proteases at or near the cell surface during secretion of the molecule. The intact C-terminal propeptide (PICP) containing intermolecular disulfide bonds can be detected in the blood as a 100-kDa component (Melkko *et al.*, 1990) and several commercial assays are now available. The assay has been used successfully to assess growth (Trivedi *et al.*, 1991), but its sensitivity to relatively small changes in bone formation, such as those accompanying menopause, has been rather limited.

Immunoassays for the N-terminal propeptide of procollagen I (PINP) have received renewed interest as this molecular domain appears to have more favorable degradative metabolism and clearance characteristics. In serum, there are components related to the N-propeptide having apparent molecular masses of about 100 and 30 kDa and, despite initial uncertainties (Orum *et al.*, 1996; Melkko *et al.*, 1996), immunoassays that measure both molecular species (Jensen *et al.*, 1998) are now accepted as good markers of bone formation. PINP assays, which are now available on automated clinical analyzers, have proved to be particular useful in monitoring bone metastases (Luftner *et al.*, 2005; Pollmann *et al.*, 2007). Animal models are important particularly for drug development research and methods to monitor PINP in rat serum have been described using immunoassay (Rissanen *et al.*, 2007) and mass spectrometry (Han *et al.*, 2007).

Cross-links and Peptide Fragments as Bone Resorption Markers

Many of the bone resorption markers currently in use are based on components or fragments of collagen type I. Such assays, comprising predominantly those for pyridinium cross-links and the N- and C-telopeptides, have generally replaced urinary hydroxyproline and urinary hydroxylysine glycosides, assays for which were both technically demanding and prone to major inaccuracies. For urinary hydroxyproline, these problems stemmed primarily from the lack of specificity for bone, extensive metabolism of the marker in liver, and the major contribution of hydroxyproline from dietary sources. Hydroxylysine glycoside markers are less prone to dietary interference and HPLC assays have developed for urine (Moro *et al.*, 1984; Yoshihara *et al.*, 1993) and serum (Al-Dehaimi *et al.*, 1999). Although an immunoassay has been described for Gal-Hyl (Leigh *et al.*, 1998) most studies of this and the disaccharide marker for soft tissue, Glc-Gal-Hyl, have used HPLC assays (Rauch *et al.*, 2002).

Pyridinium Cross-Links

As discussed previously, pyridinium cross-links are maturation products of lysyl oxidase-mediated cross-linking and their concentrations in urine; therefore, they reflect only the degradation of insoluble collagen fibers and not of any precursors. The ratio PYD:DPD in urine is similar to the ratio of these two cross-links in bone, suggesting that both of the cross-links are likely to be derived predominantly from bone. Because of its more restricted tissue distribution, generally to mineralized tissues (Eyre *et al.*, 1984; Seibel *et al.*, 1992), DPD is often described as a more bone-specific marker: this notion was reinforced by the close correlation between DPD excretion and an independent, stable isotope method for determining bone turnover rate (Eastell *et al.*, 1997). Initially, the assays for pyridinium cross-links were HPLC methods with a hydrolysis and prefractionation step (Black *et al.*, 1988); despite later automation of the procedure (Pratt *et al.*, 1992), these procedures are time-consuming. The observation that the ratio of free to peptide-bound cross-links was similar in urine from healthy individuals and from patients with a range of metabolic bone disorders (Robins *et al.*, 1990; Abbiati *et al.*, 1993) opened the way for direct analysis of urine samples without the need for the hydrolysis step. This in turn led to the development of specific immunoassays for DPD (Robins *et al.*, 1994) or for both pyridinium cross-links (Gomez *et al.*, 1996), and some of these immunoassays are now more widely available on multiple clinical analyzers. The excretion of pyridinium cross-links has been shown to be independent of dietary ingestion of these compounds (Colwell *et al.*, 1993). Changes in the metabolism of the pyridinium components can, however, give rise to problems, particularly where this leads to alterations in the proportions of free to bound cross-links. Treatment with amino-bisphosphonates appears to give particular problems in this respect, as discussed later.

Peptide Assays

Instead of using cross-links themselves as markers, several groups have developed assays based on specific antibodies raised against isolated collagen peptides containing the cross-links. The NTX and ICTP assays exemplify this type of development.

NTX Assay. The antigen for the cross-linked N-telopeptide assay was isolated from the urine of a patient with Paget disease of bone, and an immunoassay based on a monoclonal antibody was developed (Hanson *et al.*, 1992). This assay showed detectable reaction with urine from normal individuals, as well as large increases associated with elevated turnover. Although the antibody recognizes components in urine containing pyridinium cross-links (Hanson *et al.*, 1992), this type of cross-link is not essential and peptides containing pyrrolic cross-links may also be detected (Hanson and Eyre, 1996). Some form of cross-link must, however, be present for antibody recognition, thus ensuring that only degradation products of mature tissue are detected.

ICTP Assay. This assay detects fragments from the C-telopeptide region of collagen type I. The antigen was a partially purified, cross-linked peptide from a bacterial collagenase digest of human bone collagen (Risteli *et al.*, 1993). Again, the isolated peptide contained pyridinium cross-links, but this type of bond was not essential for reactivity with the rabbit antiserum used in the assay. ICTP is a serum assay and metabolism of the analyte has proved to be an important factor limiting its application. The observation that cathepsin K cleaves within the epitope for the ICTP antibody (Sassi *et al.*, 2000) appears to explain why this assay is relatively insensitive to changes in bone remodeling mediated by normal osteoclastic activity. In contrast, pathological increases in bone degradation, such as those occurring in myeloma (Elomaa *et al.*, 1992), metastatic bone disease (Aruga *et al.*, 1997), or rheumatoid arthritis (Sassi *et al.*, 2003), are well detected by the assay, as other enzyme systems, probably including MMPs, seem to be involved.

CTX Assay. Developments of the CTX assay have been discussed in connection with the occurrence of α- and β-aspartyl residues within the C-terminal portion of collagen. These forms of assay, applicable to both urine and serum and also available on multiple clinical analyzers, now represent some of the most widely used assays for monitoring bone resorption.

DISTURBANCES OF DEGRADATIVE METABOLISM

As discussed previously, treatment with amino-bisphosphonates represents a major area of uncertainty in the application of bone resorption markers. Because the proportion of free pyridinium cross-links is increased by the treatment, the apparent decrease in bone resorption indicated by these markers is less than the true value. This change is measurable and has been well documented. The pools of peptides undergoing further degradation to give free cross-links are, however, those being measured by the NTX and CTX assays. Consequently, the changes in degradative metabolism caused by bisphosphonates will result in decreased concentrations of these peptides larger than those warranted by the decrease in true bone resorption: the extent of these overestimates of bone resorption rate cannot be ascertained easily. In practical terms, these considerations have a limited impact on the applications of these markers to monitor treatment. Where more precise indications of the true changes in bone resorption rate are required, however, the use of total (hydrolyzed) pyridinium cross-links gives results less susceptible to changes in degradative metabolism.

CONCLUDING COMMENTS

In the past decade, major advances have been made in understanding the structure and metabolism of bone collagen. In terms of cross-linking, most of the structural components have been identified, although immunochemical studies have suggested that a significant proportion of mature, trifunctional cross-links within the C-terminal region of bone collagen have yet to be identified (Eriksen *et al.*, 2004). More information is needed on the functional significance of the different cross-links, and intriguing suggestions have been put forward on associations between the relative proportions of pyrrole and pyridinium cross-links and the structural organization and properties of bone trabeculae (Banse *et al.*, 2002a; Banse *et al.*, 2002b).

With the realization that BMD measurements lacked specificity in predicting fracture risk, particularly in patients treated with bisphosphonates, the concept of bone quality has attracted much attention. Although this property of bone is difficult to define precisely, many of the collagen maturation and aging changes discussed in this chapter are undoubtedly relevant to assessments of bone quality. In particular, maturation of cross-links is likely to have a profound effect on bone and the newer methods of FTIR and Raman spectroscopy now used to monitor such changes in spatially defined regions of bone may have important applications in this area of research. The isomerization and racemization of telopeptide aspartyl residues represent an additional area of research relevant to questions of bone quality and changes in the biomechanical properties of the tissue. Although the presence of such amino acid residues in bone collagen telopeptides may not contribute directly to changes in properties, these indices of age-related changes are likely to contribute to a panel of markers for bone quality, which will be the focus of future research.

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Chapter 17

Noncollagenous Bone Matrix Proteins

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INTRODUCTION

Although the organic matrix of bone is composed primarily of collagen(s) (as reviewed in a previous chapter), the existence of other noncollagenous components was first postulated by Herring and coworkers in the 1960s (Herring and Ashton, 1974). By using degradative techniques, a variety of carbohydrate-containing moieties were extracted and partially characterized. The major breakthrough in the chemical isolation and characterization of noncollagenous bone matrix proteins came with the development of techniques whereby proteins could be extracted in an intact form (Termine *et al.*, 1980, 1981). Although these procedures were suitable for the isolation of the more abundant bone matrix proteins, the advent of osteoblastic cultures that faithfully retain phenotypic traits of cells in this lineage allowed for the discovery of other proteins that are sequestered in the matrix. Although they are not as abundant as the so-called structural elements, their importance in bone physiology cannot be underestimated. This has been underscored by the identification of mutations in a number of these proteins that result in abnormal bone. Many of these low-abundance proteins are discussed in subsequent chapters.

Collagen(s) is by far and away the major organic constituent of bone matrix (Table I). However, collagen most likely is not the direct nucleator of hydroxyapatite deposition. Physicochemical studies based on predictions of the surface topography of the hydroxyapatite unit cell predict that such a nucleator would have a β-pleated sheet structure, a feature that is not found in the predicted structure of the collagen molecule (Addadi and Weiner, 1985). In addition, collagen is not present in the extracellular environment in an unbound form. There are a large number of matrix proteins that have been found to bind to collagen, thereby regulating fibril formation, and it is probable that collagen serves as scaffolding on which nucleators are oriented. Consequently, the nucleators of hydroxyapatite deposition are most likely members of the noncollagenous components of the organic bone matrix. This chapter discusses the major structural proteins (proteoglycans, glycoproteins, and γ-glutamic acid-containing proteins) found in bone matrix. These proteins have been reviewed extensively elsewhere (Zu *et al.*, 2007). This is an area that is expanding rapidly owing to the generation of better tools, such as antibodies, cDNA probes, and genomic constructs. These reagents have been quite useful in determining the pattern and regulation of expression. Furthermore, the development of transgenic animals that either overexpress or are deficient in these proteins has also provided insight into their potential function.

PROTEOGLYCANS

This class of molecules is characterized by the covalent attachment of long-chain polysaccharides (glycosaminoglycans, GAGs) to core protein molecules. GAGs are composed of repeating disaccharide units that are sulfated to varying degrees and include chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), and heparan sulfate (HS). Hyaluronic acid (HA) is also a GAG found in bone, but is not attached to a core protein. Different subclasses of proteoglycans are generally characterized by the structure of the core protein and by the nature of the GAG (Table II). Although other types of molecules can be sulfated, proteoglycans bear greater than 95% of the sulfate groups within any organic matrix (Lamoureux *et al.*, 2007).

Aggrecan and Versican (PG-100)

There are two large chondroitin sulfate proteoglycans associated with skeletal tissue that are characterized by core proteins with globular domains at the amino and carboxy termini and by binding to hyaluronan to form large

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TABLE I Characteristics of Collagen-Related Genes and Proteins Found in Bone Matrix

Collagens	Gene	Protein	Function
Type I	COL1A1 17q21.3-22 18 kb, 51 exons, 7.2 and 5.9 kb mRNA	$[\alpha l(I)_2\alpha 2(I)]$ $[\alpha 1(I)_3]$	Most abundant protein in bone matrix (90% of organic matrix), serves as scaffolding, binds and orients other proteins that nucleate hydroxyapatite deposition
	COL1A2 7q21.3-22 35 kb, 52 exons, 6.5 and 5.5 kb mRNA		
Type X	COL10A1	$[\alpha 1(x)_3]$	Present in hypertrophic cartilage but does not appear to regulate matrix mineralization
Others Type III Type V FACITS ?	COL3A1 2q24.3-q31 COL5A1 9q34.2 COL5A2 2q14 COL5A319p13.2	$[\alpha 1(III)]_3$ $[\alpha 1(V)_2\alpha 2(V)]$ $[\alpha 1(V)\alpha 2(V)\alpha 3(V)]$	Present in bone in trace amounts, may regulate collagen fibril diameter; their paucity in bone may explain the large diameter size of bone collagen fibrils

TABLE II Gene and Protein Characteristics of Proteoglycans, Leucine-Rich Repeat Proteins, and Glycosaminoglycans in Bone Matrix

	Gene	Protein	Function
Versican (PG-100)	5q 12-14 90 kb, 15 exons one splice variant 10, 9, 8 kb mRNAs	1×10^6 intact protein, ~360 kDa core, ~12 CS chains of 45 kDa, G1 and G3 globular domains with hyaluronan-binding sites, EGF- and CRP-like sequences	May "capture" space that is destined to become bone
Decorin	12q21-23 > 45 kb, 9 exons alternative promoters, 1.6 and 1.9 kb mRNA	~130 kDa intact protein, ~38–45 kDa core with 10 leucine-rich repeat sequences, 1 CS chain of 40 kDa	Binds to collagen and may regulate fibril diameter, binds to TGF-β and may modulate activity, inhibits cell attachment to fibronectin
Biglycan	Xq27 7 kb, 8 exons 2.1 and 2.6 kb mRNA	~270 kDa intact protein, ~38–45 kDa core protein with 12 leucine-rich repeat sequences, exons, 2 CS chains of 40 kDa	May bind to collagen, may bind to TGF-β, pericellular environment, a genetic determinant of peak bone mass
Fibromodulin	1q32 8.5 kb, 3 exons	59 kDa intact protein, 42 kDa core protein with leucine-rich repeat sequences, one N-linked KS chain	Binds to collagen, may regulate fibril formation, binds to TGF-β
Osteoglycin (Mimecan)	9q21,3-22 33 kb, 8 exons 3.7 kb mRNA	299 aa precursor, 105 aa mature protein leucine-rich repeat sequences	Binds to TGF-β, no GAG in bone, keratan sulfate in other tissues
Osteoadherin	9q21.3-22 4.5 kb mRNA	85 kDa intact protein, 47 kDa core protein, 11 leucine-rich repeat sequences, RGD sequence	May mediate cell attachment
Perlican	1p36.1	~660 kDa intact protein, 450 kDA core protein,3 70-100 kDA HS GAGs	Interacts with matrix components, regulates cell signaling
Glypican	Xq26	Lipid linked HSPG, 66 kDa core, 2 HS GAGs	Regulates BMP-SMAD signaling, regulates development.
Hyaluronan	Multigene complex	Multiple proteins associated outside of the cell, structure unknown	May work with versican-like molecule to capture space destined to become bone

aggregates. Aggrecan is virtually cartilage specific, but mRNA levels have been detected in developing bone (Wong *et al.*, 1992). In the nanomelic chick, there is a mutation in the aggrecan core protein such that it is not expressed in cartilage (Primorac et al., 1999). However, there is a slight effect on bones that form via the intramembranous pathway, an unexpected finding because these bones would not be expected to be affected by abnormal cartilage development. Mutation in the aggrecan gene results in a form of spondyloepiphyseal dysplasia in humans leading to premature osteoarthritis (Gleghorn *et al.*, 2005), and in mice, a mutation leading to a null allele results in cartilage matrix deficiency (cdm mice) (Watanabe *et al.*, 1994).

Closely related, but not identical, is a soft connective tissue-enriched proteoglycan termed versican, which is most localized to loose, interstitial mesenchyme in developing bone. It has been hypothesized that it captures space that will ultimately become bone (Fisher and Termine, 1985). It is this proteoglycan that is destroyed as osteogenesis progresses. It is noteworthy that the core protein of versican contains EGF-like sequences (Wight, 2002), and release of these sequences may influence the metabolism of cells in the osteoblastic lineage. As osteogenesis progresses, versican is replaced by two members of another class of proteoglycans that contain core proteins of a different chemical nature [decorin and biglycan (Fisher and Termine, 1985)]. In the *hdf* (heart defect) mouse, the versican gene is disrupted and the protein is not expressed. The mutation is embryonic lethal, and *in vitro* studies have shown defective chondrogenesis by limb mesenchyme (Williams *et al.*, 2005).

Decorin (PG-II) and Biglycan (PG-I)

The two small proteoglycans that are heavily enriched in bone matrix are decorin and biglycan, both of which contain chondroitin sulfate chains in bone, but bear dermatan sulfate in soft connective tissues. They are characterized by core proteins that contain a leucine-rich repeat (LRR) sequence, a property shared with proteins that are associated with morphogenesis such as *Drosophila* toll protein and chaoptin, the leucine-rich protein of serum and adenylyl cyclase (Fisher *et al.*, 1989). The three-dimensional structure of another protein containing this repeat sequence, ribonuclease inhibitor protein, has been determined by physicochemical methods, and the structure predicts a highly interactive surface for protein binding (Kobe and Deisenhofer, 1995). More recently, the crystal structure of decorin has been determined, and based on this analysis, it appears that the small leucine-rich repeat-containing proteoglycans (SLRPs) are not as curved as ribonuclease, and predicts that they may dimerize (McEwan *et al.*, 2006).

Although decorin and biglycan share many properties owing to the high degree of homology of their core proteins, they are also quite distinct, as best demonstrated by their pattern of expression (Bianco *et al.*, 1990). In cartilage, decorin is found in the interterritorial matrix away from the chondrocytes, whereas biglycan is in the intraterritorial matrix. In keeping with this pattern, during endochondral bone formation, decorin is widely distributed in a pattern that is virtually indistinguishable from that of type I collagen. It first appears in preosteoblasts, is maintained in fully mature osteoblasts, and is subsequently downregulated as cells become buried in the extracellular matrix to become osteocytes. However, biglycan exhibits a much more distinctive pattern of distribution. It is found in a pericellular location in unique areas undergoing morphological delineation. It is upregulated in osteoblasts and, interestingly, its expression is maintained in osteocytes and is localized to lacunae and canaliculi. It is speculated that osteocytes act as mechanoreceptors within the bone matrix (Bonewald, 2006), and that proteoglycans, possibly biglycan or cell surface-associated molecules (such as heparan sulfate proteoglycans), may act as transducers of sheer forces within canaliculi.

Transgenic mice that are deficient in decorin have primarily thin skin (Danielson *et al.*, 1997), whereas mice deficient in biglycan fail to achieve peak bone mass and develop osteopenia (Xu *et al.*, 1998). Animals that are double deficient for decorin and biglycan exhibit a phenotype reminiscent of the progeriod form of Ehlers–Danlos syndrome, whereby collagen fibrils are highly disrupted in both the dermis and in bone (Corsi *et al.*, 2002).

Although decorin and biglycan are found in soft connective tissues that do not mineralize, their presence in osteoid makes them potential candidates as nucleators of hydroxyapatite precipitation. Decorin does not appear to be a direct nucleator, because it has no effect on hydroxyapatite precipitation or crystal growth in solution assays, and it has a low affinity for calcium. In similar assays, biglycan has varying effects depending on concentration. Biglycan has a low affinity for calcium, and at low concentrations it facilitates hydroxyapatite precipitation, but inhibits precipitation at high concentrations. However, it is thought that sulfate-containing molecules must be removed prior to matrix mineralization and that they may mask sites that will ultimately act as nucleators. Consequently, it is unlikely that decorin or biglycan are initiators of matrix mineralization (reviewed in Zu *et al.*, 2007).

Both decorin and biglycan have been found to bind to transforming growth factor (TGF)-β and to regulate its availability and activity (Schonherr and Hausser, 2000). Decorin binds to collagen (decorating collagen fibrils), as does biglycan. Another activity has been demonstrated by *in vitro* cell attachment assays where decorin and biglycan were both found to inhibit bone cell attachment, presumably by binding to fibronectin and inhibiting its cell-matrix-binding capabilities (Grzesik and Robey, 1994). It is not clear how this *in vitro* phenomenon relates to normal bone cell physiology, but it points to a role for these proteoglycans in modulating cell–matrix interactions.

Other Leucine-Rich Repeat Sequence Proteins and Proteoglycans

Interestingly, there are at least 60 proteins that have been found to contain LLRs, and many of them are also proteoglycan, SLRPs (Matsushima *et al.*, 2000). One LRR found in bone is osteoglycin, previously termed osteoinductive factor and later found to be a protein bound to TGF-β (Ujita *et al.*, 1995). This molecule is similar but not identical to the proteoglycan, PG-Lb, which has now been found to be epiphycan, localized primarily in epiphyseal cartilage. More recently, another LRR, asporin, has been localized to developing bone. Unlike other LRRs, it has an aspartic acid-rich amino sequence (Henry *et al.*, 2001; Lorenzo *et al.*, 2001). Other members of the SLRP family found in bone include fibromodulin, which contains keratan sulfate and binds to collagen fibrils in regions distinctly different from those of decorin (Hedbom and Heinegard, 1993), osteoadherin, which also contains the cell attachment sequence, RGD (Sommarin *et al.*, 1998), and lumican, which may regulate collagen fibril formation. Although SLRPs appear to be "born to bind," definitive functions are not known. A mouse deficient for both biglycan and fibromodulin exhibits initial joint laxity, followed by development of extra sesmoid bones in many tendons and development of an osteoarthritis-like condition (Ameye *et al.*, 2002), the lumican/fibromodulin knockout mouse resembles a variant of Ehlers–Danlos syndrome (OMIM 130000) and has ectopic calcification (Chakravarti *et al.*, 2003). Other proteoglycans have been isolated from a variety of animal species by using varying techniques such as HAPGIII (so named for its ability to bind to hydroxyapatite) and PG-100, which has been shown subsequently to be homologous to versican as reviewed previously (Zu *et al.*, 2007).

Although not generally found in the extracellular matrix, heparan sulfate proteoglycans found associated with, or intercalated into, cell membranes may be very influential in regulating bone cell metabolism. Receptors for several growth factors (TGF-β and FGF-2, to name two) have been found to associate with heparan sulfate (either bound covalently to core proteins or as free glycosaminoglycans). Intercalated heparan sulfate proteoglycans (the syndecan family) have been postulated to regulate cell growth, perhaps through modulation of growth factor and receptor activity (Schonherr and Hausser, 2000). Perlecan has been found to interact with matrix components and to regulate cell signaling. Knockout mice have a phenotype that resembles thanatophoric dysplasia type I (OMIM 187600), and mutations in humans are associated with Schwartz–Jample syndrome (OMIM 142461). Another class of heparan sulfate proteoglycans is linked to cell membranes by phosphoinositol linkages that are cleavable by phospholipase C (glypicans). Consequently, their activity may be in the pericellular environment or in the extracellular matrix. Mutations in glypican 3 give rise to Simpson–Golabi–Behmel Syndrome (OMIM 300037), and the knockout mouse has delayed endochondral bone formation and impaired osteoclastic development. The complete cast of heparan sulfate proteoglycans present in the cellular and pericellular environment in bone is not yet complete [see Zu *et al.* (2007) for a review].

HYALURONAN

This unsulfated glycosaminoglycan is not attached to a protein core and is synthesized by a completely different pathway (see Table II). Whereas other glycosaminoglycans are formed by the transfer of growing glycosaminoglycan chains from a lipid carrier (dolichol phosphate) to a protein carrier, hyaluronan is synthesized in the extracellular environment by a group of enzymes that are localized on the outer cell membrane. Large amounts of hyaluronan are synthesized during early stages of bone formation and may associate with versican to form high molecular weight aggregates, although this association has not been demonstrated to occur in developing bone. Very little is known about the potential function of hyaluronan in bone formation, but in other tissues it is speculated to participate in cell migration and differentiation (Fedarko *et al.*, 1992).

GLYCOPROTEINS

Virtually all of the bone matrix proteins are modified post-translationally to contain either N- or O-linked oligosaccharides, many of which can be modified further by the addition of phosphate and/or sulfate (Table III). In general, compared with their soft connective tissue counterparts, bone matrix proteins are modified more extensively and in a different pattern. In some cases, differences in post-translational modifications result from differential splicing of heterogeneous nuclear RNA, but, in general, they result from differences in the activities of enzymes located along the intracellular pathway of secretion. The pattern of post-translational modifications may be cell type specific and consequently may be of use in distinguishing protein metabolism from one tissue type versus another. The development of probes and antibodies against these types of tissue-specific determinants may be of great diagnostic value.

The number of glycoproteins that have been identified in bone matrix grows by leaps and bounds every year. This, in part, is because of the explosion of sequence information from cDNA libraries, where one has the ability to pick up even the scarcest of clones. What follows next is a brief description of the more abundant bone matrix glycoproteins that most likely play major structural as well as metabolic roles. Other glycoprotein species have

TABLE III Gene and Protein Characteristics of Glycoproteins in Bone Matrix

	Gene	Protein	Function
Alkaline phosphatase	12p13.1 15 kb, 12 exons alternative promoters, one RFLP 2.5, 4.1, 4.7 kb mRNA	Two identical subunits of ~80 kDa, disulfide-bonded, tissue-specific post-translational modifications	Potential Ca^{2+} carrier, hydrolyzes inhibitors of mineral deposition such as pyrophosphates
Osteonectin	5q31-33 20 kb, 10 exons, one RFLP, 2.2, 3.0 kb mRNA	~35–45 kDa, intramolecular disulfide bonds, α-helical amino terminus with multiple low-affinity Ca^{2+} binding sites, two EF hand high-affinity Ca^{2+} sites, ovomucoid homology, glycosylated, phosphorylated, tissue-specific modifications	May mediate deposition of hydroxyapatite, binds to growth factors, may influence cell cycle, positive regulator of bone formation
Tetranectin	3p22 12 kb, 3 exons 1 kb mRNA	21 kDa protein composed of four identical subunits of 5.8 kDa, sequence homologies with asialoprotein receptor and G3 domain of aggrecan	Binds to plasminogen, may regulate matrix mineralization

been identified primarily as growth factors, produced both endogenously and exogenously, and will be covered in more detail elsewhere in this volume.

Osteonectin (SPARC, Culture Shock Protein, and BM40)

With the development of procedures to demineralize and extract bone matrix proteins without the use of degradative enzymes, osteonectin was one of the first proteins isolated in intact form. This molecule was so named owing to its ability to bind to Ca^{2+}, hydroxyapatite, and collagen and to nucleate hydroxyapatite deposition (Termine *et al.*, 1981). The osteonectin molecule contains several different structural features, the most notable of which is the presence of two EF hand high-affinity calcium-binding sites. These structures are usually found in intracellular proteins, such as calmodulin, that function in calcium metabolism (reviewed in Bhattacharya *et al.*, 2004).

Although osteonectin is highly enriched in bone, it is also expressed in a variety of other connective tissues at specific points during development, maturation, or repair processes *in vivo*. SPARC (secreted protein, acidic, rich in cysteine) was identified after induction by cAMP in teratocarcinoma cells and was found to be produced at very early stages of embryogenesis. Interestingly, if osteonectin is inactivated by the use of blocking antibodies during tadpole development, there is a disruption of somite formation and subsequent malformations in the head and trunk (Purcell *et al.*, 1993). Mice that are deficient of osteonectin present with severe cataracts (Bassuk *et al.*, 1999) and develop severe osteopenia characterized by decreased trabecular connectivity, decreased mineral content, but increased apatite crystal size (Delany *et al.*, 2000; Mansergh *et al.*, 2007).

Constitutive expression in the adult tissue is limited to cells associated intimately with mineralized tissues, such as hypertrophic chondrocytes, osteoblasts, and odontoblasts, and ion-transporting cells, such as mammary epithelium, distal tubule epithelium in the kidney, and salivary epithelium (cells associated with basement membrane, hence the name BM-40). Transient expression has been noted in other cell types, such as decidual cells in the uterus and in testis when cells are undergoing a maturation event. *In vitro*, expression appears to be deregulated rapidly, resulting in expression by cells that would not be expressing high levels *in situ*, hence its designation as a culture shock protein.

There have been numerous studies using both the intact molecule and peptides derived from different regions. Many of these structure–function studies have been performed in endothelial cell cultures, from which culture shock protein was originally isolated. From these studies, osteonectin has been implicated in regulating the progression of the cell through the cell cycle, cell shape, cell–matrix interactions, binding to metal ions, binding to growth factors, and modulating enzymatic activities (reviewed in Bradshaw and Sage, 2001). However, many of these activities have not been found or have not been tested in osteoblastic cultures. It should also be recognized that the activity of a peptide might not occur *in vivo* when it is taken out of context of the intact protein or naturally occurring degradative products.

Tetranectin

This tetrameric protein has been identified in woven bone and in tumors undergoing mineralization (Wewer *et al.*, 1994). This protein shares sequence homologies with globular domains of aggrecan and asialoprotein receptor. Mice deficient in tetranectin develop spinal deformities (Iba *et al.*, 2001), but the complete role that tetranectin plays ins osteogenesis is not known.

RGD-Containing Glycoproteins

Some of the major glycoproteins in bone matrix also contain the amino acid sequence Arg-Gly-Asp (RGD), which conveys the ability of the extracellular matrix protein to bind to the integrin class of cell surface receptors (reviewed in Luo *et al.*, 2007) (Table IV). This binding is the basis of many cell attachment activities that have been identified by *in vitro* analysis; however, it should be noted that it is not yet clear how this *in vitro* activity translates into *in vivo* physiology. The bone matrix contains a long list of RGD-containing glycoproteins (collagen(s), thrombospondin, fibronectin, vitronectin, fibrillins, osteoadherin, osteopontin, bone sialoprotein, dentin matrix protein 1, and proteins derived from the dentin sialophosphoprotein gene). Although this would appear to be a case of extreme redundancy, both *in vivo* and *in vitro* analysis indicates that the proteins are not equivalent in their abundance or pattern of expression during bone formation and in other tissues or in their *in vitro* activities (Grzesik and Robey, 1994).

Thrombospondins

Thrombospondins are a family of multifunctional proteins. Thrombospondin-1 was first identified as the most abundant protein in platelet α granules, but is found in many tissues during development, including bone (Robey *et al.*, 1989). Subsequently, five other members have been described, including COMP (cartilage oligomeric matrix protein) as thrombospondin-5 (reviewed in Adams and Lawler, 2004). In bone, all forms are present, synthesized by different cell types at different stages of maturation and development (Carron *et al.*, 1999). Thrombospondins have

TABLE IV Gene and Protein Characteristics of Glycoproteins in Bone Matrix - Continued RGD-Containing Glycoproteins

RGD-containing glycoproteins	Gene	Protein	Function
Thrombospondins	TSP-1 - 15q15 TSP-2 - 6q27 TSP-3 - 1q21-24 TSP-4 - 5q13 COMP - 19p13.1 4.5–16 kb, 22 exons 4.5–6.1 kb mRNA	∼450 kDa molecule, three identical disulfide-linked subunits of ∼150–180 kDa, homologies to fibrinogen, properdin, EGF, collagen, von Willebrand, P. *falciparum* and calmodulin, RGD at the C-terminal globular domain	Cell attachment (but usually not spreading), binds to heparin, platelets, type I and V collagens, throm fibrinogen, laminin, bin, plasminogen and plasminogen activator inhibitor, histidine-rich glycoprotein, TSP-2 is a negative regulator of bone formation
Fibronectin	2p14-16, 1q34-36 50 kb in chicken, 50 exons, multiple splice forms, 6 RFLPs,7.5 kb mRNA	∼400 kDa with two nonidentical subunits of ∼200 kDa, composed of type I, II, and III repeats, RGD in the 11th type III repeat 2/3 from N terminus	Binds to cells, fibrin heparin, gelatin, collagen
Vitronectin	17q ∼ 70 kDa, RGD close to N 4.5 kb, 8 exons, 1.7 kb mRNA	Cell attachment protein, terminus, homology to somatomedin B, rich in cysteines, sulfated, phosphorylated	Binds to collagen, plasminogen and plasminogen activator inhibitor, and to heparin
Fibrillin	15q15-23, 5 (two different genes), 110 kb, 65 exons, 10 kb mRNA	350 kDa, EGF-like domains, RGD, cysteine motifs	May regulate elastic fiber formation
Osteopontin	4q13-21 8.2 kb, 7 exons, multiple alleles, one RFLP, one splice variant, several alleles 1.6 kb mRNA	∼44–75 kDa, polyaspartyl stretches, no disulfide bonds, glycosylated, phosphorylated, RGD located 2/3 from the N terminus	Binds to cells, may regulate mineralization, may regulate proliferation, inhibits nitric oxide synthase, may regulate resistance to viral infection, a regulator of bone resorption
Bone sialoprotein	4q13-21 15 kb, 7 exons, 2.0 mRNA	∼46–75 kDa, polyglutamyl stretches, no disulfide bonds, 50% carbohydrate, tyrosine-sulfated, RGD near the C terminus	Binds to cells, may initiate mineralization
BAG-75	Gene not yet isolated, mRNA not yet cloned	∼75 kDa, sequence homologies to phosphophoryn, osteopontin and bone sialoprotein, 7% sialic acid, 8% phosphate	Binds to Ca^{2+}, may act as a cell attachment protein (RGD sequence not yet confirmed), may regulate bone resorption

many proposed activities, including binding to a large number of matrix proteins and cell surface proteins. *In vitro*, it mediates bone cell adhesion in an RGD-independent fashion, indicating the presence of other sequences in the molecule that are required. Furthermore, cell spreading requires the synthesis of other proteins. The thrombospondin-2-deficient mouse has been found to have increased cortical thickness compared with normal littermates, and to exhibit abnormalities in response to mechanical loading (Hankenson *et al*., 2006).

Fibronectin

Fibronectin is synthesized by virtually all connective tissue cells and is a major component of serum. There are a large number of different mRNA splice variants such that the number of potential forms is quite high. Consequently, bone matrix could contain fibronectin that originates from exogenous as well as endogenous sources (reviewed in Pankov and Yamada, 2002). The precise form that is present in cells in the osteoblastic lineage is unknown. Fibronectin is produced during early stages of bone formation and has been found to be highly upregulated in the osteoblastic cell layer. Interestingly, bone cell attachment to fibronectin *in vitro* is in an RGD-independent fashion (Grzesik and Robey, 1994). However, this correlates well with the expression of the fibronectin receptor, $\alpha_4\beta_1$, which binds to a sequence other than RGD in the fibronectin molecule and is also expressed by some osteoblastic cells. Cell–matrix interactions mediated by fibronectin-$\alpha_4\beta_1$ binding may play a role in the maturation sequence of cells in the osteoblastic lineage.

Vitronectin

This serum protein, first identified as S-protein owing to its cell-spreading activity, is found at low levels in mineralized matrix (Grzesik and Robey, 1994). Its cell surface receptor, $\alpha_v\beta_3$, is distributed broadly throughout bone tissue. There may also be endogenous synthesis of a related form (Seiffert, 1996). In addition to cell attachment activity, it also binds to and affects the activity of the plasminogen activator inhibitor (Schvartz *et al*., 1999).

Fibrillins

In addition to the RGD sequence, fibrillin-1 and fibrillin-2 are glycoproteins that also contain multiple EGF-like repeats. They are major components of microfibrils, and mutations in these genes lead to Marfan's syndrome, which exhibits abnormalities in bone growth (reviewed in Ramirez and Dietz, 2007). It is not yet known whether they are produced at a specific stage of bone formation, remodeling, or turnover; however, it is known that they associate with LTBP (latent TGF-β-binding protein) in microfibrils and regulate TGF-β bioactivity (Chaudhry *et al*., 2007).

Small Integrin-Binding Ligands with N-linked Glycosylation (SIBLINGs)

Several bone matrix proteins are characterized not only by the inclusion of RGD within their sequences, but also by the presence of relatively large amounts of sialic acid. Interestingly, they are clustered at 4q21-23 and appear to have arisen by gene duplication. For this reason, the family has been termed SIBLINGs (Fisher and Fedarko, 2003). The family includes osteopontin (OPN) and bone sialoprotein (BSP), the two best characterized proteins of the family, along with dentin matrix protein-1 (DMP-1), dentin sialoprotein (DSP), and dentin phosphoprotein (DPP), which are coded for by the same gene, now termed dentin sialophosphoprotein (DSPP), matrix extracellular glycoprotein (MEPE), and the more distantly related protein, enamelin (ENAM). Although the SIBLINGs were initially thought to be specific for mineralized tissues, it is now apparent that many of them are expressed in metabolically active epithelial cells (Ogbureke and Fisher, 2004, 2005). Interestingly, three of the family members bind to and activate specific matrix metalloproteinases (BSP–MMP-2, OPN–MMP-3, DMP1–MMP-9) (Fedarko *et al*., 2004).

Osteopontin (Spp, BSP-I)

This sialoprotein was first identified in bone matrix extracts, but it was also identified as the primary protein induced by cellular transformation. In bone, it is produced at late stages of osteoblastic maturation corresponding to stages of matrix formation just prior to mineralization. *In vitro*, it mediates the attachment of many cell types, including osteoclasts. In osteoclasts, it has also been reported to induce intracellular signaling pathways as well. In addition to the RGD sequence, it also contains stretches of polyaspartic acid and it has a fairly high affinity for Ca^{2+}; however, it does not appear to nucleate hydroxyapatite formation in several different assays. The osteopontin-deficient mouse develops normally, but has increased mineral content, although the crystals are smaller than normal. The role of osteopontin in skeletal homeostasis is covered in greater detail in another chapter in this volume, and there are numerous reviews available for its role in cancer and immune function (for example, Chakraborty *et al*., 2006; Scatena *et al*., 2007)].

Bone Sialoprotein (BSP-II)

The other major sialoprotein is bone sialoprotein, composed of 50% carbohydrate (12% is sialic acid) and stretches of polyglutamic acid (as opposed to polyaspartic acid in osteopontin). The RGD sequence is located at the carboxy terminus of the molecule, whereas it is located centrally in osteopontin. The sequence is also characterized by multiple tyrosine sulfation consensus

TABLE V Gene and Protein Characteristics of γ-Carboxy Glutamic Acid-Containing Proteins in Bone Matrix

	Gene	Protein	Function
Matrix Gla protein	12p 4 exons, 3.9 kb	~15 kDa, five Gla residues, one disulfide bridge, phosphoserine residues	May function in cartilage metabolism, a negative regulator of mineralization
Osteocalcin	4 exons, 1 1.2 kb	~5 kDa, one disulfide bridge, Gla residues located in α-helical region	May regulate activity of osteoclasts and their precursors, may mark the turning point between bone formation and resorption
Protein S	3q11.2	~75 kDa	May be made primarily in the liver, protein S deficiency may result in osteopenia

sequences found throughout the molecule, in particular, in regions flanking the RGD (Fisher *et al.*, 1990). Sulfated BSP has been isolated in a number of animal species, but the levels appear to be variable.

Bone sialoprotein exhibits a more limited pattern of expression than osteopontin. In general, its expression is tightly associated to mineralization phenomena (although there are exceptions). In the skeleton, it is found at low levels in chondrocytes, in hypertrophic cartilage, in a subset of osteoblasts at the onset of matrix mineralization, and in osteoclasts (Bianco *et al.*, 1991). Consequently, BSP expression marks a late stage of osteoblastic differentiation and an early stage of matrix mineralization. Outside of the skeleton, BSP is found in trophoblasts in placental membranes, which in late stages of gestation fuse and form mineralized foci. A BSP-deficient mouse has been generated, but reportedly does not exhibit a skeletal phenotype, possibly because of compensation of BSP function by other SIBLINGs.

BSP may be multifunctional in osteoblastic metabolism. It is very clear that it plays a role in matrix mineralization as supported by the timing of its appearance in relationship to the appearance of mineral and its Ca^{2+}-binding properties. BSP has a very high affinity for calcium. The polyglutamyl stretches were thought to be solely responsible for this high affinity; however, studies using recombinant peptides suggest that although the polyglutamyl stretches are required, they are not the sole determinants (Stubbs *et al.*, 1997). Unlike osteopontin, BSP does nucleate hydroxyapatite deposition in a variety of assays.

It is also clear from *in vitro* assays that BSP is capable of mediating cell attachment, most likely through interaction with the somewhat ubiquitous $\alpha_v\beta_3$ (vitronectin) receptor. Bone cells attach to the intact molecule in an RGD-dependent fashion. However, when BSP is fragmented, either endogenously by cells or using commercially available enzymes, the fragment most active in cell attachment does not contain the RGD sequence (Mintz *et al.*, 1993). Studies indicate that the sequence upstream from the RGD mediates attachment (in an RGD-independent fashion) and suggest that the integrin-binding site is more extended than had been envisioned previously. Sequences flanking the RGD site are often tyrosine sulfated. However, it is not known how sulfation influences BSP activity, as *in vitro*, unsulfated BSP appears to be equivalent in its activity. Once again, it is not clear if currently available *in vitro* assays are sufficiently sophisticated to determine what influence post-translational modifications, such as sulfation, have on the biological activity. In addition to sulfation, conformation of the RGD site may also influence the activity of the protein. Although the RGD region in fibronectin is found in a looped-out region that is stabilized by disulfide bonding, there are no disulfide bonds in BSP. However, the flanking sequences most likely influence the conformation of the region. The cyclic conformations also appear to have a higher affinity for cell surface receptors than linear sequences (van der Pluijm *et al.*, 1996).

Dentin Matrix Protein 1 (DMP1)

Although DMP1 was originally thought to be specific to dentin, it was subsequently found to be synthesized by osteoblasts as well (D'Souza *et al.*, 1997). However, its function in bone metabolism is not presently known. The DMP1-deficient mouse has craniofacial and growth plate abnormalities, along with rickets and osteomalacia owing to increased secretion of the phosphate-regulating hormone, FGF-23. The overproduction of FGF-23 is hypothesized to be caused by abnormal osteocyte function (Feng *et al.*, 2006). Mutations in DMP1 have been identified in patients with dentinogenesis imperfecta (OMIM 600980) and in forms of autosomal recessive hypophosphatemic rickets (OMIM 241520).

TABLE VI Gene and Protein Characteristics of Serum Proteins Found in Bone Matrix

Serum proteins	Gene	Protein	Function
Albumin	4q11-22 17 kb, 15 exons	69 kDa, nonglycosylated, one sulfhydryl, 17 disulfide bonds, high-affinity hydrophobic binding pocket	Inhibits hydroxyapatite crystal growth
α_2HSglycoprotein	3 two RFLP 1.5 kb mRNA	Precursor protein of fetuin, cleaved to form A and B chains that are disulfide linked, Ala-Ala and Pro-Pro repeat sequences, N-linked oligosaccharides, cystatin-like domains	Promotes endocytosis, has opsonic properties, chemoattractant for monocytic cells, bovine analogue (fetuin) is a growth factor

Matrix, Extracellular, Phosphoglycoprotein (MEPE)

MEPE is expressed in bone and bone marrow, but also at high levels in the brain and low levels in the lung, kidney, and placenta. This gene is also highly expressed by tumors that induce osteomalacia, and it has been regarded as a potential phosphate-regulating hormone (Rowe *et al.*, 2000). It is thought that the C-terminal portion of MEPE (ASARM), together with PHEX, regulates mineralization and renal phosphate metabolism (Rowe, 2004). Conversely, animals deficient in MEPE have significantly increased bone mass owing to increased numbers of trabeculae and increased cortical thickness (Gowen *et al.*, 2003).

γ-Carboxy Glutamic Acid (GLA)-Containing Proteins

There are three proteins found in bone matrix that undergo γ-carboxylation via Vitamin K-dependent enzymes: matrix-gla-protein (MGP) (Price *et al.*, 1983), osteocalcin (bone gla-protein, BGP) (Price *et al.*, 1976), both of which are made by bone cells, and protein S (made primarily in the liver but also made by osteogenic cells) (Maillard *et al.*, 1992) (Table V). The presence of di-carboxylic glutamyl (gla) residues confers calcium-binding properties to these proteins.

MGP is found in many connective tissues and is highly expressed in cartilage. It appears that the physiological role of MGP is to act as an inhibitor of mineral deposition. MGP-deficient mice develop calcification in extraskeletal sites such as in the aorta (Luo *et al.*, 1997). Interestingly, the vascular calcification proceeds via transition of vascular smooth muscle cells into chondrocytes, which subsequently hypertrophy (El-Maadawy *et al.*, 2003). In humans, mutations in MGP have been also been associated with excessive cartilage calcification (Keutel syndrome, OMIM 245150).

Whereas MGP is broadly expressed, osteocalcin is somewhat bone specific, although messenger RNA (mRNA) has been found in platelets and megakaryocytes (Thiede *et al.*, 1994). Osteocalcin-deficient mice are reported to have increased bone mineral density compared with normal (Ducy *et al.*, 1996). In human bone, it is concentrated in osteocytes, and its release may be a signal in the bone-turnover cascade (Kasai *et al.*, 1994). Osteocalcin measurements in serum have proved valuable as a marker of bone turnover in metabolic disease states. Interestingly, it has been recently suggested that osteocalcin also acts as a hormone that influences energy metabolism by regulating insulin secretion, β-cell proliferation, and serum triglyceride (Lee *et al.*, 2007).

SERUM PROTEINS

The presence of hydroxyapatite in the bone matrix accounts for the adsorption of a large number of proteins that are synthesized elsewhere and brought into the vicinity via the circulation (Delmas *et al.*, 1984). Most of these proteins are synthesized in the liver and hematopoietic tissue and represent classes of immunoglobulins, carrier proteins, cytokines, chemokines, and growth factors. Interestingly, some of these proteins are also synthesized endogenously by cells in the osteoblastic lineage. It is not known if the origin of a particular factor (and hence proteins with potentially different post-translational modifications) affects biological activity or not.

Although serum proteins are not synthesized locally, they may have a significant impact on bone metabolism (Table VI). Albumin, which is synthesized by the liver, is concentrated in bone severalfold above levels found in the circulation. It is not known whether it plays a structural role in bone matrix formation but it does have an influence on hydroxyapatite formation. In *in vitro* assays, albumin inhibits hydroxyapatite growth by binding to several faces of the seed crystal (Garnett and Dieppe, 1990). In addition to this inhibitory activity, it also inhibits crystal aggregation.

Another serum protein, α_2-HS-glycoprotein, is even more highly concentrated in bone than albumin (up to 100 times more concentrated). It is known that α_2-HS-glycoprotein is the human analogue of bovine fetuin (Ohnishi *et al.*, 1993). This protein is synthesized as a precursor that contains a disulfide bond linking the amino- and carboxy-terminal regions. Subsequently, the midregion is cleaved and removed from the molecule, yielding the A and B peptides (much in the same way that insulin is processed). In rat, the midregion is not removed and the molecule consists of a single polypeptide. This protein also contains cystatin-like domains (disulfide-linked loop regions), and another member of this family has been identified in bone matrix extracts.

α_2-HS-glycoprotein has many proposed functions that may also be operative in bone cell metabolism. In other cell culture systems, it has been proposed to promote endocytosis and to have opsonic properties. It is also a chemoattractant for monocytic cells, and consequently, it may influence the influx of osteoclastic precursor cells into a particular area (Nakamura *et al.*, 1999). Furthermore, it is a transforming growth factor-β type II receptor mimic and cytokine antagonist (Demetriou *et al.*, 1996). Fetuin, the bovine homologue, has been found to be a major growth-promoting factor in serum, and results *in vitro* suggest that fetuin, along with transforming growth factor-β, inhibit osteogenesis (Binkert *et al.*, 1999). Interestingly, deletion of the fetuin gene in mice leads to widespread ectopic calcification throughout the body (Jahnen-Dechent *et al.*, 1997). Consequently, this protein may play a very important role in bone cell metabolism irrespective of whether or not it is synthesized locally.

OTHER PROTEINS

In addition to the proteins described earlier there are representatives of many other classes of proteins in the bone matrix, including proteolipids, enzymes and their inhibitors (including metalloproteinases and tissue inhibitors of metalloproteinase [TIMPs], plasminogen activator and plasminogen activator inhibitor, matrix phosphoprotein kinases, and lysosomal enzymes), morphogenetic proteins, and growth factors (Zu *et al.*, 2007). Although their influence on bone cell metabolism is highly significant and they may cause significant alterations of the major structural elements of bone matrix, they are not necessarily part of the structural scaffold of bone matrix (with the possible exception of proteolipids). Important aspects of many of these classes of proteins are reviewed elsewhere.

CONTROL OF GENE EXPRESSION

In vivo and *in vitro* analysis clearly indicates that the timing and location of bone matrix protein expression are controlled by cells in the osteoblastic lineage as they progress toward maturation. The sequence of molecular events that regulate this progression is mediated by *cis*- and *trans*-acting factors present in the nucleus. *cis*-acting factors (also known as response elements) are present in the promoter region of the gene (the sequence upstream from the gene transcription start site). *cis*-acting factors can be roughly separated into two types: those that serve as binding sites for DNA polymerases (TATA, CAAT) and those that serve as binding sites for *trans*-acting (nuclear binding) factors. The interaction of *cis*-acting sequences within the promoter and *trans*-acting factors thereby modulates the activity of DNA polymerases, resulting in either activation or suppression of gene activity.

Utilizing both *in vitro* and *in vivo* analysis, virtually all of the promoters of the bone matrix protein genes have been characterized. Numerous *cis*-acting elements have been identified in all of the genes by direct sequence analysis, and their activity in serving as binding sites for *trans*-acting nuclear factors has been tested in DNA footprint and mobility assays. The most reliable information appears to be derived from studies utilizing transgenic animals that have been engineered to contain parts of the promoter, either wild type or mutated, linked to a reporter molecule, such as chloramphenicol transferase, β-galactosidase, or luciferase. This stance stems from studies of type I collagen and alkaline phosphatase gene expression whereby sequences identified as active by *in vitro* analysis were not active when placed in the animal. Transgenic animals of this sort have been generated for many of the bone matrix protein promoters and have provided a great deal of information on what factors are controlling both the timing and the location of bone matrix protein expression during bone formation. Salient features of the bone matrix protein promoters are listed in Table VII.

Although not complete, the mechanism by which the pattern of gene expression is controlled during osteogenesis is becoming clearer. This owes in large part to the identification of RUNX2/CBFA1, a transcription factor that is required for bone formation during development and for modeling and remodeling after birth (Ducy *et al.*, 1997). Deletion of this gene resulted in the generation of mice that were completely devoid of bone (Komori *et al.*, 1997). The promoters of bone matrix proteins expressed at late stages of osteoblastic maturation, osteopontin, bone sialoprotein, and osteocalcin, all have RUNX2/CBFA1-binding sites (Ducy *et al.*, 1996), although it appears that CBFA1 represses bone sialoprotein expression (Javed *et al.*, 2001). Transcriptional control of osteoblastic differentiation and of their secretory products is presented in detail elsewhere in this volume.

BONE MATRIX GLYCOPROTEINS AND ECTOPIC CALCIFICATIONS

The development of sensitive radiographic techniques, in addition to histological observations, has lead to the description of ectopic calcifications in many different pathological disorders. Although dystrophic mineralization has long been noted, it was not thought that bone matrix proteins

TABLE VII Promoter Characteristics of Bone-Related Genes

Protein	Polymerase-binding sites	*cis*-acting factors	*trans*-acting factors
Collagens			
α1(I)	TATA, CCAAT, CT rich, AG rich	SP1, VDRE, NF1	CAAT-binding protein, two silencers, VDR
α2(I)	CCAAT, CT rich	NF1	CAAT-binding protein, CTF/NF1
Proteoglycans			
Versican	TAATA, CCAAT		CAAT-binding factor
Decorin	Two promoters 1a- GC rich 1b -two TATA, one CAAT		
Biglycan	GC rich	+/− elements, XRE, SP1, CRE	
Fibromodulin	Analysis unavailable	AP1, AP5, NF-κ b, Pu/Py mirror repeat	
Glycoproteins		SP1, AP1, AP2, NF1, NF- κb	
Alkaline phosphatase	Two promoters, GC rich, TATA	3 SP1s	
Osteonectin	GA repeats, S1 sensitve	SP1, AP1, CRE, GHE, HSE, MRE, 1st intron, four CCTG repeats	VDR, RAR GGA-binding protein
Tetranectin	Analysis unavailable		
Thrombospondin	Three genes, TATA, GC rich, inverted CAAT	AP1, AP2, SP1, NFY, SRE, Egr1 Egr1	c-Jun
Fibronectin	TATA, CAAT, GC rich	CRE, SP1	ATF2
Vitronectin	Analysis not available		
Fibrillin	TATA, CCAAT, GC rich		
Osteopontin	Inverted CCAAT, TATA, GC box	+/− elements, SP1, AP, AP4, AP5, RAE, TPA, PEA3, THR, GHV, VDRE	VDR, CBFA1-binding site
Bone sialoprotein	Inverted TATA, inverted CCAAT	AP1, CRE, Homeobox, RARE, p53, GRE, VDRE, 1st intron, poly Py, poly AC, YY-1, supressor?	VDR, CBRA1-binding sites
BAG-75	Not yet cloned		
Gla-proteins			
Matrix Gla protein	TATA, CAAT	RARE, VDRE	VDR, RA
Osteocalcin	TATA, CCAAT, overlaps with other elements	OC Box, AP1, AP2, VCE, VDRE, CRE, GRE, NF1, MSX, VA	VDR, c-Fos, CBFA1-binding site
Protein S	Analysis not available		
Serum proteins			
Albumin	TATA, CCAAT	PGRBS, GRE	C/EBP, NHF1, FTF, NF1
α_2HS glycoprotein	Analysis unavailable		

played a role in generating this type of mineralization. Dystrophic mineralization (such as in traumatic muscle injury) is brought about by cell death (perhaps in the form of apoptosis) and not by the physiological pathways mediated by collagen or matrix vesicles. However, bone matrix proteins have been identified in mineralized foci in several different pathological states. Osteonectin, osteopontin, and bone sialoprotein have been found in mineralized foci in primary breast cancer (Bellahcene and Castronovo, 1997). Bone sialoprotein has also been found in other cancers, such as prostate, thyroid, and lung (Bellahcene *et al.*, 1997, 1998; Waltregny *et al.*, 1998). Although it is possible that, in some cases, the area mineralizes dystrophically and bone matrix proteins are adsorbed from the circulation because of their affinity to hydroxyapatite, mRNAs for the bone matrix proteins are expressed and it appears that the proteins are actually synthesized by resident cells that have been triggered (by factors that have yet to be identified).

Given the fact that bone matrix proteins are expressed by a number of different cancers, the next question is why? The processes by which cancer cells invade the surrounding normal tissue, gain entry into the circulation, and metastasize to other tissues is complex, but recently, members of the SIBLING family have emerged as active players in tumorigenesis and metastasis. SIBLINGs and their proteolytic fragments, along with their partner MMPS (for BSP, OPN, and DMP1, at least) may modulate a tumor cell's adhesion via specific integrins, matrix degradation, and migration (reviewed in Bellahcene *et al.*, 2008).

Another example of ectopic calcification is seen in atherosclerosis, again, associated with the production of bone matrix proteins (Bini , 1999). Unlike dystrophic calcification, vascular calcification appears to form in a regulated fashion similar to what is seen in bone formation in the skeleton. As mentioned earlier in relationship to the MGP-deficient mouse, it appears that vascular smooth muscle cells are emerging as the culprit. Factors that stimulate the expression of bone matrix proteins are not yet known but may include changes in cell–cell interactions, serum lipid composition, and phosphate concentrations, and apoptosis of vascular smooth muscle cells may initiate the process (Trion and van der Laarse, 2004). It may also be that a population of stem cells exists that are normally quiescent, but then are induced to become osteogenic, again, by factors that are not known. The aorta has its own vasculature, which may harbor these stem cells. Supporting this hypothesis, pericytes from the retinal vasculature have been shown to undergo bone formation *in vitro* and *in vivo* (Canfield *et al*., 2000).

SUMMARY

Bone matrix proteoglycans and glycoproteins are proportionally the most abundant constituents of the noncollagenous proteins in bone matrix. Proteoglycans with protein cores composed of the leucine-rich repeat sequences (decorin, biglycan, fibromodulin, and osteoadherin) are the predominant form found in mineralized matrix, although hyaluronan-binding forms (in particular, versican) are present during early stages of osteogenesis. They participate in matrix organization and in regulating growth factor activity. Glycoproteins such as alkaline phosphatase, osteonectin, RGD-containing proteins (osteoadherin, thrombospondin, fibronectin, vitronectin, osteopontin, and bone sialoprotein), fibrillin, and tetranectin are produced at different stages of osteoblastic maturation. They exhibit a broad array of functions ranging from control of cell proliferation, cell–matrix interactions, and mediation of hydroxyapatite deposition. The ectopic expression of bone matrix proteins may also play a significant role in pathological states such as bone metastasis in certain forms of cancer and atherosclerosis.

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Chapter 18

Osteopontin

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Osteopontin (OPN) is one of the noncollagenous proteins present in bone matrix (Mark *et al.*, 1987). Independently, it was found to be present in the plasma of patients bearing highly metastatic tumors (Brown *et al.*, 1994). Another line of study revealed that the same molecule was expressed at high levels by activated T cells (Singh *et al.*, 1990). OPN was also characterized as a molecule that regulates the calcification of urinary stones because an antibody raised against it was able to block the formation of calcium oxalate-based stones (Shiraga *et al.*, 1992). The presence of OPN in various organs, including those with and without matrix, and also in plasma, suggests that this molecule could act both as a structural molecule and as a humoral factor, or cytokine (Nanci, 1999; Rittling and Denhardt, 1999; Denhardt *et al.*, 2001). The recent accumulation of a body of new data has opened a new era of studies on OPN function. This chapter focuses on these novel features of OPN.

STRUCTURE OF OSTEOPONTIN

The amino acid sequence of OPN has been determined for a number of species (Sodek *et al.*, 2000a). The conservation of much of the sequence suggests that this molecule has had a fundamental role in biological systems during evolution. OPN consists of about 300 amino acids. Importantly, an RGDS (arginine-glycine-aspartate-serine) motif is located in the midportion of the molecule. A thrombin cleavage site is located just carboxyl-terminal to the RGDS motif (Senger *et al.*, 1994; Bautista *et al.*, 1994); the products of thrombin cleavage can be observed when serum preparations are analyzed by gel electrophoresis.

However, intact OPN is more abundant than the cleavage product in blood (Kon *et al.*, 2000). Therefore, to assay the intact OPN concentration in human blood, plasma rather than serum should be prepared to avoid the effect of thrombin, which is activated in the process of serum preparation. Consideration has been given to measuring circulating OPN levels by ELISA to identify people with high risk for diseases such as osteoporosis or to evaluate the response of patients to particular clinical treatments. However, the fact that OPN is sequestered by factor H may be a complication (Fedarko *et al.*, 2000).

Clinical measurements of OPN in the circulatory system are not restricted only to patients with involutional bone diseases; such measurements may also be relevant to the evaluation of patients with metastatic tumors, with certain kinds of immunodeficiencies, with neuronal diseases, and with urinary stones. At this point, however, it is not certain what would be the contribution of intact OPN to such a diagnosis; also the significance of the levels of its cleavage products or the function of the cleavage products in each situation is unclear.

OPN is modified post-translationally by phosphorylation, the addition of sugars, such as sialic acid, and sulfation (Nagata *et al.*, 1989; Beninati *et al.*, 1994; Sørensen *et al.*, 1995; Neame and Butler, 1996; Zhu *et al.*, 1997; Safran *et al.*, 1998). The levels of glycosylation, sulfation, and phosphorylation vary depending on the organs and the time after synthesis when OPN modification is assessed. Phosphorylation modulates osteoblastic and osteoclastic functions and has been suggested to affect the efficiency of binding to various cell types (Saavedra, 1994; Lasa *et al.*, 1997; Katayama *et al.*, 1998; Ashkar *et al.*, 2000). Sulfation can affect the formation of mineralized bone nodules in culture (Nagata, 1989). So far, these data are mostly *in vitro*, and therefore, the physiological significance of such post-translational modifications has not been elucidated. It appears that differences in the post-translational modification of OPN expressed by different cell types regulate how it impacts on target cells (Razzouk *et al.*, 2002; Gericke *et al.*, 2005; Kazanecki *et al.*, 2007). Transgenic

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("knock-in") mice having mutations in sites of post-translational modifications may help elucidate the specific function of each of the modifications.

OPN is encoded in seven exons (Craig and Denhardt, 1991; Hijiya *et al.*, 1994; Crosby *et al.*, 1995); however, additional, or alternative, exons are sometimes expressed because more than one mRNA species has been observed in Northern analyses. In the case of human OPN, alternative splicing may produce isoforms (Young *et al.*, 1990; Crivello and Delvin, 1992; Parrish and Ramos, 1997). Functional difference among the isoforms, as well as the difference in the expression patterns in various tissues, has not yet been clearly documented. As mentioned earlier, OPN has been identified in many tissues, and in these tissues this molecule could mediate communication between cells. Thus, OPN could be regarded as a cytokine. Modification and/or splicing would allow more opportunities for this molecule to function differently under particular conditions, thus contributing to the specificity of the signaling between cells.

OPN interacts with the molecules constituting bone matrix. Proteins in the bone matrix are 90% type I collagen and 10% a variety of noncollagenous proteins. OPN is known to bind covalently to fibronectin via transglutamination, and transglutamination of OPN increases its binding to collagen (Beninati *et al.*, 1994; Kaartinen *et al.*, 1999). Other molecules, such as bone sialoprotein (BSP), also bind covalently to type I collagen. Osteocalcin suppresses trans-glutaminase-catalyzed cross-linking of OPN (Kaartinen *et al.*, 1997). Such a network and mutual regulation among matrix proteins in bone may facilitate conformational changes of the molecules and hence could add additional functions or activation/inactivation switches to the molecules depending on the sites and composition of the interaction between the molecules. So far, however, whether such covalent bonding between matrix proteins and OPN or other noncollagenous molecules plays any role in the physiological maintenance of bone during the remodeling cycle or in pathological situations such as osteoporosis or osteopenia requires further elucidation of the functional aspects of this interaction.

Another unique structural property of OPN is a run of 10–12 aspartic acid residues. This motif gives rise to a localized high negative charge that may be important for the binding of OPN to bone mineral. OPN has a strong affinity to calcified matrix, such as bone, and also to pathological calcifications, such as those seen in sclerotic glomeruli and atherosclerosis. The high affinity of OPN for calcium has been suggested to modulate the nucleation of calcium phosphate during mineralization (Boskey, 1995; Contri *et al.*, 1996; Srivatsa *et al.*, 1997; Sodek *et al.*, 2000b); however, initial studies on the OPN-deficient mouse failed to indicate the presence of any major defect in mineralization (Rittling *et al.*, 1998). Possibly, the role of OPN in bone mineralization is compensated for by other regulatory systems for mineralization.

The molecular conformation of OPN may be altered by the binding of several molecules (Jain *et al.*, 2002). In the case of calcium, it is in a manner dependent on the concentration of the Ca^{2+} ion. It has been proposed that depending on such calcium ion-dependent conformational changes, OPN may reveal binding motifs such as the RGD sequence to its cognate receptors or to any other interactive extracellular matrix protein (Singh *et al.*, 1993; Bennett *et al.*, 1997). The dependence of the structure of OPN on the calcium concentration is an attractive feature of this molecule with regard to modulation of its function, e.g., during bone resorption by osteoclasts. For instance, when osteoclasts resorb bone, there are significant changes in the calcium concentration in the secondary lysosome-like closed space underneath the resorbing osteoclast. This calcium concentration may render signals through the calcium-sensing receptor (CasR), a seven membrane-spanning type receptor (Kameda *et al.*, 1998; Kanatani *et al.*, 1999). In addition to such calcium signaling, OPN may change its conformation depending on the calcium levels, thereby affecting cell function through its binding to the receptors expressed on the surface of the osteoclasts, such as $\alpha_v\beta_3$. However, it remains to be seen whether there is any functional significance of putative calcium-dependent structural changes in the OPN molecule.

OPN was first found as a secreted protein, and consequently one of the many names that have been given to it is secreted phosphoprotein (SPP) (Denhardt and Guo, 1994). In fact, when osteoblast-like cells such as ROS17/2.8 cells were stained for OPN protein, no major signal can be observed inside the cells. However, OPN mRNA expression in these cells and protein expression in the medium were easily detectable and were regulated by calciotropic agents and cytokines (Noda *et al.*, 1990; Farrington *et al.*, 1998; Hullinger *et al.*, 2001; Shi *et al.*, 2001; Shen and Christakos, 2005). A major part of the OPN protein moves out of the cell immediately after its synthesis. However, the presence of an intracellular form of OPN has been shown (Zohar *et al.*, 1998, 2000).

Whether the intracellular form of OPN is different from other forms of OPN with regard to alternative splicing or post-translational modifications is not known. The intracellular form of OPN was co-localized with CD44 in extensions of the osteoclasts known as podosomes, but not in the perinuclear regions where BSP has been observed (Suzuki *et al.*, 2000). Therefore, CD44 and OPN as well as the co-localized $\alpha_v\beta_3$ receptors probably form a complex that facilitates osteoblast movement. As osteoblasts produce OPN and $\alpha_v\beta_3$, migration of osteoblasts may also be dependent on the intracellular form of OPN, CD44, and/or $\alpha_v\beta_3$ integrins (Suzuki *et al.*, 2000). Like CD44, OPN may promote the multinucleation of osteoclast precursors because it was observed in OPN-null mice thatmononuclear cells are more abundant than multinucleated cells, similar to the situation in the CD44-deficient mouse. Migration experiments conducted *in vitro* using the Boyden chamber system indicated that the presence of OPN is required for efficient migration through the membrane pores. Furthermore, this migration was dependent on the presence of ezrin and hyaluronan. OPN also acts after its binding to $\alpha_v\beta_3$ integrins through Rho to stimulate gelsolin-associated phosphatidylinositol 3-kinase activity, podosome assembly, stress fiber formation, osteoclast motility, and bone resorption (Chellaiah *et al.*, 2000c).

RECEPTORS FOR OPN

OPN binds to $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_4\beta_1$, α_5, and $\alpha_9\beta_1$ integrins (Denhardt and Noda, 1998; Duong *et al.*, 2000; Zheng *et al.*, 2000; Barry *et al.*, 2000). With regard to CD44 binding to OPN, the domain in OPN that interacts with CD44 is not the glycine-arginine-aspartic acid-serine (GRGDS) motif (Katagiri *et al.*, 1999). It has been found that some melanoma cells bind in a non-RGD dependent manner to the v6/v7 isoform of CD44. During the interaction, CD44 may bind to another cell surface molecule, integrins β, and that association may in turn provide the optimal interaction for CD44 to OPN (Katagiri *et al.*, 1999). Details of the interaction of CD44 with OPN remain to be characterized. The presence of additional cell receptors, the various isoforms of CD44, and variable post-translational modifications (phosphorylation and glycosylation) of OPN and CD44 are all complicating factors.

As OPN binds to the $\alpha_v\beta_3$ integrin, this integrin is considered to be responsible for major signals in response to the binding of OPN (Miyauchi *et al.*, 1991; Zimolo *et al.*, 1994). Postreceptor signaling via the $\alpha_v\beta_3$ integrin is dependent on the cellular background (Zheng *et al.*, 2000). In addition to regulating osteoclastic activity, OPN binding to the $\alpha_v\beta_3$ integrin activates osteoprotegerin expression and protects endothelial cells from apoptosis (Malyankar *et al.*, 2000).

Studies on β_3-deficient mice are relevant to understanding at least part of the function of OPN. Cells prepared from the bone marrow of β_3 knockout mice were able to differentiate into osteoclasts with efficiencies similar to the wild type (McHugh *et al.*, 2000). This observation indicates that $\alpha_v\beta_3$ is not required for osteoclast formation. The β_3 knockout mice were relatively normal while they were young but they revealed osteosclerosis radiographically by four months, suggesting that aging is one of the factors that reveals a phenotype in these mice (again, somewhat similar to OPN-deficient mice). Also, similar to OPN-deficient mice, there was a 3.5-fold increase in osteoclast number, which would appear to compensate for the mild hypocalcemia in the mice. Osteoclasts developed from the bone marrow cells of these mice were less efficient than wild-type cells in excavating pits on dentin slices, showing some inability to resorb bone, again similar to osteoclasts derived from OPN-deficient mice. The difference appears to reside in the cytoskeleton, which is abnormal in the β_3 knockout mice, suggesting a defect in intracellular signaling compared to osteoclasts derived from OPN-deficient mice where formation of the cytoskeleton and actin rings appeared to be normal when the osteoclasts were developed by culturing in the presence of RANKL and M-CSF (McHugh *et al.*, 2000; Ihara *et al.*, 2001). Overall, these findings on β_3 knockout mice further support the notion that the OPN signaling integrin through β_3 integrin pathway is important in regulation of osteoclastic activities.

OSTEOPONTIN AND CELL ATTACHMENT

OPN promotes the attachment of fibroblasts to plastic or glass substrates or other substrate (Somerman *et al.*, 1988; Reinholt *et al.*, 1990; Helfrich *et al.*, 1992; Cheng *et al.*, 2001; Standal *et al.*, 2004; Lim *et al.*, 2005). In bone, OPN is expressed in osteoblasts and its expression is enhanced by vitamin D (Prince *et al.*, 1987). Osteoclasts also express OPN when they are vigorously resorbing bone in human osteoarthritis specimens (Merry *et al.*, 1993; Dodds *et al.*, 1995; Connor *et al.*, 1995) and adhere to osteopontin (Bernards *et al.*, 2007). Osteoclasts express $\alpha_v\beta_3$ integrin at high levels (Horton *et al.*, 1995; Duong *et al.*, 2000). Although the $\alpha_v\beta_3$ integrin is not a specific marker of osteoclasts, monoclonal antibodies raised against osteoclasts appear to specifically visualize osteoclasts in bones due to its high abundance. Therefore, $\alpha_v\beta_3$ integrin can be used as one of the markers of osteoclasts.

Immunoelectron microscopic examination using colloidal gold particles indicated that OPN was observed underneath the clear zone of osteoclasts (Reinholt *et al.*, 1990). As clear zones are involved in the attachment of osteoclasts to the bone matrix, the location of OPN appeared to fit its hypothesized function. However, later experiments indicated that OPN may bind to $\alpha_v\beta_3$ integrin expressed on the basolateral surface of the osteoclasts and this binding also generates signals to modulate osteoclastic functions (Zimolo *et al.*, 1994; Zheng *et al.*, 2000). Therefore, including the intracellular form of OPN, osteoclasts could be regulated through more than one pathway of OPN signaling. However, it is not clear whether the intracellular form of OPN is the same in terms of its post-translational modification as the form released into the extracellular environment that binds to the cells in an autocrine or paracrine manner. With regard to osteoblasts, some reports indicated the presence of the $\alpha_v\beta_3$ integrin on the surface of osteoblasts (Gronthos *et al.*, 1997) and, therefore, there could be a certain commonality in OPN function in osteoblasts and osteoclasts.

OPN is deposited along the cement line and lamina limitans after the cessation of bone resorption by osteoclasts (McKee and Nanci, 1996). This OPN may provide a signal to the osteoblasts that are attracted to the bone resorption sites to deposit bone matrix to fill the cavity. Although OPN-deficient mice do not contain OPN in the cement lines, the bone architecture by itself was not largely different from that of wild-type mice (Rittling *et al.*, 1998; Rittling and Denhardt, 1999). Therefore, significance of the signaling by OPN deposited at the cement line or lamina limitans may be minor *in vivo*. However, when the osteoclasts were cultured individually in an *in vitro* system, the absence of OPN resulted in reduced osteoclast function, indicating a role for this molecule, at least in these cells (Chellaiah *et al.*, 2000b; Ihara *et al.*, 2001; Chellaiah *et al.*, 2003; Chellaiah and Hruska, 2003) whereas osteoclasts resorb protein-free mineral efficiently in the absence of OPN (Contractor *et al.*, 2005). In arthritis experiments, OPN's role may be related to adherion although it would depend on the model of rheumatoid arthritis (Yumoto *et al.*, 2002; Gravallese, 2003; Jacobs *et al.*, 2004; Ishii *et al.*, 2004).

OSTEOPONTIN-DEPENDENT INTRACELLULAR SIGNALING

RGD-containing molecules, such as OPN, bind to integrins on the surface of osteoclasts and induce integrin clustering (Hruska *et al.*, 1995; Rodan and Rodan, 1997; Chellaiah *et al.*, 2000a, 2000c; Duong *et al.*, 2000). This binding initiates intracellular signaling by the phosphorylation of tyrosine residues, including tyrosine 402 on PYK2. The phosphorylation of tyrosine residues leads to binding of Src via its SH2 domain, which then further increases phosphorylation of PYK2 at other sites (Duong *et al.*, 1998, 2000; Duong and Rodan, 1999). Such phosphorylation amplifies the signal, attracting other adaptor molecules to bind PYK2, thereby eliciting signals that activate cellular functions, including adhesion and cytoskeletal structure formation needed for osteoclastic actions, such as sealing zone formation and intracellular trafficking (Nakamura *et al.*, 1999). PYK2 also binds to CAS; however, this interaction is independent of tyrosine phosphorylation (on both of the two molecules).

In addition to interactions between kinases and adaptor molecules at focal adhesion sites, p21GTPase activity is also important in OPN-dependent signaling. One of the targets of rho, mDial, which appears to be involved in the formation of the actin ring, associates with gelsolin located in the podosomes of osteoclasts (Chellaiah *et al.*, 2000b). In osteoclasts isolated from OPN-deficient mice, podosome structures were similar to those in wild-type mice; however, the mDial and gelsolin association was not observed and there was a reduction in osteoclast motility in response to vitronectin (Chellaiah *et al.*, 2000b). Relative to wild-type osteoclasts, OPN-deficient osteoclasts exhibited a decrease in CD44 expression on the cell surface. This defect in the surface expression of CD44 and dissociation between mDial and gelsolin was reversed by the addition of exogenous OPN. Thus, it was suggested that OPN-deficiency induces impairment in the motility of osteoclasts by the suppression of CD44 expression on the cell surface as well as a disruption of the association between mDial and gelsolin, thereby suppressing podosome assembly. However, exogenously added OPN only stimulated the motility of osteoclasts, without correcting the depth of the pits formed on the dentin slices. Thus, the two phenomena of reduced CD44 expression and suppression of the formation of podosomes could be causing the osteoclastic cells to be hypomotile (Chellaiah *et al.*, 2000b). This may be the explanation for the inefficiency of osteoclasts in OPN-deficient mice.

PHENOTYPE OF OSTEOPONTIN-DEFICIENT MICE

Because OPN-deficient mice produced independently in two laboratories do not show any structural alterations in bones at birth and during their subsequent growth period, there does not appear to be a requirement for OPN for normal development (Rittling *et al.*, 1998; Liaw *et al.*, 1998). In these OPN-deficient mouse strains, skeletal defects were not observed, whereas altered wound healing was noted by Liaw *et al.* (1998). Several possibilities have been proposed to explain the apparent lack of major bone phenotype. The first one was that in the absence of OPN, other related molecules, such as those containing RGDS, can compensate for the missing OPN. However, in OPN/vitronectin double knockout mice, no bone abnormalities were noted (Liaw *et al.*, 1998). Another possibility could be that the T-cell-based type I immune response deficiency that results from the absence of OPN (Ashkar *et al.*, 2000) could modify the response of bone due to the alteration in the cytokine network that is involved in the maintenance of both cellular immune responses and the mineralized skeleton.

OSTEOPONTIN PLAYS A ROLE IN ESTROGEN DEPLETION-INDUCED BONE LOSS

Although compensation may account for the normal development and normal maintenance of bone in OPN-deficient mice, the difference between the absence and the presence of OPN could be overt in circumstances of accelerated bone turnover, such as osteoporosis. A mouse osteoporosis model made by the ovariectomy-induced depletion of estrogen provided clues to answer the question on the role of OPN in the regulation of bone metabolism (Yoshitake *et al.*, 1999). After ovariectomy, both wild-type mice and OPN-deficient mice exhibited a similar reduction in uterine weight within 4 weeks, suggesting that the hormonal system in OPN-deficient mice was similar to that in wild-type mice, at least in terms of the uterine response to estrogens. That the estrogen system in OPN-deficient mice is normal is suggested by the normal rate of sexual maturation and growth, as well as the normal fertility and littermate size in OPN-deficient mice.

Micro-CT analysis indicated that trabecular bone was lost and that the porosity in the epiphyseal portion of the long bones was decreased by about 60% in ovariectomized wild-type mice (Yoshitake *et al.*, 1999). Micro-CT analysis of the epiphyseal region of the long bones in sham-operated OPN-deficient mice revealed a slight increase in the trabecular bone volume compared to sham-operated wild-type mice. Fig. 1 shows that in contrast to the clear reduction in trabecular bone volume seen in wild-type mice after ovariectomy, no major reduction is observed in OPN-deficient mice. The preservation in the levels of bone volume even after ovariectomy could be due to the increase in bone formation, decrease in bone resorption, or both. Dynamic parameters for bone formation in these mice, such as bone formation rate/bone volume (BFR/BV), were increased in ovariectomized wild-type mice as shown previously. In the case of OPN-deficient sham-operated mice, the value was similar to sham-operated wild-type mice. However, no significant increase in BFR was observed in OPN-deficient mice, indicating the absence of high turnover status in bone metabolism even after ovariectomy. Similar basal levels of BFR suggest that bone formation activity in OPN-deficient mice is basically normal.

Morphological examination also supported the suppression of ovariectomy-induced bone resorption in OPN-deficient mice. The number of osteoclasts was increased about threefold 4 weeks after ovariectomy in wild-type mice. In contrast, basal levels of osteoclast number were relatively high in OPN-deficient mice and were not increased even after depletion of estrogen. The large number of osteoclasts, together with the increased bone volume in OPN-deficient mice, appears paradoxical. However, it could be due to a feedback system in the body that maintains serum calcium levels tightly by increasing osteoclast number to compensate for the reduced efficiency of the osteoclasts to resorb calcium from bone. Even with such compensation, a defect in the ability of osteoclasts to resorb bone is suggested by the relatively large trabecular bone volume in sham-operated OPN-deficient mice (Fig. 1; Yoshitake *et al.*, 1999).

OSTEOPONTIN FACILITATES RESORPTION AND ANGIOGENESIS OF ECTOPICALLY IMPLANTED BONE DISCS

Angiogenesis is important for bone resorption because osteoclast progenitors are derived from hematopoietic precursor cells. However, it is not known whether OPN promotes bone resorption by stimulating angiogenesis or by stimulating bone resorption via signaling through the bone matrix. Studies of ectopic bone (disc-shaped pieces punched out of the calvaria) implantation revealed a relationship between OPN and bone resorption associated with vascularization (Asou *et al.*, 2001). Wild-type bone

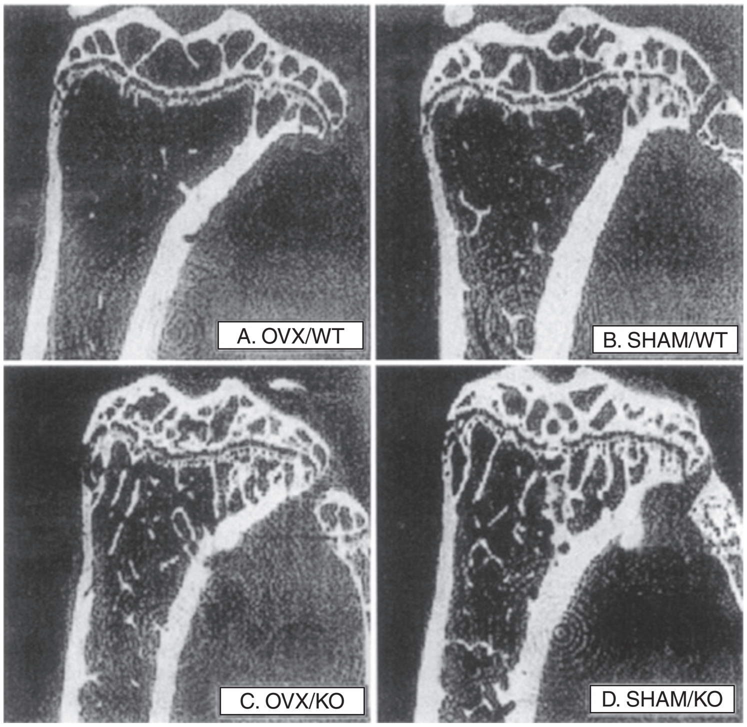

FIGURE 1 OPN deficiency suppresses ovariectomy-induced bone loss. Micro-CT images of the dissected tibia from ovariectomized and sham-operated mice, both wild-type and OPN-deficient, are shown. See Yoshitake *et al.* (1999) for further details.

implanted intramuscularly in the back of the wild-type mice was resorbed by about 25%. In contrast, bone from OPN-deficient mice implanted into OPN-deficient mice exhibited significantly less resorption (5%). Thus, about fivefold more bone was resorbed in the presence of OPN; this is illustrated in Fig. 2. The promotion of the resorption of ectopically implanted bone by OPN was associated with a larger number of osteoclasts attached to the surface of the wild-type bone than that in OPN-deficient bone. Furthermore, the number of CD31-positive vessels in the vicinity of bones implanted in OPN-deficient mice was reduced compared to the number of vessels in wild-type bones, suggesting that OPN deficiency may lead to a reduction in neovascularization of the ectopically implanted bones, and consequently a reduction in the number of osteoclasts and subsequent bone resorption efficiency. It is also possible that OPN may promote the survival of endothelial cells on the bone matrix.

Implantation of bone into muscle is suitable for the evaluation of bone resorption because of the higher vascularity of the tissues. However, detailed examination of the vascularization is difficult in intramuscular implantation experiments. Therefore, subcutaneous implantation was used to examine vascularization without making histological sections (Asou *et al.*, 2001). Significant vascularization was observed in both wild-type and OPN-deficient mice. Interestingly, the length of the blood vessels and the number of branch points in the vasculature on the surface of the implanted bone were both decreased in the OPN-deficient bone relative to wild-type bone. This observation further supports the notion that OPN facilitates vascularization of bone tissue. In these subcutaneous implantation experiments the absence of OPN reduced the TRAP-positive area on the bone disc. These observations indicate that OPN *in vivo* facilitates vascularization in association with osteoclast recruitment, thereby stimulating bone resorption.

Cross-mixing of the genotypes of implanted bone disc and host mouse indicated that when either the implanted bone disc or the host mouse was deficient in OPN, then bone resorption, as well as vascularization efficiency, was reduced to a value intermediate between wild-type and OPN-deficient mice. Angiogenic cytokines, such as FGF, upregulates OPN in epiphyseal growth plate (Weizmann *et al.*, 2005). Overall, data indicated the importance of OPN for vascularization during bone resorption. Because the growth plate in OPN-deficient mice is mostly normal it is intriguing to know how the mechanisms involved in vascularization and/or chondroclast accumulation in growth plate metabolism are different from those involved in bone resorption and the related vascularization.

PARATHYROID HORMONE-INDUCED BONE REGULATION AND OSTEOPONTIN

As ovariectomy experiments suggest that OPN-deficient mice are resistant to bone loss and ectopic bone implantation experiments indicate that these mice exhibit a reduced efficiency in bone resorption, it was suspected that OPN deficiency may cause a direct suppression of osteoclastic activity to resorb bone matrix. However, direct action of OPN in the process of bone resorption cannot be verified conclusively by ovariectomy experiments or ectopic bone resorption experiments per se. In this regard, bones in organ culture stimulated by parathyroid hormone have shown that OPN is directly responsible for bone resorption in the microenvironment of bone without influences from other humoral factors or vascularization (Ihara *et al.*, 2001). In these experiments, the release of $^{45}Ca^{2+}$ into the medium from $^{45}Ca^{2+}$-labeled forelimb bones excised from newborn OPN-deficient mice and cultured in the presence or absence of parathyroid hormone was measured.

The basal level of calcium release from organ cultures of forelimb bones of OPN-deficient mice was similar to that of wild-type bones. As reported previously, the presence of parathyroid hormone increased Ca^{2+} release from

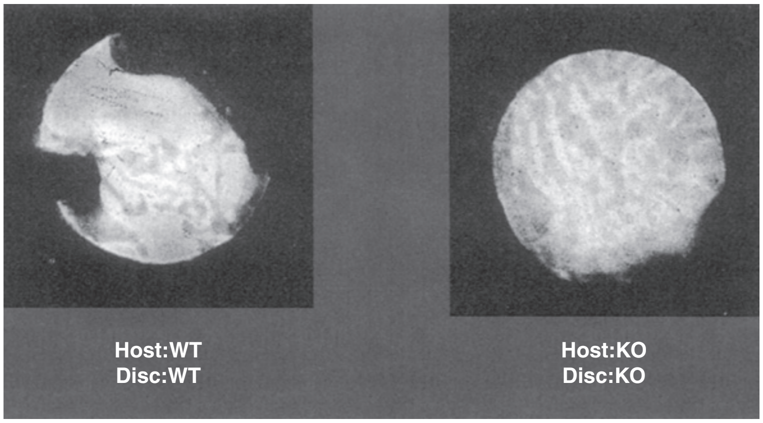

FIGURE 2 OPN is required for ectopic bone resorption. Bone discs derived from the calvaria were implanted intramuscularly as described by Asou *et al*. (2001). Four weeks after implantation, the discs were removed and examined using soft X-rays.

the cultured wild-type bones. However, as shown in Fig. 3, in the organ cultures of OPN-deficient forelimb bones, the increase in calcium release was not observed even in the presence of parathyroid hormone. Because parathyroid hormone increases osteoclast activities via stimulation of the expression of receptor activator of NFκB ligand (RANKL), soluble RANKL in combination with M-CSF was used to stimulate bone resorption. However, OPN-deficient bones failed to respond to RANKL and M-CSF, indicating that the deficiency is downstream of RANKL. Analysis of TRAP-positive cells in the cultured bones indicated that PTH treatment increased the number of these cells in wild-type bones. However, such an increase was not observed in the case of OPN-deficient bones, suggesting that the deficit resided in the inability to increase osteoclast number in the local environment of the bone rudiments. Because bone marrow cells or spleen cells taken from OPN-deficient mice were able to generate similar numbers of osteoclast-like TRAP-positive multinucleated cells with normal morphology compared to wild-type cells in culture in the presence of RANKL and M-CSF, the intrinsic ability of the progenitors to develop into osteoclasts per se is apparently not impaired in OPN-deficient mice. Actin ring formation and the distribution of Src appeared similar in osteoclasts developed in the presence of soluble RANKL and M-CSF in cultures of spleen cells regardless of the presence or absence of OPN. These observations suggest that in the absence of OPN in the microenvironment, PTH is unable to stimulate the formation of TRAP-positive cells; enabling PTH action could therefore be one mechanism by which OPN promotes bone resorption.

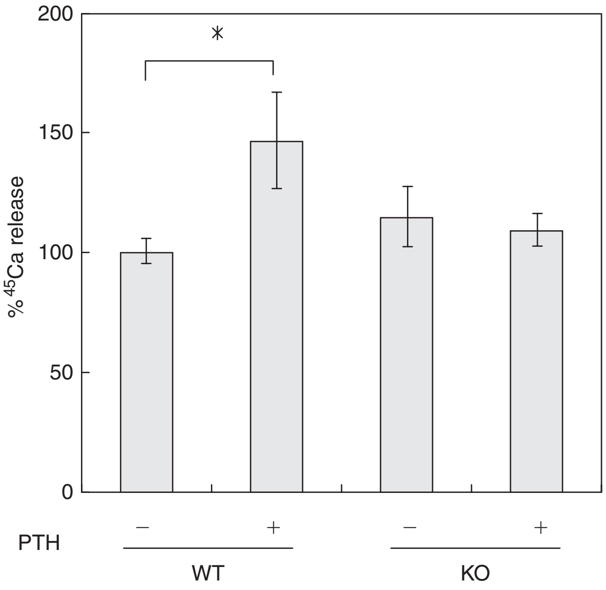

FIGURE 3 PTH fails to stimulate bone resorption in organ culture in the absence of OPN. Fetal forearm bones labeled with 45Ca^{2+} were incubated in the presence of 10–7 M parathyroid hormone. After 6 days the amount of 45Ca^{2+} in the medium was quantified. The percentage calcium released is determined relative to control cultures incubated in the absence of hormone (Ihara *et al*., 2001).

Parathyroid hormone has been used to treat severe osteoporosis patients. However, how PTH affects bones appears to be dependent on several signaling events and the full picture of the mechanism has not yet been understood. Because OPN expression is enhanced by PTH, the role of OPN in the case of PTH-induced increase in bone mass regulation was examined in the mice deficient in OPN. PTH treatment in the paradigm of intermittent administration increased bone mass levels in wild-type mice as known before. In the absence of osteopontin, however, intermittent PTH treatment further enhanced the levels of bone mass. Analysis of bone formation and bone resorption based on histomorphometry revealed that mineral apposition rate (MAR) and bone formation rate (BFR) were enhanced by PTH and this enhancement was further strengthened by the absence of OPN. Intermittent PTH treatment also enhanced the levels of osteoclast number and osteoclast surface in wild-type mice; however, the absence of OPN did not significantly alter the levels of such osteoclastic parameters in bone. Therefore, OPN acts as a negative signal to suppress the bone formation activity during PTH-induced enhancement of bone mass.

Whether such PTH-induced phenomena in the case of osteopontin deficiency were due to the PTH signal in bone or in tissues other than bone was tested by utilizing transgenic mice where constitutively active PTH/PTHrP receptor was specifically in osteoblasts using Collagen promoter. As known before, these transgenic mice exhibited high bone mass levels. When these transgenic mice were crossed with osteopontin-deficient mice, further enhancement of the increase in cancellous bone levels was observed. These data indicated that osteopontin function to suppress PTH signals was at least in part in osteoblastic compartment. Recent observations indicate that PTH signal would also be important for niche and OPN as well as osteoblasts are involved in the niche formation (Huang *et al.,* 2004; Nilsson *et al.,* 2005; Stier *et al.*, 2005).

Phosphate and mineralization-related events are also linked to OPN function as seen in *in vivo* models (Giachelli and Steitz, 2000; Steitz *et al.,* 2002; Boskey *et al.,* 2002; Harmey *et al.,* 2004; Harmey *et al.,* 2006). Osteopontin is present in bone; however, it is expressed in many other extra skeletal tissues. One of the tissues that express high levels of osteopontin is kidney. It is known that osteopontin expression in cells is enhanced by the presence of high levels of phosphate in the medium. In the body, blood phosphate levels are regulated by the function of kidney. High levels of blood phosphate could be seen in the case of chronic kidney diseases and such condition could lead to secondary hyperparathyroidism and resulting renal osteodystrophy, suggesting a link between OPN and phosphate *in vivo*. The role of osteopontin in such high phosphate-induced bone loss was tested in osteopontin-deficient mice. When the wild-type mice were fed a high phosphate diet, phosphate levels in the urine increased and high levels

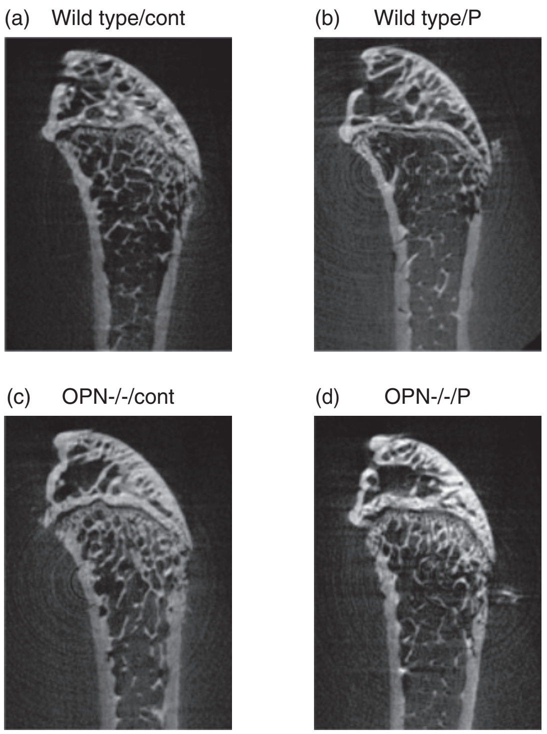

FIGURE 4 High phosphate diet-induced bone loss was prevented by osteopontin deficiency (Koyama *et al*., 2006).

of PTH were observed in association with resulting bone loss as known before. In the absence of osteopontin, however, although the levels of phosphate in the urine were high and PTH levels were also elevated, bone loss was not observed. Analysis based on histomorphometry indicated that a high phosphate diet induced an increase in the levels of osteoclast number and osteoclast surfaces were no longer observed in the absence of osteopontin. On the other hand, bone formation parameters were not significantly altered. These observations indicated that in the case of a high phosphate diet, osteopontin is highly expressed in kidney and ends up with the increase of osteoclastic activity to reduce bone due to the elevated levels of PTH. Thus, osteopontin could act as the signal to link the function of kidney to the downstream organs including bones Fig. 4; (Koyama *et al.,* 2006).

OSTEOPONTIN AND METASTATIC DISEASE

Many types of tumor cells express OPN. OPN levels in the serum of patients bearing highly metastatic tumors have been known to be high (Koeneman *et al.*, 1999; Liaw and Crawford, 1999; Carey *et al.*, 1999; Goodison *et al.*, 1999; Hotte *et al.,* 2002; Wai and Kuo, 2004; Cook *et al.,* 2006; Ramankulov *et al.,* 2007). It has been suggested that tumor cells produce OPN that could protect tumor cells themselves against the attack by macrophages by suppressing their production of nitric oxide (NO), which can kill tumor cells (Hwang *et al.*, 1994; Denhardt and Chambers, 1994; Feng *et al.*, 1995; Rollo *et al.*, 1996). Another possibility is that OPN may be a positive factor for the attachment of tumor cells and/or may promote proliferation of the tumor cells. Interestingly, in the absence of OPN, the bone loss associated with tumor growth at bone sites proceeds rapidly despite the osteoclast defects in OPN-deficient mice (Natasha *et al.,* 2006). Melanoma cells are known to be highly metastatic, and the prognosis of patients bearing those tumors is poor. Bone is one of the sites of melanoma tumor metastases. Once tumor cells metastasize to bones, the mass due to the growth of the tumor causes severe pain and eventually destroys bone tissue, resulting in debilitating fractures. Thus, elucidation of the role of OPN in the process of tumor metastasis is important.

B16 murine melanoma cells attached more effectively to a culture dish coated with recombinant OPN than with glutathione *S*-transferase (GST) control, while their proliferation was not affected by the presence of OPN (Nemoto *et al.*, 2001). The β_4 integrin and CD44 were detected in B16 melanoma cells, consistent with a previous report that these molecules may be involved in B16 cell attachment. An experimental metastasis assay based on the injection of B16 melanoma cells via an intracardiac route revealed a reduction in the number of melanoma tumors in the bones (5.4 ± 1.7) of OPN-deficient mice compared to the number in wild-type mice (11.5 ± 2.5). Injection of B16 cells into the left ventricle, which also gives rise to metastasis in nonskeletal tissues, yielded 6.5 ± 2.8 tumors in the adrenal glands of OPN-deficient mice and 17.8 ± 5.5 in wild-type animals. In the liver, the number of melanoma metastasis was 102.6 ± 53.0 for wild-type mice and 62.2 ± 32.2 for OPN-deficient mice although the difference was not statistically significant (Nemoto *et al.*, 2001).

As reported previously, a different injection route for experimental metastasis ends up with the different efficiency of metastasis to the different tissues in OPN-deficient mice. When B16 melanoma cells were injected via the femoral vein, most of the metastases were found in the lung. The number of lung metastases in OPN-deficient mice was 37.8 ± 11.4, whereas the number in wild-type mice reached up to 126.7 ± 42, again indicating suppression by the absence of OPN ($p < 0.05$). Overall, these experimental metastasis data clearly indicated that the presence of OPN promotes the metastasis of B16 melanoma cells to bone as well as to soft tissues such as lung regardless of the route of injection. In B16 cells, expression of OPN per se was very low and hardly detectable by Northern blot analyses compared to MC3T3-E1, an osteoblastic cell line that expresses high levels of OPN. Although the possibility that B16 melanoma cells still produce sufficient OPN to contribute to the metastatic process cannot be excluded, the clear difference in the number of experimental metastases seen in wild-type host animals compared to OPN-deficient host animals indicates that at least the presence of OPN in the host makes a difference in the metastatic process (Nemoto *et al.*, 2001).

Metastasis of a tumor in an animal is a complex event initiated by the detachment of the cells from the primary tumor, followed by invasion into the vasculature (or lymphatics) and movement to other locations in the body where the tumor cells extravasate and establish themselves at a new site. Proliferation of the cells at that site and vascularization of the resulting tumor by the host animal produce an expanding tumor mass. Experimental metastasis by injection into the vascular system does not test the first steps in this process, steps that may also involve OPN. Even in the latter part of the process, how OPN functions to promote metastasis remains to be elucidated. Among vascular tissues, endothelial cells express $\alpha_v\beta_3$ integrin. Thus, vascularization may be one of the steps affected by the absence of OPN. Unfortunately, our injection model did not allow us to examine the vascularization process because the animals started to die before the tumor foci were large enough to see an effect of vascularization. Within the limit of this model, it appears that OPN can promote the metastasis of tumor cells to various skeletal and nonskeletal sites. It is known that the efficiency of metastasis can vary depending on the tumor cells and tissues. In fact, implantation of B16 cells in bone could reveal the importance of OPN (Ohyama *et al.*, 2004). The reduction of melanoma metastases in OPN-deficient mice observed in bone and lung suggests the involvement of a common mechanism operating in both tissues, for instance, possibly based on host macrophages. When tumor cells invade, host stromal cells have been suggested to produce OPN to attract macrophages, which in turn may suppress tumor formation (Crawford *et al.*, 1998). OPN also promotes migration of melanoma cells (Hayashi *et al.*, 2007). Our data suggest that OPN may be required in the initial attachment phase when the tumor cell is colonizing a new site.

ROLE OF OSTEOPONTIN IN MEDIATING MECHANICAL STRESS

OPN is expressed in cells of the osteoblastic lineage, and possibly those including osteocytes (Noble and Reeve, 2000), which are exposed to mechanical stress (Terai *et al.*, 1999). Because chondrocytes express receptors for cell attachment molecules, i.e., $\alpha_v\beta_3$ integrins, they are also candidates for the perception of mechanical stress *in vivo* (Loeser, 2000). In addition, proximal kidney tubules express OPN in response to renin–angiotensin following mechanical stimulation, such as cell stretch. When an antisense oligonucleotide was introduced to block angiotensinogen or angiotensin 2 type I receptor expression, there was a significant decrease in OPN mRNA expression compared to unstretched cells (Ricardo *et al.*, 2000).

Smooth muscle cells also express osteopontin and respond to mechanical stress. Pulsatile pressure increases the proliferation of differentiated smooth muscle cells; in contrast, cells expressing low levels of smooth muscle cell differentiation markers exhibit decreased cell growth and decreased MAPK signaling in response to the mechanical stress (Cappadona *et al.*, 1999).

Integrin-binding forces in intact cells have been measured by using atomic-force microscopy. In cells attached to hexapeptides, 32–97 pN were measured. In contrast, for larger molecules such as OPN and BSP, the experiments showed different binding affinities. Therefore, the context of the RGD sequence has considerable influence on the final binding strength of the receptor interaction (Lehenkari and Horton, 1999). OPN may affect nanomechanics in bone (Kavukcuoglu *et al.*, 2007).

In terms of tooth movement, OPN has been studied in rodent and human tissues (Lao *et al.*, 2006; Fujihara *et al.*, 2006; Wongkhantee *et al.*, 2007; Chung *et al.*, 2007; Aguiar and Arana-Chavez, 2007). Only 3.3% of the osteocytes in the interradicular septum of rats expressed OPN in the absence of the pressure. However, upon the application of pressure, the number was increased to 87.5% within 48 hours after initiation of the tooth movement. This movement was followed by a seventeen-fold increase in the number of osteoclasts on the pressure side. These responses were inhibited upon injection of an RGD peptide (Terai *et al.*, 1999). In another model of bone stress, distraction osteogenesis, chondrocyte-like cells in the osteotomized area expressed OPN, osteocalcin, and alkaline phosphatase; this region also includes many osteoblastic cells and preosteoblastic cells that are also expressing OPN at the boundary between fibrous tissue and new bone. The levels of OPN, osteonectin, bone matrix Gla protein, and osteocalcin mRNA expression were enhanced remarkably by the distraction force (0.25 mm/12 hours) (Sato *et al.*, 1998). Osteopontin has been known to be expressed in the alveolar bone and expression of osteopontin was enhanced along with the pressure application to the tooth in an experimental setting. Osteopontin levels in the bone-facing tooth are enhanced upon pressure application to the tooth. Osteopontin signal in the traction side was observed in the existing alveolar bone mainly on a cement line; however, osteopontin signal was not observed in the newly formed bone that appeared after the application of pressure to the tooth. This observation also coincided with the observation that osteopontin may play a role in suppression of bone formation. Thus, the presence and absence of osteopontin appear to be functionally related to bone remodeling in alveolar bone upon the movement of tooth (Fig. 5; Chung *et al.*, 2007).

In chondrogenic cells, induction of OPN expression by mechanical stress was found to be dependent on integrin receptors because OPN expression and the response to mechanical stimuli were blocked by the absence of

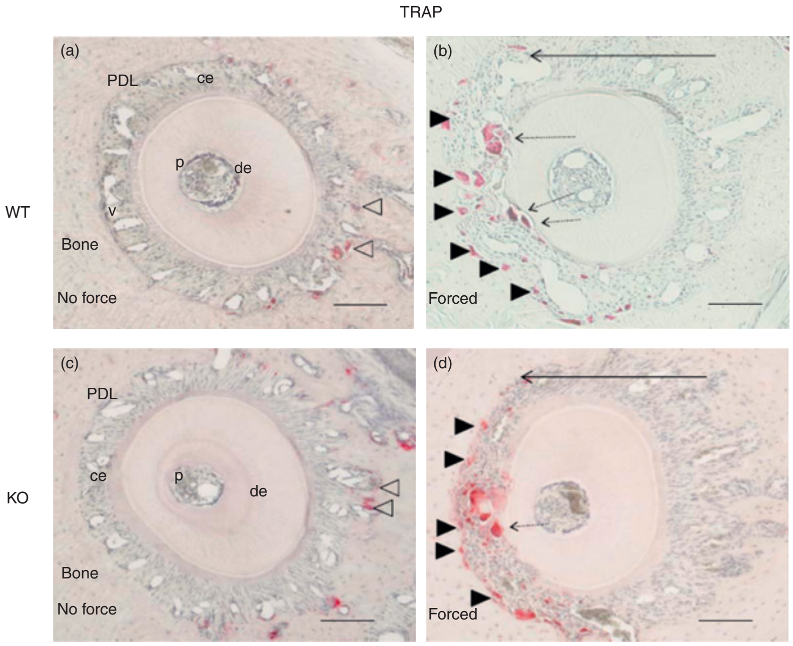

FIGURE 5 Odontoclast number, as well as tooth root resorption, were reduced in the absence of osteopontin in mouse tooth movement model. See Chung *et al*. (2007) for further details. (See plate section)

fibronectin, and by the presence of an RGD competitor (Carvalho *et al.*, 1998). These data indicate that osteopontin expression is enhanced in response to mechanical stimuli and that it is mediated by certain integrins recognizing fibronectin.

As mentioned, OPN expression is regulated by mechanical stress, whereas OPN itself is also involved in bone resorption, and possibly in bone formation. These observations suggest that OPN plays one or more roles during metabolic changes in response to mechanical loading. The question of whether OPN is involved in the mechanical stress-mediated regulation of bone metabolism was tested in a tail suspension model, which is one of the representative models to study unloading effects on bone metabolism (Vico, 1998; Vico *et al.*, 1998, 2000; Bikle and Halloran, 1999; Marie *et al.*, 2000). Unloading causes suppression of bone formation in growing rodents; however, it is not clear whether the bone resorption side is affected by tail suspension-based unloading in rodents.

To examine the role of OPN in regulation of bone loss induced by unloading, OPN-deficient mice were subjected to tail suspension. Micro-CT analysis of the metaphyseal region of the long bones indicated an increase in sparsity in wild-type mice after unloading as expected. However, no such increase in sparsity was observed in OPN-deficient mice. Quantification of the fractional trabecular bone volume indicated about 50% reduction in the wild-type animals. In contrast, no such reduction in bone volume was observed in OPN-deficient mice (Ishijima *et al.*, 2001). Biochemically, a reduction in bone volume was reflected by the increase in the bone resorption marker deoxypyridinolin, which is secreted in the urine of the mice. In wild-type mice, deoxypyridinolin secretion was increased. However, no such increase was detected in the tail-uspended OPN-deficient mice, indicating that systemic bone resorption due to tail suspension was suppressed in OPN-deficient mice.

The cellular basis for the alteration in unloading-induced bone loss in OPN-deficient mice was revealed by histomorphometric analysis. As expected, the number of osteoclasts was increased by about 150% in wild-type mice; this increase was not observed in OPN-deficient mice. In parallel to the number of osteoclasts, the osteoclast surface was also increased in wild-type but not in OPN-deficient mice. This inability of osteoclasts to respond to unloading may be due to a defect in the signaling system to support osteoclastogenesis. However, *in vitro* osteoclastogenesis experiments using RANKL and M-CSF indicated that TRAP-positive multinucleated cell formation was similar regardless of unloading or loading and/or difference in genotypes. Thus, suppression of the response to unloading in the case of osteoclastogenesis in OPN-deficient mice would be due to the signaling prior to osteoclastogenesis, as the intrinsic ability for osteoclastogenesis in the precursor cells per se does not seem to be impaired (Ihara *et al.*, 2001). Therefore, certain extracellular signaling could be lost in OPN-deficient mice. This possibility was also suggested by the analysis of the osteoblastic cells.

Osteoblasts are regarded as central players in regulating bone metabolism because they express receptors for parathyroid hormone, prostaglandins, and vitamin D—all major humoral factors that regulate bone metabolism. In addition, they are thought to be the cells that respond to mechanical stress. Analysis of osteoblastic activity in tail-suspended mice indicated suppression of bone formation rate as well as mineral apposition rate in wild-type mice, as has been reported previously. However, a reduction in the values of these two parameters of osteoblastic bone formation did not occur when OPN-deficient mice were subjected to unloading by tail suspension (Ishijima *et al.*, 2001). Because reduction of bone formation was not affected in experiments where bisphosphonate was administered to tail-suspended animals to block bone resorption due to unloading, the two phenomena appear to be regulated independently; alternatively, the bone resorption aspect could be downstream of the bone formation aspect. However, this may not be the case as increase in bone could be observed earlier than resorption after tail suspension relative to alterations in bone formation.

The inability of osteoblasts to respond to unloading in OPN-deficient mice suggests that OPN is involved in mediating the signaling induced by tail suspension to suppress the function of osteoblastic cells. This suppression, in turn, could give another signal to increase osteoclastic activities during the loss of bone mass in tail-suspended mice. Bone resorption due to unloading occurs immediately after the exposure of animals and human to unloading conditions. However, it is not known whether such an early response of the osteoclasts to unloading is a direct or indirect phenomenon. Because osteoblastic activity, i.e., bone formation, can be detectable morphologically only after a relatively long stimulation period (a week or two) compared to bone resorption, and because the bone formation rate, though slow to change, is the most reliable marker for bone formation *in vivo*, it is possible that initial signals elicited by osteoblasts in the early period of time after exposure to loading and/or unloading have not yet been recognized by the current techniques.

To obtain further insight into the mechanism of the effects of mechanical stress, events occurring immediately after loading or unloading must be investigated by using methodology suitable to detect small changes in the metabolism or to detect signals elicited by the cells in the local environment. By such analyses it is still to be elucidated whether the absence of OPN by itself could impair sensing of the mechanical stress in the case of OPN-deficient mice directly in the osteoblasts or indirectly by prohibiting osteoblastic cells via producing other possible loading-induced signals or by delivering the molecular messenger molecules to activate osteoclasts in response to unloading. Analyses of the events that are taking place at the interface between cells and extracellular components, as well as in the intracellular signaling process resulting in modified gene expression or protein function in the early period of unloading in the case of OPN-deficient mice, may yield clues to long-standing questions regarding how bone mass is lost in response to unloading. Studies have suggested that adherent bone cells unable to synthesize OPN tended to have a defect in their ability to respond to a fluid flow stimulus or orthodontic force (Ishijima *et al.*, 2000; You *et al.*, 2001; Ishijima *et al.*, 2002; Morinobu *et al.*, 2003; Denhardt *et al.*, 2005; Ishijima *et al.*, 2006; Ishijima *et al.*, 2007). Prostaglandins induced by mechanical stress may also be important as EP4 agonist action in bone is modulated by OPN (Kato *et al.*, 2007).

The immune system has been known to be involved in the regulation of bone metabolism. Many cytokines whose functions are critical for the immunoregulatory system also play a role in the regulation of bone resorption and bone formation. It is intriguing to note that tail suspension is used as a disuse model but it is also used as a stress model. Three days tail suspension reduces body weight as well as spleen and thymus weight. In the absence of osteopontin, such reduction of body weight and atrophy of spleen and thymus were less evident. In addition T and B cells were reduced by tail suspension in wild-type but not in OPN-deficient mice. This change is associated with the tail suspension-induced increase in corticosterone and the increase in apoptotic cells in the spleen and thymus in the wild type and these changes were not observed in osteopontin-deficient mice. Whether such changes in the immune system were relevant to OPN effects on bone is still to be determined (Wang *et al.*, 2007).

SUMMARY

As reviewed in the chapter, OPN plays a critical role in the maintenance of bone, especially as a molecule involved in the response of bones to external stress. It is also involved in other homeostatic defense mechanism in the mammalian organism. Further investigations are required to elucidate the molecular mechanisms of OPN action in mediating responses to inflammation, mechanical stress, angiogenesis, and accelerated bone resorption. Understanding the pathways of OPN signaling will contribute to the development of novel measures to cure patients suffering from many bone diseases and other afflictions in our aging modern society.

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Chapter 19

Bone Proteinases

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INTRODUCTION

This chapter surveys our knowledge of the proteinases expressed in bone. Although previously the osteoclast was considered to be the main producer of proteinases in bone, it has become increasingly clear that osteoblasts play a significant role in the production of many of these proteinases. For example, it is true that the osteoclast secretes abundant lysosomal cysteine proteinases, especially cathepsin K (Vaes, 1988; Xia *et al.*, 1999; Sahara *et al.*, 2003) and produces some of the neutral proteinases, e.g., matrix metalloproteinase-9 (MMP-9; Wucherpfennig *et al.*, 1994; Delaissé *et al.*, 2000). However, osteoblasts, like their related cells fibroblasts, are able to secrete a host of proteinases, including neutral proteinases such as serine proteinases, plasminogen activators, and metalloproteinases such as collagenase-3, as well as lysosomal proteinases, e.g., cathepsins. Thus, osteoblasts, like fibroblasts, have the capacity to not only synthesize a range of matrix proteins, including type I collagen, but also have the ability to remodel their own extracellular matrix by the secretion of a range of proteinases.

Proteinases can be classified into four groups: metalloproteinases e.g., collagenase-3; serine proteinases, e.g., plasminogen activator; cysteine proteinases, e.g., cathepsin K; and aspartic proteinases, e.g., cathepsin D. This subdivision is based on the structure and the catalytic mechanism of the active site involving particular amino acid residues and/or zinc. In the following review of the proteinases synthesized in bone, we deal with each group according to this subdivision in the order just given. For some, much more is known than for others and they have warranted their own section.

METALLOPROTEINASES

Matrix metalloproteinases (MMPs) are an important group of neutral proteinases thought to be involved in bone growth and bone remodeling. According to structural and functional characteristics, human MMPs can be classified into at least six different subfamilies of closely related members: collagenases (MMP-1, -8, -13, and -18), type IV collagenases (gelatinases, MMP-2 and -9), stromelysins (MMP-3, -10, and -11), matrilysins (MMP-7 and -26), membrane-type MMPs (MT-MMPs, MMP-14, -15, -16, -17, -24, and -25), and other MMPs (MMP-12, -19, -20, and -27) (Matrisian, 1992; Vu and Werb, 2000). All matrix metalloproteinases are active at neutral pH, require Ca^{2+} for activity and contain Zn^{2+} in their active site. The catalytic domain of MMPs contains the conserved sequence HEXGH, which is believed to be the zinc-binding site. Metalloproteinases are secreted or inserted into the cell membrane in a latent form caused by the presence of a conserved cysteine residue in the prosegment, which completes the tetrad of zinc bound to three other residues in the active site. Cleavage of this propiece by other proteolytic enzymes (e.g., trypsin, plasmin, cathepsins, MT-MMPs, or other unknown activators) causes a loss of ~10 kDa of the pro-piece; this disrupts the cysteine association with the zinc and results in a conformational change in the enzymeyielding activation. Metalloproteinases all have homology to human fibroblast collagenase (collagenase-1, MMP-1) and all are inactivated by tissue inhibitors of metalloproteinases (TIMPs) (Varghese, 2006). It should be noted that MMPs, originally characterized

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and named "gelatinases," clearly function as collagenases *in vitro* and could potentially function as collagenases *in vivo*. MMP-2, for example, when free of TIMPs, cleaves native collagens, to yield the typical 3/4 to 1/4 fragments (Ames and Quigley, 1995; Seandel *et al.*, 2001).

Activation of MMPs can occur via the plasminogen activator/plasmin pathway. Plasminogen activators convert plasminogen to plasmin, which subsequently can activate prostromelysin to stromelysin and procollagenase to collagenase. The activated MMPs can then degrade collagens and other extracellular matrix proteins. MT-MMP is necessary for MMP-2 activation in fibroblasts (Ruangpanit *et al.*, 2001), and MT-1 MMP mediated MMP-2 activation is important for invasion and metastasis of tumors (Mitra *et al.*, 2006).

Apart from the regulation of secretion, activation, and/or inhibition, MMPs are substantially regulated at the transcriptional level (Matrisian, 1992; Crawford and Matrisian, 1996). Several MMPs contain specific regulatory elements in their promoter sequences. Human and rat stromelysin-1 and -2 contain activator protein-1 (AP-1) and polyoma enhancer activator-3 (PEA-3)-binding sites that may be important for basal levels and inducibility. AP-1 and PEA-3 consensus sequences have also been found in human, rabbit, and rat collagenase genes (Brinckerhoff, 1992; Selvamurugan *et al.*, 1998; Tardif *et al.*, 2004). The transcription factors Fos and Jun form heterodimers and act through the AP-1 sequence (Lee *et al.*, 1987; Chiu *et al.*, 1988), whereas c-*ets* family members bind at the PEA-3 sequence (Wasylyk *et al.*, 1993). The urokinase plasminogen activator gene also contains AP-1 and PEA-3-binding sites and, as a result, agents acting through these sites could lead to coordinate expression of many of these genes (Matrisian, 1992; Hsieh *et al.*, 2006). Moreover, TIMP-1 is controlled by AP-1 transcription factor in the brain, along with a role for AP-1 in regulation of the neuronal MMP-9 gene (Kaczmarek *et al.*, 2002). Glucocorticoids and retinoids can suppress metalloproteinase synthesis at the transcriptional level (Brinckerhoff, 1992) by forming a complex with AP-1 transcription factors and inhibiting their action (Schroen *et al.*, 1996).

A second transcription factor-binding site was identified in the MMP-13 (collagenase-3) promoter as well as in bone-specific genes such as osteocalcin (Shah *et al.*, 2004; Selvamurugan *et al.*, 1998). This site is referred to as the runt domain (RD)-binding site or polyomavirus enhancer-binding protein-2A/osteoblast-specific element-2/nuclear matrix protein-2 binding site (Geoffroy *et al.*,1995; Merriman *et al.*, 1995). Members of the core-binding factor (CBF) protein family (renamed Runx by the Human Genome Organization), such as the osteoblastic transcription factor, Runx2 (Cbfa1), bind to these RD sites (Kagoshima *et al.*, 1993). Runx proteins are capable of binding to DNA as monomers, but can also heterodimerize with CBFbeta, a ubiquitously expressed nuclear factor (Kanno *et al.*, 1998). Runx2 is essential for the maturation of osteoblasts, and targeted disruption of the Runx2 gene in mice produces skeletal defects that are essentially identical to those found in human cleidocranial dysplasia (Banerjee *et al.*, 1997; Ducy *et al.*, 1997; Mundlos *et al.*, 1997; Otto *et al.*, 1997).

Stromelysin

Stromelysin-1 (MMP-3) degrades fibronectin, gelatin, proteoglycans, denatured type I collagen, laminin, and other extracellular matrix components (Chin *et al.*, 1985). Mesenchymal cells, such as chondrocytes and fibroblasts, are commonly found to secrete stromelysin-1 (Matrisian, 1992). Transin, the rat homologue of human stromelysin, was originally discovered in fibroblasts transformed with the polyoma virus (Matrisian *et al.*, 1985). One importance of stromelysin comes from its implication in the direct activation of procollagenases, including MMP-1, -8, -9, and -13 (Murphy *et al.*, 1987; Knauper *et al.*, 1993), and the enzyme is thought to play a role, together with collagenases, in the destruction of connective tissues during disease states (Brinckerhoff, 1992; Posthumus *et al.*, 2000). Recently, it has been identified to have a crucial role in MMP-mediated cartilage damage in osteoarthritis, as MMP-3 knockout mice were shown to have reduced MMP-mediated cartilage breakdown after induction of osteoarthritis (Blom *et al.*, 2007).

Stromelysin-1 is regulated by growth factors, oncogenes, cytokines, and tumor promoters. Epidermal growth factor (EGF) has been shown to increase stromelysin transcription through the induction of Fos and Jun, which interact at the AP-1 site in the promoter (McDonnell *et al.*, 1990). Platelet-derived growth factor is also important in the induction of stromelysin (Kerr *et al.*, 1988a). The protein kinase C activator, phorbol myristate acetate (PMA), is a notable stimulator of stromelysin transcription (Brinckerhoff, 1992; Prontera *et al.*, 1996). Transforming growth factor-β (TGF-β), however, causes an inhibition of transin (rat stromelysin) expression (Matrisian *et al.*, 1986; Kerr *et al.*, 1988b) through a TGF-β inhibitory element (Kerr *et al.*, 1990). Recent studies show that BMP-4 represses MMP-3 and -13 gene expression, but does not induce adipocyte differentiation in C3H10T1/2 cells (Otto *et al.*, 2007). IL-4 and -13 were shown to inhibit MMP-3 synthesis in human conjunctival fibroblasts (Fukuda *et al.*, 2006; Stewart *et al.*, 2007).

In bone, stromelysin-1 has been shown to be produced by normal human osteoblasts (Meikle *et al.*, 1992) after stimulation with PTH or monocyte-conditioned media (cytokine-rich). Similarly, Rifas *et al.* (1994) have shown that two human osteosarcoma cell lines (MG-63 and U2OS) secrete stromelysin and this may be increased by treatment with PMA, interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α), but these authors were not able to find the enzyme in medium conditioned by cultured normal human osteoblasts. Mouse osteoblasts and osteoblastic cell lines also produce stromelysin-1 and demonstrate enhanced expression with 1,25$(OH)_2D_3$, interleukin-1, or interleukin-6 treatment (Thomson *et al.*, 1989; Breckon *et al.*, 1999; Kusano *et al.*, 1998; Le Maitre *et al.*, 2005). There have also been reports that this stromelysin is expressed by osteoclasts (Witty *et al.*, 1992). Despite the many papers on stromelysin-1, it is not clear what its phy-siological substrate is, nor has its role in physiological and pathophysiological skeletal resorption been established. The course of experimental arthritis and bone and cartilage destruction is not altered by null mutations in the mouse stromelysin-1 gene (Mudgett *et al.*, 1998).

Type IV Collagenases (Gelatinases)

Type IV collagenases or gelatinases are neutral metalloproteinases requiring Ca^{2+} for activity and are involved in the proteolysis and disruption of basement membranes by degradation of type IV, V, and denatured collagens. There are two types of gelatinases: 72-kDa gelatinase (gelatinase A) or MMP-2 (Collier *et al.*, 1988) and 92-kDa gelatinase (gelatinase B) or MMP-9 (Wilhelm *et al.*, 1989). There are very distinct differences between the two gelatinases. The 72-kDa gelatinase has been found complexed to TIMP-2 (Stetler-Stevenson *et al.*, 1989), whereas the 92-kDa gelatinase has been found complexed to TIMP-1 (Wilhelm *et al.*, 1989). Regulation of the two gelatinases is also very distinct. Analysis of the genomic structure and promoter of the 72-kDa gelatinase has revealed that this gene does not have an AP-1 site or TATA box in the 5′ promoter region as all the other MMPs have been shown to have (Huhtala *et al.*, 1990). This enzyme is also not regulated by PMA and, in many cases, seems to be expressed constitutively rather than in a regulated fashion. In contrast, the 92-kDa gelatinase has a promoter very similar to the other MMPs and is regulated similarly (Huhtala *et al.*, 1991). Nevertheless, expression and activity of both types of gelatinase are markedly stimulated by interleukin-1 (Kusano *et al.*, 1998).

In bone, as is to be expected, MMP-2 is expressed constitutively by many osteoblastic preparations (Overall *et al.*, 1989; Rifas *et al.*, 1989, 1994; Meikle *et al.*, 1992) and is unchanged by treatment with any of the agents tested. The zymogen form of MMP-2 is also resistant to activation by serine proteases, but MT1-MMP can initiate the activation of MMP-2 by cleavage of the Asn66-Leu peptide bond (Sato *et al.*, 1994). In 2001, Martignetti *et al.* (2001) described a form of multicentric osteolysis (Winchester/Torg syndrome) with striking tarsal and carpal bone resorption, accompanied by arthropathy, osteoporosis, subcutaneous nodules, and a distinctive facies in large, consanguineous Saudi Arabian families. They localized the gene to 16q12–21 and demonstrated two homoallelic, family-specific mutations in the region that encodes MMP-2. Nonsense and missense mutations have been documented consistent with decreased levels of MMP-2 (Al-Aqeel, 2005). *Mmp2*-null mice were first reported by Itoh *et al.* (1997) to have no phenotype except for some shortening of limb bones. Subsequent work by this group documented osteoporosis in older mice as well as altered remodeling of the canalicular system with osteocyte apoptosis, fewer canaliculi and decreased canalicular connectivity (Inoue *et al.*, 2006). They also described striking defects in formation/maintenance of osteocyte networks and connectivity in the collagenase-resistant (r/r) mice; the r/r mice had previously been shown to have osteocyte and osteoblast apoptosis and prominent emptying of osteocyte lacunae (Zhao *et al.*, 2000). We emphasize, however, that although the *Mmp2*-null mice had osteoporosis the characteristic nodulosis and severe focal osteolysis of the human NAO syndrome with mutations in *MMP-2* were not found in the *Mmp2-/-* mice. Recently, Mosig *et al.* (2007) reported that *Mmp2*-null mice obtained from Itoh *et al.* (1997) and described earlier display progressive loss of bone mineral density, articular cartilage destruction, and abnormal long bone and craniofacial development. These mice had 50% fewer osteoblasts and osteoclasts compared to control littermates at 4 days, whereas there was almost no difference after 4 weeks of age. In addition, inhibition of MMP-2 via siRNA in human SaOS2 and murine MC3T3 osteoblast cell lines caused a decrease in cell proliferation rates. These findings imply that MMP-2 is critical for normal skeletal and craniofacial development, as well as bone cell growth and proliferation. Mosig *et al.* (2007) did not comment on focal osteolysis of the NAO human syndrome in the *Mmp2*-null mice they studied nor did they examine the canalicular networks using approaches similar to those of Inoue *et al.* (2006).

Mmp7-/- (matrilysia) mice were reported to have several abnormalities such as decreased intestinal tumorigenesis but no obvious skeletal defects (Wilson *et al.*, 1997). More recent work from this group demonstrated in a prostate cancer model that MMP-7 produced by osteoclasts at the tumor-bone interface has the capacity to process cell-bound RANKL to a soluble form that further promotes osteoclast activation (Lynch *et al.*, 2005). In *Mmp7*-deficient mice, there was reduced RANKL processing and reduced tumor-induced osteolysis. It appears, however, that *Mmp7-/-* mice have no physiological abnormality in physiological skeletal remodeling (i.e., no abnormality in the absence of bone metastasis).

The 92-kDa gelatinase (MMP-9) is secreted by three osteosarcoma cell lines (TE-85, U2OS, and MG-63) (Rifas *et al.*, 1994) and, in some of the cell lines, can be stimulated by PMA, IL-1β, and TNF-α, analogous to these authors' observations regarding stromelysin. Similarly, they were unable to identify secreted MMP-9 in the media of normal human osteoblasts or the human osteosarcoma cell line SaOS-2, which has been shown to have retained many characteristics of highly differentiated osteoblasts. Likewise, Meikle *et al.* (1992) found very little immunohistochemical staining for MMP-9 in normal human osteoblasts. In fact, this enzyme has been found to be highly expressed by rabbit and human osteoclasts (Tezuka *et al.*, 1994a; Wucherpfennig *et al.*, 1994; Vu *et al.*, 1998). Indeed, a lack of expression of MMP-9 in mature osteoclasts of c-*fos*-null mice may be one of the reasons the animals exhibit an osteopetrotic phenotype (Grigoriadis *et al.*, 1994). Furthermore, studies of mice with a targeted inactivation of the gene indicate that MMP-9 plays a role in regulating endochondral bone formation, particularly of the primary spongiosa, possibly by mediating capillary invasion. Mice containing a null mutation in the MMP-9 gene exhibit delays in vascularization, ossification, and apoptosis of the hypertrophic chondrocytes at the skeletal growth plates (Vu *et al.*, 1998). These defects result in an accumulation of hypertrophic cartilage in the growth plate and lengthening of the growth plate. The defects are reversible, and by several months of age the affected mice have an axial skeleton of normal appearance. It was postulated that MMP-9 is somehow involved in releasing angiogenic factors such as vascular endothelial growth factor (VEGF) that is normally sequestered in the extracellular matrix (Gerber *et al.*, 1999). Extracellular galectin-3 could be an endogenous substrate of MMP-9 that acts downstream to regulate hypertrophic chondrocyte death and osteoclast recruitment during endochondral bone formation. Thus, the disruption of growth plate homeostasis in MMP-9 null mice links galectin-3 and MMP-9 in the regulation of the clearance of late chondrocytes through regulation of their terminal differentiation (Ortega *et al.*, 2005).

Membrane-Type Matrix Metalloproteinases

Although most matrix metalloproteinases are secreted, a subtype called membrane-type matrix metalloproteinases (MT-MMPs) are inserted into the cell membrane (Sato *et al.*, 1997; Pei, 1999). These proteases contain a single transmembrane domain and an extracellular catalytic domain. Characteristically, MT-MMPs have the potential to be activated intracellularly by furin or furin-like proteases through recognition of a unique amino acid sequence: Arg-Arg-Lys-Arg111 (Sato *et al.*, 1996). To date, six MT-MMPs have been described, four transmembrane proteins and two glycosylphosphatidylinositol (GPI)-anchored ones. MT1-MMP, MT2-MMP, and MT3-MMP have been shown to have a wide range of activities against extracellular matrix proteins (Pei and Weiss, 1996; Velasco *et al.*, 2000). MT1-MMP is involved in endothelial cell migration and invasion (Galvez *et al.*, 2000; Collen *et al.*, 2003), and MT2-MMP and MT3-MMP are also involved in cell migration and invasion, depending on the cell type (Hotary *et al.*, 2000; Shofuda *et al.*, 2001). In a collagen-invasion model, MT1-MMP appears to be the critical MMP (Sabeh *et al.*, 2004).

A mouse cDNA homologue to MT4-MMP (mMT4-MMP) has been cloned (English *et al.*, 2000). MT4-MMP has the least degree of sequence identity to the other family members and has TNF-α convertase activity but does not activate pro-MMP2 (Puente *et al.*, 1996). Conversely, MT5-MMP and MT6-MMP may facilitate tumor progression through their ability to activate pro-MMP2 at the membrane of cells from tumor tissue (Llano *et al.*, 1999; Velasco *et al.*, 2000). As mentioned earlier, MT1-MMP (MMP-14) serves as a membrane receptor or activator of MMP-2 and possibly other secreted MMPs (Sato *et al.*, 1994). Furthermore, studies indicate that MT1-MMP may also function as a fibrinolytic enzyme in the absence of plasmin and facilitate the angiogenesis of endothelial cells (Hiraoka *et al.*, 1998). MT1-MMP is highly expressed in embryonic skeletal and periskeletal tissues and has been identified in osteoblasts by *in situ* hybridization and immunohistochemistry (Apte *et al.*, 1997; Kinoh *et al.*, 1996). Targeted inactivation of the MT1-MMP gene in mice produces several skeletal defects that result in osteopenia, craniofacial dysmorphisms, arthritis, and dwarfism (Holmbeck *et al.*, 1999; Zhou *et al.*, 2000). Several of the notable defects in bone formation include delayed ossification of the membranous calvarial bones, persistence of the parietal cartilage vestige, incomplete closure of the sutures, and marked delay in the postnatal development of the epiphyseal ossification centers characterized by impaired vascular invasion. Histological observation suggested that the progressive osteopenia noted in these animals may be attributed to excessive osteoclastic resorption and diminished bone formation. This finding was supported by evidence that osteoprogenitor cells isolated from the bone marrow of these mutant mice demonstrate defective osteogenic activity. MT1-MMP is also associated with osteoclast-mediated bone resorption in rheumatoid arthritis (Pap *et al.*, 2000).

COLLAGENASES

Collagenases generally cleave fibrillar native collagens I-III at a single helical site at neutral pH (Matrisian, 1992). The resultant cleavage products denature spontaneously at 37°C and become substrates for many enzymes, particularly gelatinases. The collagenase subfamily of human MMPs consists of three distinct members: fibroblast collagenase-1 (MMP-1), neutrophil collagenase-2 (MMP-8), and collagenase-3 (MMP-13) (Goldberg *et al.*, 1986; Freije *et al.*, 1994). An additional collagenase, called collagenase-4 (MMP-18), has been identified in *Xenopus laevis* (Stolow *et al.*, 1996), but a human homologue of this enzyme has not been identified. At the present time, only one rat/mouse interstitial collagenase has been studied thoroughly and shown to be expressed by a range of cells, including osteoblasts. This collagenase has a high degree of homology (86%) to human collagenase-3 and is aptly named collagenase-3 (Quinn *et al.*, 1990). Rat collagenase-3 is secreted by osteoblasts, smooth muscle cells, and fibroblasts, in proenzyme form at 58 kDa, and is subsequently cleaved to its active form of 48 kDa (Roswit *et al.*, 1983). Two murine orthologues of human collagenase-1, called murine collagenase-like A (Mcol-A) and murine collagenase-like B (Mcol-B), were first identified by nucleotide sequence similarity to human MMP-1, but only Mcol-A was able to degrade native type I and II collagens, casein, and gelatins (Balbin *et al.*, 2001). In this report, the expression of Mcol-A was limited to early embryos. A murine ortholog of collagenase-2 (MMP-2) has been identified by two groups (Lawson *et al.*, 1998; Balbin *et al.*, 1998). A role for Mcol-A, Mcol-B, or murine collagenase-2 in bone cell function has not been demonstrated, although human collagenase-2 is expressed in chondrocytes and other skeletal cells. *Mmp8*-/- mice (Balbin *et al.*, 2003) have no skeletal abnormalities during development; skeletal changes in adults have not yet been reported.

As noted previously**,** it has also been shown that other MMPs [MMP-2 (gelatinase A (GelA or 72-kDa gelatinase)), and MMP-14 (MT1-MMP)] can function as collagenases *in vitro* (Aimes and Quigley, 1995**;** Seandel *et al.*, 2003; Ohuchi *et al.*, 1997). These MMPs (-1, -2, -8, -13, and -14) all cleave each of the triple helical interstitial collagens at the same locus and therefore must also be considered to be collagenases.

Collagenase-3

In developing rat calvariae, we have found ample amounts of collagenase-3 by immunohistochemistry 14 days after birth (Davis *et al.*, 1998). These are always in select areas, mostly associated with sites of active modeling. At the cellular level, staining is associated with osteocytes and bone-lining cells that have the appearance of osteoblasts. Originally, there was controversy regarding the cellular origin of bone collagenase. The osteoclast was reported to show immunohistochemical staining for collagenase (Delaissé *et al.*, 1993), but it was not determined whether this was a gene product of the osteoclast or was, perhaps, produced by osteoblasts/osteocytes and bound by the osteoclast through a receptor (see below). However, *in situ* hybridization of 17- to 19-gestational-day rat fetal long bones showed collagenase-3 (MMP-13) expression only in chondrocytes, bone surface mononuclear cells, and osteocytes adjacent to osteoclasts; there was no evidence of expression in osteoclasts (Fuller and Chambers, 1995). Similarly, Mattot *et al.* (1995) showed expression of mouse collagenase-3 in hypertrophic chondrocytes and in cells of forming bone from humeri of mice at the 18th gestational day. In human fetal cartilage and calvaria, collagenase-3 transcripts were detected in hypertrophic chondrocytes, osteoblasts, and periosteal cells by *in situ* hybridization, whereas no expression of collagenase-3 was detected in osteoclasts (Johansson *et al.*, 1997). In addition, it has been known for some time that bone explants from osteopetrotic mice (lacking active osteoclasts) continue to produce abundant collagenolytic activity, either unstimulated or stimulated by bone-resorbing hormones (Jilka and Cohn, 1983; Heath *et al.*, 1990). These studies demonstrate that the osteoblast/osteocyte and hypertrophic chondrocytes are the source of collagenases in skeletal tissue, whereas the osteoclast does not appear to express these genes. It should also be noted that the expression of MMP-13 assayed by *in situ* hybridization was strikingly reduced (Lanske *et al.*, 1996) in the distal growth plate and midshafts of bones from PTH/PTHrP receptor -/- mouse embryos (Lanske *et al.*, 1998).

The remodeling of the fracture callus mimics the developmental process of endochondral bone formation. Excess tissue accumulates as callus prior to endochondral ossification followed by osteoclast repopulation. In collaboration with Dr. Mark Bolander, we demonstrated profuse concentrations of metalloproteinases in the fracture callus of adult rat long bones (Partridge *et al.*, 1993). The predominant cells observed to stain for collagenase-3 are hypertrophic chondrocytes during the phase of endochondral ossification; marrow stromal cells (putative osteoblasts) when the primary spongiosa is remodeled; and osteoblasts/osteocytes at a time when newly formed woven bone is being remodeled to lamellar bone. This indicates that the adult long bone has the ability to produce profuse levels of collagenase-3, but only when challenged, e.g., by a wound-healing situation. Recently, it was shown that collagenase-3 (MMP-13) null mice have delayed bone fracture healing, characterized by a retarded cartilage response in the fracture callus (Kosaki *et al.*, 2007). The consistent observation here is a role for this enzyme when a collagenous matrix must undergo substantial, rapid remodeling.

Liu *et al.* (1995) have demonstrated that targeted mutation around the collagenase cleavage site in both alleles of the endogenous mouse type I collagen gene Colla1, which results in resistance to collagenase cleavage, leads to dermal fibrosis and uterine collagenous nodules. These animals are able to develop normally to adulthood, while some of the major abnormalities only become apparent with increasing age. Studies of these mice revealed that homozygous mutant (r/r) mice have diminished PTH-induced bone resorption, diminished PTH-induced calcemic responses, and thicker bones (Zhao *et al.*, 1999). These observations imply that collagenase activity is necessary not only in older animals for rapid collagen turnover, but also for PTH-stimulated bone resorption. In the r/r mice, empty osteocyte lacunae were evident in the calvariae and long bones as early as 2 weeks of age, with the number of empty lacunae increasing with age, and an increase in apoptosis was observed in osteocytes, as well as periosteal cells (Zhao *et al.*, 2000). Thus, normal osteocytes (and osteoblasts) and osteoclasts might bind to cryptic epitopes that are revealed by the collagenase cleavage of type I collagen by liganding the $\alpha_v\beta_3$ integrin and, if such signals are not induced (as postulated for the osteoclastic defect in r/r mice), they would undergo apoptosis and their lacunae would empty. Young r/r mice are also noted to develop thickening of the calvariae through the deposition of new bone predominantly at the inner periosteal surface; an increased deposition of endosteal trabecular bone was found in long bones in older r/r mice. Thus, the failure of collagenase to cleave type I collagen in r/r mice was associated with increased osteoblast and osteocyte apoptosis, and paradoxically, increased bone deposition as well.

To elucidate the functional roles of collagenase-3 during skeletal development *in vivo*, Inada *et al.* (2004) generated collagenase-3 null mice. The mice deficient for collagenase-3 were found to have lengthened growth plates, owing to an increase in the hypertrophic chondrocyte zone, as well as delayed ossification at the primary centers. Abnormalities of growth plates were apparent in the early stages of embryonic development and persisted throughout adulthood. This abnormality is most likely owing to a decrease in degradation of extracellular matrix cartilage, as was shown by the significant increase in the area of type X collagen deposition, although an increase in synthesis of type X collagen also remains a possible cause. These observations suggest that collagenase-3 plays a critical role in collagen degradation during growth plate development and endochondral ossification. Similar to these findings, Stickens *et al.* (2004) found that deletion of collagenase-3 caused abnormal endochondral bone development as a result of impaired extracellular matrix remodeling. These collagenase-3-deficient mice were viable, fertile, and had a normal life span, with no gross phenotypic abnormalities. However, an increase in the hypertrophic chondrocyte zone of the skeletal growth plate was observed, as a result of the delayed exit of chondrocytes from the growth plate. In addition, unlike the late phenotype of the collagenase-resistant mice, these mice showed an early increase in trabecular bone that persisted for months. This was owing to the absence of collagenase expression in osteoblasts, not chondrocytes, as was shown by tissue-specific knockouts. The crucial role of collagenase-3 in bone formation and remodeling is further demonstrated by a missense mutation, F56S, in the proregion domain of MMP-13 in a form of chondrodysplasia in humans. This mutation, the substitution of an evolutionarily conserved phenylalanine residue for a serine, causes the Missouri type of spondyloepimetaphyseal dysplasia (SEMD(MO)), an autosomal dominant disorder characterized by defective growth and modeling of vertebrae and long bones (Kennedy *et al.*, 2005). This is thought to be owing to intracellular autoactivation and degradation of the mutant enzyme.

Related to work in whole animals, we have shown, together with Drs. Jane Lian and Gary Stein, that collagenase-3 is expressed late in differentiation in an *in vitro* mineralizing rat osteoblast culture system (Shalhoub *et al.*, 1992; Winchester *et al.*, 1999, 2000). When osteoblasts derived from fetal rat calvariae are grown in this culture system, they undergo development from an immature preosteoblast to a mature, differentiated osteoblast, which exists within a mineralized extracellular matrix [reviewed in Stein and Lian (1993) and Stein *et al.* (1990)]. The appearance of the enzyme in late differentiated osteoblasts may correlate with a period of remodeling of the collagenous extracellular matrix.

These observations regarding the differentiation of rat osteoblasts may explain the very low levels of MMP-1 observed in cultures of normal human osteoblasts (Rifas *et al.*, 1989), where mRNAs and proteins were isolated from cells at confluence, but apparently not from mineralized cultures. Alternatively, the cultures may predominantly express MMP-13 (rather than MMP-1), which has been shown to be expressed by osteoblasts, chondrocytes, and in synovial tissue, particularly in pathologic conditions such as osteoarthritis (Johansson *et al.*, 1997; Mitchel *et al.*, 1996; Reboul *et al.*, 1996, Wernicke *et al.*, 1996). At the time that Rifas and colleagues conducted the work on human osteoblasts, human MMP-13 had not been identified.

Canalis' group has conducted considerable research on the hormonal regulation of collagenase-3 in rat calvarial osteoblasts, including demonstrating stimulation by retinoic acid (Varghese *et al.*, 1994). They have also demonstrated that triiodothyronine (T_3), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF) all stimulate collagenase-3 transcription (Pereira *et al.*, 1999, Rydziel *et al.*, 2000; Varghese *et al.*, 2000). Interestingly, they have also shown that insulin-like growth factors (IGFs) inhibit both basal and retinoic-stimulated collagenase expression (Canalis *et al.*, 1995) by these cells. Recently, we have shown that TGF-beta 1 stimulates collagenase-3 expression in the rat osteoblastic cell line UMR 106-01 (Selvamurugan *et al.*, 2004).

We have conducted many studies with the clonal rat osteosarcoma line UMR 106-01, which has been described as osteoblastic in phenotype (Partridge *et al.*, 1980, 1983). This cell line responds to all of the bone-resorbing hormones by synthesizing collagenase-3 (Partridge *et al.*, 1987; Civitelli *et al.*, 1989). In contrast to the physiological regulation of collagenase in fibroblasts (Woessner, 1991), synoviocytes (Brinckerhoff and Harris, 1981), and uterine smooth muscle cells (Wilcox *et al.*, 1994), the control of expression of collagenase-3 in bone and osteoblastic cells appears to have some distinct differences. First, it is stimulated by all the bone-resorbing hormones (Partridge *et al.*, 1987; Delaissé *et al.*, 1988), which act through different pathways, including protein kinase A (PKA; PTH and PGs), protein kinase C (PKC; PTH and PGs), tyrosine phosphorylation (EGF), and direct nuclear action [$1,25(OH)_2D_3$; retinoic acid]. Second, glucocorticoids do not inhibit stimulation by PTH (Delaissé *et al.*, 1988; T. J. Connolly, N. C. Partridge, and C. O. Quinn, unpublished observations) whereas retinoic acid stimulates collagenase-3 expression rather than inhibiting it (Delaissé *et al.*, 1988; Connolly *et al.*, 1994; Varghese *et al.*, 1994). Last, in rat osteosarcoma cells, PMA is unable to elicit a pronounced stimulatory effect on collagenase-3 gene expression.

Among the bone-resorbing agents tested, PTH is the most effective in stimulating collagenase-3 production by UMR cells. A single 10^{-7} M PTH dose significantly stimulates transient collagenase-3 secretion with maximal enzyme concentrations achieved between 12 and 24 hours (Partridge *et al.*, 1987; Civitelli *et al.*, 1989). This level is maintained at 48 hours, decreases to 20% of the maximum by 72 hours, and is ultimately undetectable by 96 hours. We hypothesized that collagenase-3 was removed from the media through a cell-mediated binding process because the enzyme is stable in conditioned medium and experiments showed that this disappearance was not owing to extracellular enzymatic degradation. Binding studies conducted with ^{125}I-collagenase-3 revealed a specific receptor with high affinity (K_d = 5 nM) for rat collagenase-3 (Omura *et al.*, 1994). Further studies showed that binding of collagenase-3 in this fashion is responsible for its rapid internalization and degradation. The processing of collagenase-3 in this system requires receptor-mediated endocytosis and involves sequential processing by endosomes and lysosomes (Walling *et al.*, 1998). In addition to UMR cells, we identified a very similar collagenase-3 receptor on normal, differentiated rat osteoblasts, rat and mouse embryonic fibroblasts, and human and rabbit chondrocytes (Walling *et al.*, 1998; Barmina *et al.*, 1999; Raggatt *et al.*, 2006). These results indicate that the function of these receptors is to limit the extracellular abundance of collagenase-3 and, consequently, breakdown of the extracellular matrix.

Further investigation of the collagenase-3 receptor system has led us to conclude that collagenase-3 binding and internalization requires a two-step mechanism involving a specific collagenase-3 receptor and a member of the low-density lipoprotein (LDL) receptor-related superfamily. Ligand blot analyses demonstrate that ^{125}I-labeled collagenase-3 specifically binds two proteins (approximately 170 and 600 kDa) in UMR 106-01 cells (Barmina *et al.*, 1999). Of these two binding proteins, the 170 kDa protein appears to be a high-affinity primary-binding site, and the 600 kDa protein appears to be the low-density lipoprotein receptor-related protein-1 responsible for mediating internalization. The LDL receptor superfamily represents a diverse group of receptors, including the LDL receptor, the low-density lipoprotein-related receptor protein (LRP-1), the VLDL receptor, megalin (LRP-2) and LRP 5/6 (Herz and Bock, 2002). All of these plasma membrane receptors have a single membrane-spanning domain and several stereotyped repeats, both complement-like (for ligand binding) and EGF-like (for ligand dissociation). Most receptors in this family participate in receptor-mediated endocytosis, whereby the receptor-ligand complex is directed (via an NPXY signal in the receptor) to clathrin-coated pits and then internalized. Ligands of these receptors include LDL, VLDL, uPA-or tPA-PAI-1 complexes, tPA, lactoferrin, activated α_2-macroglobulin/proteinase complexes, apolipoprotein E-enriched β-VLDL, lipoprotein lipase, *Pseudomonas* exotoxin A, Wnts, and vitellogenin (Herz and Bock, 2002).

The striking stimulation of collagenase-3 secretion by bone-resorbing agents in UMR cells was shown to be paralleled by an even more striking induction of collagenase-3 mRNA. Northern blots using a cDNA clone to rat collagenase-3 as a probe showed a ~180-fold induction of collagenase-3 mRNA 4 hours after PTH treatment (Scott *et al.*, 1992), with an initial lag period between 0.5 and 2 hours before collagenase-3 mRNA levels rose above basal. Nuclear run-on studies showed a comparable increase in transcription of the gene 2 hours after treatment with PTH. The PTH-induced increase in collagenase-3 transcription was completely inhibited by cycloheximide, whereas the transcriptional rate of β-actin was unaffected by inclusion of the protein synthesis inhibitor (Scott *et al.*, 1992). These results demonstrate that the PTH-mediated stimulation of collagenase-3 involves transcription, and requires *de novo* synthesis of a protein factor(s).

PTH treatment was found to increase the transcription of collagenase-3 in rat osteoblastic osteosarcoma cells primarily by stimulation of the cAMP signal transduction pathway (Scott *et al.*, 1992). Second messenger analogs were used to test which signal transduction pathway is of primary importance in the PTH-mediated transcriptional induction of the collagenase-3 gene. The cAMP analogue, 8BrcAMP, was capable of inducing collagenase-3 transcription to levels close to that of PTH. In contrast, neither the PKC activator, PMA, nor the calcium ionophore, ionomycin, when used alone, resulted in any increase in collagenase-3 gene transcription similar to that elicited by PTH after 2 hours of treatment. Furthermore, this effect requires protein synthesis and a 1- to 1.5-hour lag period, suggesting that the transcriptional activation of the collagenase-3 gene may be the result of interactions with immediate early gene products. PTH treatment was also found to transiently increase the mRNA expression of the AP-1 protein subunits *c-fos* and *c-jun* (Clohisy *et al.*, 1992). Both mRNA species were maximally induced within 30 minutes, well before the maximal transcription rate at 90 minutes for collagenase-3. Later, it was determined that PTH is responsible for phosphorylation of the cAMP response element-binding (CREB) protein at serine 133 (Tyson *et al.*, 1999). Once phosphorylated, the CREB protein binds a cAMP response element (CRE) in the *c-fos* promoter and activates transcription (Pearman *et al.*, 1996).

The collagenase-3 gene has 10 exons (Rajakumar *et al.*, 1993), encoding a mRNA of ~2.9 kb, which in turn encodes the proenzyme with a predicted molecular weight of the core protein of 52 kDa (Quinn *et al.*, 1990). A series of deletion and point mutants of the promoter region were generated to identify the PTH-responsive region and subsequently the primary response factors, which convey the hormonal signal and bind to this region(s) of the collagenase-3 gene. The minimum PTH regulatory region was found to be within 148 bp upstream of the transcriptional start site (Selvamurugan *et al.*, 1998). This region contains several consensus transcription factor recognition sequences including SBE (Smad binding element), C/EBP (CCAAT enhancer-binding protein site), RD (runt domain-binding sequence), p53, PEA-3 (polyoma enhancer activator-3), and AP (activator protein)-1 and -2. The AP-1 site is a major target for the Fos and Jun families of oncogenic transcription factors (Chiu *et al.*, 1988; Lee *et al.*, 1987; Angel and Karin, 1991). The RD site is a target for core-binding factor proteins, specifically Runx2. Mice containing a targeted disruption of the Runx2 gene die at birth and lack both skeletal ossification and mature osteoblasts (Ducy *et al.*, 1997; Komori *et al.*, 1997; Otto *et al.*, 1997). These mutant mice also do not express collagenase-3 during fetal development, indicating that collagenase-3 is one of the target genes regulated by Runx2 (Jimenez *et al.*, 1999).

Additional experiments on the collagenase-3 promoter determined that both native AP-1 and RD sites and their corresponding binding proteins, AP-1 and Runx2-related proteins, are involved in PTH regulation of collagenase-3 transcription. Using gel-shift analysis, we further showed enhanced binding of Fos and Jun proteins at the AP-1 site upon treatment with PTH (Selvamurugan *et al.*, 1998), although there was no significant change in the level of Runx2 binding to the RD site. We determined that PTH induces PKA-mediated post-translational modification of Runx2 and leads to enhanced collagenase-3 promoter activity in UMR cells (Selvamurugan *et al.*, 2000b). The binding of members of the AP-1 and Runx families to their corresponding binding sites in the collagenase-3 promoter also appears to regulate collagenase-3 gene expression during osteoblast differentiation (Winchester *et al.*, 2000). As discussed earlier, collagenase-3 expression is regulated by a variety of growth factors, hormones, and cytokines, but the effects of these compounds appear to be cell type specific. Data obtained in breast cancer and other cell lines suggest that the differential expression of and regulation of collagenase-3 in osteoblastic compared to nonosteoblastic cells may depend on the expression of AP-1 factors and post-translational modifications of Runx2 (Selvamurugan and Partridge, 2000; Selvamurugan *et al.*, 2000a). The close proximity of the AP-1 and RD sites and their cooperative involvement in the activation of the collagenase-3 promoter suggests that the proteins binding to these sites physically interact; Runx2 was found to directly bind Fos and Jun in both *in vitro* and *in vivo* experiments (D'Alonzo *et al.*, 2002). To determine the importance of these regulatory sites in the expression of MMP-13 *in vivo*, transgenic mice containing the *E. coli* lacZ reporter fused to either wild-type MMP-13 promoter or that with mutated AP-1 and RD sites were generated (Selvamurugan *et al.*, 2007). The wild-type transgenic lines expressed higher levels of bacterial β-galactosidase in bone, teeth, and skin compared to the mutant and transgenic lines. Thus, the AP-1 and RD sites of the promoter most likely regulate and are necessary for gene expression *in vivo* in bone, as well as teeth and skin.

PLASMINOGEN ACTIVATORS

The plasminogen activator (PA)/plasmin pathway is involved in several processes, including tissue inflammation, fibrinolysis, ovulation, tumor invasion, malignant transformation, tissue remodeling, and cell migration. The PA/plasmin pathway is also thought to be involved in bone remodeling by osteoblasts and osteoclasts. The pathway results in the formation of plasmin, another neutral serine proteinase, which degrades fibrin and the extracellular matrix proteins fibronectin, laminin, and proteoglycans. In addition, plasmin can convert matrix metalloproteinases, procollagenase, and prostromelysin to their active forms (Eeckhout and Vaes, 1977). Plasminogen has been localized to the cell surface of the human osteosarcoma line MG63, where its activity was enhanced by endogenous cell bound urokinase-type plasminogen activator (uPA) (Campbell *et al.*, 1994).

The PA/plasmin pathway is regulated by members of the serpin family in addition to various hormones and cytokines. The primary function of this family of inhibitors is to neutralize serine proteinases by specific binding to the target enzyme. Serpins are involved in the regulation of several processes, including fibrinolysis, cell migration, tumor suppression, blood coagulation, and extracellular matrix remodeling (Potempa *et al.*, 1994). Members of this pathway involved in the regulation of the PA/plasmin pathway are plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2), which regulate uPA and tissue type plasminogen activator (tPA); protease nexin-1, which regulates thrombin, plasmin, and uPA; and α_2-antiplasmin, which regulates plasmin. Active PAI-1 combines with uPA and tPA, forming an equimolar complex, exerting its inhibition through interactions with the active site serine. PAI-1 has been detected in media of cultured human fibrosarcoma cells (Andreasen *et al.*, 1986) and primary cultures of rat hepatocytes and hepatoma cells. PAI-1 was also detected from conditioned medium of rat osteoblast-like cells and rat osteosarcoma cells (Allan *et al.*, 1990). The expression of uPA, tPA, type I receptor for uPA, PAI-1, -2 and the broad-spectrum serine proteinase inhibitor, protease nexin I is induced by PTH treatment in primary mouse osteoblasts. The regulation of these various enzymes within bone tissue may determine the sites where bone resorption will be initiated (Tumber *et al.*, 2003).

Urokinase-Type Plasminogen Activator

The urokinase-type plasminogen activator (u-PA) is secreted as a precursor form of ~55 kDa (Nielsen *et al.*, 1988; Wun *et al.*, 1982). It is activated by cleavage into a 30-kDa heavy chain and a 24-kDa light chain, joined by a disulfide bond, with the active site residing in the 30-kDa fragment. Urokinase has a Kringle domain, serine proteinase-like active site, and a growth factor domain (GFD). The noncatalytic NH_2-terminal fragment contains the GFD and Kringle domain and is referred to as the amino-terminal fragment (ATF). The u-PA and PAI-1 are involved in regulation of the first steps of angiogenesis (Pepper, 1997). Rabbani *et al.* (1990) demonstrated that ATF stimulated proliferation and was involved in mitogenic activity in primary rat osteoblasts and the human osteosarcoma cell line, SaOS-2. The GFD of the ATF is necessary for the binding of uPA to its specific receptor.

Tissue-Type Plasminogen Activator

The tissue-type plasminogen activator is secreted as a single-chain glycosylated 72-kDa polypeptide. This enzyme has been found in human plasma and various tissue extracts, as well as in normal and malignant cells. The cleavage of tPA forms a 39-kDa heavy chain and a 33-kDa light chain linked by a disulfide bond. The heavy chain has no proteinase activity, but contains two Kringle domains that assist in binding fibrin to plasminogen (Banyai *et al.*, 1983; Pennica *et al.*, 1983). Furthermore, the heavy chain contains a finger domain involved in fibrin binding (van Zonneveld *et al.*, 1986) and a GFD with homology to human and murine epidermal growth factor. Recently, it has been shown that tPA is expressed in osteoblastic cells with nicotine or PTH treatments (Katano *et al.*, 2006; Tumber *et al.*, 2003).

Plasminogen Activators in Bone

Plasminogen activator activity is increased in normal and malignant osteoblasts as well as calvariae by many agents, including PTH, $1,25(OH)_2D_3$, PGE_2, IL-1α, fibroblast growth factor, and EGF (Hamilton *et al.*, 1984, 1985; Thomson *et al.*, 1989; Pfeilschifter *et al.*, 1990; Cheng *et al.*, 1991; Leloup *et al.*, 1991; De Bart *et al.*, 1995). It should be noted that work suggests that PAs are not necessary for PTH- and $1,25(OH)_2D_3$-induced bone resorption (Leloup *et al.*, 1994). Expression of tissue-type plasminogen activator, urokinase-type plasminogen activator, plasminogen activator inhibitor-1, plasminogen activator inhibitor-2, protease nexin, and urokinase receptor isoform 1 (uPAR1) were detected in mouse osteoclasts using the reverse transcriptase–polymerase chain reaction (RT-PCR) (Yang *et al.*, 1997). Deletion of tPA, uPA, PAI-1, and plasminogen genes in mice can lead to fibrin deposition, some growth retardation, and inhibition of osteoclast ability to remove noncollagenous proteins *in vitro* (Carmeliet *et al.*, 1993, 1994; Bugge *et al.*, 1995; Daci *et al.*, 1999). Moreover, lack of the plasminogen activators leads to elongation of the bones and increased bone mass. Osteoblast differentiation and formation of a mineralized bone matrix are enhanced in osteoblast cultures derived from tPA-/- and uPA-/- mice (Daci *et al.*, 2003).

There are conflicting data as to whether the increase in osteoblastic PA activity is owing to an increase in the total amount of one or both of the PAs, or to a decline in the amount of PAI-1. All possible results have been observed, depending on which osteoblastic cell culture system is used or the method of identification of the enzymes. The latter have been difficult to assay categorically because there have not been abundant amounts of specific antibodies available for each of the rat PAs. Similarly, different groups have found the predominant osteoblastic PA to be uPA whereas others have obtained results indicating it to be tPA.

A range of agents have also been found to inhibit the amount of osteoblastic PA activity. These include glucocorticoids, TGF-β, bFGF, leukemia inhibitory factor, and IGF-I (Allan *et al.*, 1990, 1991; Cheng *et al.*, 1991; Forbes *et al.*, 2003; Lalou *et al.*, 1994; Pfeilschifter *et al.*, 1990). Where it has been examined, in many of these cases the decline is owing to a substantial increase in PAI-1 mRNA and protein. Nevertheless, some of these agents also markedly enhance mRNA abundance for the PAs (Allan *et al.*, 1991), although the net effect is a decline in PA activity.

CYSTEINE PROTEINASES

The major organic constituent of the ECM of bone is fibrillar type I collagen, which is deposited in intimate association with an inorganic calcium/phosphate mineral phase. The presence of the mineral phase not only protects the collagen from thermal denaturation but also from attack by proteolytic enzymes (Glimcher, 1998). The mature osteoclast, the bone-resorbing cell, has the capacity to degrade bone collagen through the production of a unique acid environment adjacent to the ruffled border through the concerted action of a vacuolar proton pump ([V]-type H^+-ATPase) (Chakraborty *et al.*, 1994; Bartkowicz *et al.*, 1995; Teitelbaum, 2000) and a chloride channel of the Cl-7 type (Kornak *et al.*, 2001). Loss-of-function mutations in the genes that encode either this proton pump (Li *et al.*, 1999; Frattini *et al.*, 2000; Kornak *et al.*, 2000) or the chloride channel (Kornak *et al.*, 2001) lead to osteopetrosis. At the low pH in this extracellular space adjacent to the ruffled border, it is possible to leach the mineral phase from the collagen and permit proteinases that act at acid pH to cleave the collagen (Blair *et al.*, 1993). Candidate acid-acting proteinases are cysteine proteinases such as cathepsin K. Cathepsin K is highly expressed in osteoclasts (Drake *et al.*, 1996; Bossard *et al.*, 1996). Cysteine proteinases contain an essential cysteine residue at their active site that is involved in forming a covalent intermediate complex with its substrates (Bond and Butler, 1987). The enzymes are either cytosolic or lysosomal. The latter have an acidic pH optimum and make up the majority of the cathepsins. These enzymes are regulated by a variety of protein inhibitors, including the cystatin superfamily (Turk and Bode, 1991) and α_2-macroglobulin (Barrett, 1986). Their extracellular abundance must consequently be regulated by cell surface receptors for α_2-macroglobulin as well as the lysosomal enzyme targeting mannose-6-phosphate/IGF-II receptors.

Involvement of lysosomal cysteine proteinases in bone resorption has been indicated by many studies showing that inhibition of these enzymes prevents bone resorption *in vitro* as well as lowering serum calcium *in vivo* (Delaissé *et al.*, 1984; Montenez *et al.*, 1994). The more recently identified cathepsin, cathepsin K (Tezuka *et al.*, 1994b), was found to have substantial effects on bone. Mice containing a targeted disruption of cathepsin K were developed and found to exhibit an osteopetrotic phenotype characterized by excessive trabeculation of the bone marrow space (Saftig *et al.*, 1998, 2000). Cathepsin K–deficient osteoclasts are capable of demineralizing the extracellular matrix, but are unable to fully remove the demineralized bone (Gowen *et al.*, 1999). Additionally, cathepsin K mutations have been linked to pycnodysostosis, a hereditary bone disorder characterized by osteosclerosis, short stature, and defective osteoclast function (Gelb *et al.*, 1996). Recent studies show that the expression of MMP-9, TRACP for osteoclastic enzymes and osteoblastic proteases (MMP-13, MMP-14), and receptor activator of nuclear factor κB ligand (RANKL) are increased in cathepsin K – deficient mice (Kiviranta *et al.*, 2005). Moreover, cathepsin K – deficient osteoclasts compensate the lack of this enzyme by using MMPs in the resorption of bone matrix (Everts *et al.*, 2006).

Cathepsin inhibitors may be therapeutically beneficial in the treatment of osteoporosis and rheumatoid arthritis to stimulate cortical bone formation and inhibit bone resorption (Xiang *et al.*, 2007). However, cathepsin K deficiency reduces atherosclerotic plaque and induces plaque fibrosis (Lutgens *et al.*, 2006a). Use of a cathepsin K inhibitor as a possible therapeutic target for atherosclerosis and bone diseases has to be evaluated with care because cathepsin K inhibition may lead to a profibrotic, but also to a more lipogenic, plaque phenotype (Lutgens *et al.*, 2006b).

Immunohistochemistry revealed that the majority of the cysteine proteinases (cathepsins B, K, and L) and the aminopeptidases (cathepsins C and H) are products of osteoclasts (Ohsawa *et al.*, 1993; Yamaza *et al.*, 1998; Littlewood-Evans *et al.*, 1997), although immunoreactive staining for cathepsins B, C, and H was also seen in osteoblasts and osteocytes. It is notable that the most potent collagenolytic cathepsin at acid pH, cathepsin L, was strongly expressed in osteoclasts and very weakly in osteoblasts. Mathieu *et al.* (1994), however, have detected both cathepsins B and L as proteins secreted by their immortalized osteogenic stromal cell line, MN7. Everts *et al.* (2006) have shown that cathepsin L is involved in modulating MMP-mediated resorption by calvarial osteoclasts.

Oursler *et al.* (1993) have also demonstrated that normal human osteoblast-like cells produce cathepsin B and that dexamethasone can increase expression and secretion of this lysosomal enzyme by these cells. Interestingly, they also showed that dexamethasone treatment causes activation of TGF-β and, by the use of lysosomal proteinase inhibitors, ascribed a role for cathepsins B and D to activation of this growth factor.

ASPARTIC PROTEINASES

These lysosomal proteinases contain an aspartic acid residue at their active site and act at acid pH. Very little investigation has been conducted on these enzymes in bone cells except for the observations that cathepsin D, a member of this family, can be found by immunohistochemical staining in osteoblasts and osteocytes (Ohsawa *et al.*, 1993), and expression of this enzyme is increased markedly by dexamethasone treatment of human osteoblasts in culture (Oursler *et al.*, 1993). Cathepsin D is secreted into the resorbing area of human odontoclasts in order to participate in degradation of mineralized tooth matrix (Gotz *et al.*, 2000).

CONCLUSIONS

The osteoblast has the ability to produce proteinases of all four classes, but far more is known about their production of collagenase and plasminogen activators, at least *in vitro.* We still do not know the absolute role of any of these osteoblastic enzymes *in vivo.* Further work with knockouts of the respective enzymes is likely the only way we will determine their required functions. These roles may not be restricted to assisting in the resorption process but may include functions to regulate bone development. Additionally, the osteoclast produces MMP-9 and cathepsin K, which appear to have similar roles in the two diverse processes.

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Chapter 20

Integrins and Other Cell Surface Attachment Molecules of Bone Cells

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INTRODUCTORY REMARKS: ADHESION AND BONE CELL FUNCTION

Bone (re)modeling (see Chapters 1, 3, and 19) involves the coordinated response of osteoblasts, osteocytes, and osteoclasts. Osteoblasts (see Chapter 4) and bone-lining cells form a near-continuous layer covering the periosteal, endosteal, and trabecular bone; interactions between these cells and the organic matrix of bone are important determinants of osteoblast proliferation and differentiation. Osteocytes (see Chapter 6) are found in lacunae, set within the bone matrix, and are joined both to their neighbors and cells lining the bone surfaces by cytoplasmic processes, which pass through fine channels or canaliculi. Together, this interconnecting network of osteoblasts, bone-lining cells, and osteocytes provides a possible mechanism for the detection of physical or mechanical changes and the coordination of osteosynthetic and resorptive activity leading to remodeling. Cell–cell and cell–matrix communication is central to this process, and by inference, cell adhesion molecules will be key players in these events, both in normal skeletal homeostasis, growth, and development and in pathological situations where the balance between resorption and remodeling becomes disturbed (see Table I).

Connective tissue cells in general, and bone and cartilage cells in particular, are surrounded by an abundance of extracellular matrix. Chondroblasts, osteoblasts, and, to a lesser extent, osteocytes are responsible for the synthesis of the majority of the organic components of this matrix, whereas osteoclasts mainly degrade the matrix. The function of bone and cartilage cells reflects the matrix components that surround them; conversely, the composition of the matrix, i.e., the structure of cartilage and bone, is highly dependent on the cellular function of chondroblasts, osteoblasts, and osteoclasts.

Osteoclasts are the main cells responsible for the breakdown of the extracellular matrix of bone during normal and pathological bone turnover (see Chapters 7 and 8; Teitelbaum, 2007). Osteoclastic bone resorption involves a series of developmental and regulatory steps that include the proliferation and homing to bone of hemopoietic progenitor cells; their differentiation into postmitotic osteoclast precursors, which express features of mature osteoclasts; fusion to form multinucleated cells; and migration of osteoclasts to the area of bone to be remodeled, where they attach to the bone surface. Adhesion of mature osteoclasts to bone is critical for their resorptive capacity and is dependent on expression of functional adhesion receptors. In addition, many of the steps preceding resorption involve adhesion between mature osteoclasts, osteoclast precursors, and other cell types in the bone/bone marrow compartment and with components of the extracellular matrix of bone. Some of these possible functional events are summarized in Table I. Once attached to bone, osteoclasts polarize to create three discrete areas of plasma membrane: (1) the "basolateral membrane," which faces the marrow space and is not in contact with the bone; (2) the "sealing zone," or "clear zone" that is closely apposed to the bone matrix; and (3) the "ruffled border," a highly convoluted area of plasma membrane that faces the bone matrix and is surrounded by the sealing zone. The sealing zone forms a diffusion barrier and permits the localized accumulation of high concentrations of protons and proteases secreted via the ruffled border into an extracellular resorption compartment underneath the cell (discussed in more detail later).

Cell–matrix interactions associated with osteoclastic bone resorption have been researched extensively (reviewed in Väänänen and Horton, 1995; Horton and Rodan, 1996; Duong *et al.*, 2000; Helfrich and Horton, 2006). Much less is known about cell–cell and

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TABLE I Summary of Possible Functions of Cell Adhesion Receptors in Bone

Osteoclast development and function
Migration of committed osteoclast precursors from the bone marrow to sites of future resorption, exiting via specialized endothelial barriers
Homing to "bone" (using chemo-, haptotactic signals) and ingress across vascular endothelium
Recognition of, adhesion to, and migration upon "bone" matrix proteins
Fusion of postmitotic osteoclast precursors
Regulatory intercellular interactions with osteoblasts, leucocytes, and other cell types in marrow space; presentation of growth factors from extracellular matrix stores
Signal transduction (and control of osteoclast function) by interaction with matrix (via RGD and other sequences)
Cellular polarization, cytoskeletal (re)organization, tight sealing zone formation, and bone resorption
Cessation of resorption by detachment from matrix, cell migration, and regulation of osteoclast survival versus apoptosis
Osteoblasts
Transduction of mechanical signals within skeleton to regulate cell function
Adhesion to, and migration on, bone matrix, including unmineralized osteoid
Regulation of osteoblast maturation from mesenchymal stem cells
Regulation of mature cell function (gene expression, matrix synthesis, protease secretion, etc.)
Interaction with other bone cells (e.g., osteoclasts) and cells in the bone marrow compartment (e.g., marrow stroma, leukocytes)
Osteocytes
Mechanosensing and mechanotransduction
Recognition of and adhesion to matrix
Interaction with other cells (osteocytes, osteoblasts, ?osteoclasts)
Chondrocytes
Response to mechanical forces (e.g., in articular cartilage)
Maintenance of tissue integrity by matrix synthesis and assembly
Regulation of chondrocyte maturation from mesenchymal stem cells
Regulation of mature chondrocyte function (gene expression, matrix synthesis, proliferation and cell survival, etc.)
Mediation of response in cartilage to injury and disease

cell–matrix interactions in osteoblasts and related populations, although there has been considerable progress since the first published analysis (Horton and Davies, 1989; Helfrich and Horton, 2006). The best defined of these adhesive interactions are mediated by a particular class of cell adhesion molecule, the integrin receptors. Integrins are now known to be major functional proteins of osteoclasts and have become targets for potential therapeutic intervention in bone diseases such as osteoporosis. The balance of this chapter somewhat reflects this bias. However, the nature and function of other adhesion proteins and cell adhesion receptors is becoming increasingly clear and in skeletal tissues there is an increased focus outside osteoclasts and integrins. Moreover, the essential roles of adhesion molecules in skeletal function are increasingly coming from the investigation of patients with genetic conditions and from knockout or transgenic mice – data arising from such studies will also be reviewed.

OVERVIEW OF CELL ADHESION MOLECULE STRUCTURE

Adhesion Receptors and Their Ligands

Molecular and immunological approaches have led to considerable advances in our understanding of the range of cell membrane molecules that are capable of mediating cell adhesion. Detailed sequence and structural analysis have enabled many of them to be grouped into "families," with related structure based on their content of highly homologous domains. The major groupings of adhesion receptor families are summarized in Table II; this

TABLE II Classes of Cell Adhesion Receptors and Their Ligands

Family	Homology region in receptor	Examples	CD No.	Ligands	Recognition motif in ligand/counterreceptor	Extracellular matrix components with shared homology domain
Integrin	PEGG (all β chains)	gpIIbIIIa	CD41/61	Blood proteins	RGD, KQAGDV	Collagen VI, von Willebrand factor, cartilage matrix protein (integrin I domain)
	I domain (CD11, $\alpha1\alpha2$)	LFA-1	CD11/18	ICAM counterreceptor	ICAMs, etc.	
		$\alpha_v\beta_3$	CD51/61	Matrix, blood proteins, matricellular (CNN) proteins	RGD	
		$\alpha_2\beta_1$	CD49b/29	Collagen	DGEA, GER	
		$\alpha_4\beta_1$	CD49d/29	Fibronectin	EILDV	
Ig superfamily	Ig fold	ICAMs, VCAM	CD54, etc.	Heterophilic interaction	Multiple	Perlecan (Ig fold)
		N-CAM		Homophilic	KYSFNYDGSE	Fibronectin, tenascin, thrombospondin (N-CAM type III repeat)
		CD2		LFA-3 counterreceptor		
Selectins	C-type lectin, EGF repeat Complement regulatory protein domain	L-, P-selectin	CD62	Glycam-1, PSGL-1, CD34, etc.	Sialyl Le^x (CD15), etc.	Aggrecan, versican (lectin) Laminin, tenascin, thrombospondin, aggrecan, veriscan (EGF repeat)
Cadherins	LDRE repeat (110 amino acid module)	E-, N-cadherin		Homophilic	HAV	
Leucine-rich glycoproteins (LRG)	Leucine repeat (24 amino acid repeat)	Platelet gpIb	CD42b	Blood proteins	von Willebrand factor, thrombin	Biglycan, decorin
Mucins	Mucin side chain	Leukosialin	CD43 CD34	Selectins		Muc-1
CD36 family		Platelet gpIV	CD36	Thrombospondin, collagen	SVTCG (for thrombospondin)	Aggrecan, versican, link protein
CD44	Hyaluronidate-binding site		CD44	Hyaluronic acid, etc.		Aggrecan, versican, link protein

identifies some specific examples, their regions of homology by which they are defined, their ligands, and the nature and specificity of their interactions with receptors. Individual members of the families have a diverse range of structures, tissue distribution, and functions, and it is outside the scope of this chapter to provide information other than in outline. The reader is referred to Barclay *et al.* (1997) and Isacke and Horton (2000) for further details, but some basic structural information is provided here.

Similar methods have been applied to elucidate the structure of the molecules recognized by cell adhesion proteins, i.e., their ligands. These include components of the extracellular matrix and plasma proteins and cell-associated matrix proteins (Table II; Ayad *et al.,* 1998; Humphries *et al.,* 2006) and cell membrane-associated "counterreceptors" (e.g., the "ICAMs," Table II). As with adhesion receptors, a range of structural domains are recognizable within their ligands, some of which have clearly defined functions; e.g., the well-characterized Arg-Gly-Asp (RGD) peptide motif, originally described in the protein fibronectin and now known to be present widely in many matrix proteins (Pierschbacher and Ruoslahti, 1984; Ruoslahti, 1996, 2003). Interestingly, some of the domains that have been found in extracellular matrix proteins can also be identified in adhesion receptors (Table II), suggesting a shared function; e.g., hyaluronidate-binding sites have been found in the matrix proteoglycan, versican, and the "homing" receptor, CD44.

The diversity of the types and combinations of cellular receptors and the complexity of the molecular structure of the extracellular matrix are reflected in the large number of functions that have been ascribed to "cell adhesion molecules." These include both true adhesive interactions, which are clearly seen in, for instance, cell-to-cell interactions regulating the immune response and the integrity of epithelial barriers, or via the increasingly identified signaling pathways mediated by adhesion receptors, including integrins and cadherins. This includes events mediated through linkages to the F-actin cytoskeleton, leading to changes in cell shape and motility, and activation of Src family and other tyrosine kinases or mobilization of intracellular calcium, resulting in other functional changes downstream, such as activation of early response genes or protease secretion (termed "outside-in" signaling). Similarly, intracellular events can lead to the modification of receptor affinity and activity ("inside-out" signaling; e.g., the platelet integrin gpIIbIIIa will only bind fibrinogen after alterations in integrin conformation following activation on ligand binding to other non-integrin receptors such as via the thrombin receptor).

Integrin Structure

Integrins (Hynes, 2002; see also Isacke and Horton, 2000) are heterodimeric proteins whose constituent polypeptide chains, α and β, are linked noncovalently. Although originally identified by antibodies or direct purification, the primary structure of most integrin subunits has been deduced by cDNA cloning. To date, 18 different mammalian α subunits and 8 β subunits have been identified, forming 24 distinct heterodimers. Both integrin subunits are transmembrane, N-glycosylated glycoproteins with a large extracellular domain, a single hydrophobic transmembrane region, and a short cytoplasmic domain (apart from β_4, which has a large intracellular domain not found in other integrins). Electron microscopy of several purified integrin dimers shows an extended structure with dimensions of approximately 10 by 20 nm, formed by an N-terminal globular "head" composed by the association of the two subunits, connected to the membrane by two "stalks."

α subunits vary in size from 120 to 180 kDa, and analysis of their cDNA sequences reveals several features in common. All contain seven homologous repeating domains, folded into a propeller domain with three or four of the "blades" containing divalent cation binding sites. These cation binding sites, together with one such site in the β subunit, are critically important for ligand binding – note how both the α and the β subunit contribute to the ligand binding site – and also play a role in receptor stability. Some subunits (the collagen-binding α_1, α_2, α_{10}, and α_{11} chains, α_E and the leukocyte integrins α_D, α_X, α_M, and α_L) contain an additional 200 amino acids inserted between repeats 2 and 3, known as I domain (Table II). The I domain contains a characteristic metal ion-dependent adhesion site (MIDAS) motif, which can bind Mn^{2+} and Mg^{2+} ions and is also critical in ligand binding (reviewed in detail in Luo *et al.,* 2007). Integrin α subunits without I domains are cleaved post-translationally near the transmembrane domain and disulfide bonded.

β subunits are 90 to 110 kDa in size, apart from the 210 kDa β_4 chain. Their cDNA sequences show a high cysteine content (e.g., 56 Cys residues in β_3), largely concentrated in four 40 amino acid long segments that are internally disulfide bonded. Several conserved motifs in β chains are involved in ligand binding and interaction with cytoskeletal elements. β subunits also contain an I domain with a MIDAS motif, which in physiological circumstances bind an Mg^{2+} ion (for details see Luo *et al.,* 2007).

The first electron microscopic studies to define the structure of the fibronectin receptor $\alpha_5\beta_1$ (Nermut *et al.,* 1988) showed that the large extracellular domain is organized as a globular head supported by a stalk, composed of one α and one β subunit leg with overall dimensions of $\sim 10 \times$ x20 nm.This basic three-dimensional structure has now been confirmed in a number of integrins and more recently has been refined by detailed analysis of the crystal structure of the extracellular domain of the $\alpha_v\beta_3$ integrin, both with and without bound ligand (Xiong *et al.,* 2001, 2002). These studies revealed that the integrins not only exist as straight stalk structures, but can also bend over with the globular head facing toward the C-termini of the legs projecting from the plasma membrane. There has been extensive research to understand how the conformation of integrins changes upon cation binding. This generally results in "activation," a conformation in which the receptor can bind ligand and is important especially for the β_2- and β_3-containing integrins. The majority of data suggests that the bent form of the integrin is the inactive, nonligand bound state, whereas straightening is associated with activation and ligand binding. These switches between active and inactive conformations are comprehensively reviewed elsewhere (Luo *et al.*, 2007). Post-translational modification of integrins include the cleavage of the α subunits lacking I domains, with the exception of α_4, into heavy and light fragments that are disulphide linked, and glycosylation of α and β chains.

Ligands for integrins include a wide range of extracellular matrix proteins and Ig family members such as VCAM and ICAM (reviewed in Plow *et al.,* 2000; Humphries *et al.,* 2006). Most integrins can bind more than one ligand and often this is through recognition of a common sequence. In many extracellular matrix molecules this is an RGD peptide sequence, although in other proteins, sequences such as DGEA (in collagen) or LDV (in fibronectin) are recognized.

Cross-linking studies using radioactively labeled RGD peptide probes for the integrins $\alpha_v\beta_3$ and gpIIbIIIa, respectively (see Isacke and Horton, 2000), and mutational analysis have shown the ligand-binding site to be composed of distinct, relatively short elements in the N termini of both α and β subunits. When taken with the requirement for an "I" domain for ligand binding in some integrins, these data suggest that the interaction site depends on the composite structure formed by interplay of the two chains of the receptor, with ligand specificity reflecting subunit usage.

Integrins are linked to the F-actin cytoskeleton via interaction of the β subunit with actin-binding proteins, including α-actinin, vinculin, and talin. The cytoplasmic domain of the β subunit also associates with a signaling complex comprising kinases and phosphatases and various adaptor proteins. Ligand binding leads to the activation of one or more intracellular signal transduction pathways, which, in turn, contribute to the regulation of differentiation, cytoskeletal organization, and other aspects of cell behavior. Much information on signaling via integrins has come from studies in cells such as fibroblasts and osteosarcoma cell lines that produce focal contacts *in vitro* containing the focal adhesion kinase (FAK), which associates directly with the β subunit. Upon occupation and clustering of the integrin, FAK is targeted to focal adhesions, where it associates with the cytoskeleton and is activated by autophosphorylation. Downstream signaling pathways include association of Src family kinases with phosphorylated FAK and engagement and activation of Rho-like GTPases, Erk and Jnk signaling pathways, ultimately leading to cytoskeletal rearrangement (van der Flier and Sonnenberg, 2001). Similar pathways operate in osteoclasts, discussed in general later and in detail in Chapter 9.

Cadherins

Cadherins are a rapidly growing family (>80 members) of calcium-dependent proteins that play prominent roles in morphogenesis and the maintenance of adhesive contacts in solid tissues. They are divided into five subsets of receptors: the classical cadherins types I and II, the latter directly linked to the actin cytoskeleton, desmocollins and desmogleins, protocadherins, and a number of other, more distantly related cadherins (Patel *et al.,* 2003). Cadherins are calcium-dependent single chain single pass transmembrane glycoproteins that share "cadherin domains," which contain specific amino acid motifs that have essential roles in calcium binding and dimerization of the receptors (Yagi and Takeichi, 2000; Patel *et al.,* 2003). In the context of this review the classical cadherins are the most important subgroup. The type I cadherins (members E-, N-, M-, P-, and R- cadherin) all share a common structure and have highest homology in the short cytoplasmic domain. They have an extracellular domain containing five repeats of around 110 amino acids that contain the negatively charged, calcium-binding motifs and conserved cysteine residues in the fifth repeat. They have molecular weights of around 100–130 kD. The ligand-binding site of type I cadherins is the conserved HAV motif located at the N-terminal region of the molecule in the first conserved extracellular repeat. Type II cadherins (cadherin 5–12) share the basic cadherin structure, but have lower amino acid homology with type I cadherins. They also lack the cell adhesion HAV motif.

Cadherins are mediators of cell–cell adhesion and bind ligand mainly in a homophilic manner, although heterophilic binding between different cadherin molecules is possible, but appears restricted to cadherins from the same class (Patel *et al.,* 2003). As with integrins, cells often express a repertoire of different cadherins simultaneously, although a particular feature of cadherins is their restricted expression at specific stages of embryonic and cellular development. On the cell surface, cadherins tend to be concentrated at cell–cell junctions. The cytoplasmic tail of cadherins binds to catenins and this complex is important in gene transcription as well as regulation of adhesion. β-catenin is also the key player in the Wnt signaling pathway, a pathway important in regulation of bone mass (Westendorf *et al.,* 2004). Current work is trying to unravel interconnections between Wnt signaling and cadherin-mediated cell adhesion through regulation of free β-catenin levels in cells (Nelson and Nusse, 2004). Signal transduction through cadherins is complex and dependent upon cell type and in addition to phosphorylation of β-catenin can include activation of small GTPases and tyrosine kinase pathways (Wheelock and Johnson, 2003; Lilien and Balsamo, 2005; Perez-Moreno and Fuchs, 2006).

Immunoglobulin Superfamily

The immunoglobulin (Ig) family of receptors all share a basic motif consisting of an Ig fold of between 70 and 110 amino acids (reviewed in Barclay *et al.,* 1997; Isacke and Horton, 2000) organized into two anti-parallel β sheets that seem to serve as a scaffold on which unique determinants can be displayed. There is considerable variation in the primary structure of the members of this family and hence in molecular weights, but their tertiary structure is well conserved. There are now over 700 human genes known that share Ig motifs, making this one of the largest superfamilies in the human genome (Brummendorf and Lemmon, 2001). Many of these are splice variants of cell adhesion molecules and Ig genes, but overall the cell adhesion molecules constitute a large part of this superfamily. Here we are concerned only with the cell adhesion molecules. Their functions are wide ranging with some members functioning as true signal-transducing receptors, whereas others have predominantly adhesive functions. Ligands for Ig family members include other Ig family members (identical, as well as non-identical members) such as NCAM binding to itself, but also members of the integrin family (such as for the ICAMs, which bind β_2 integrins) and components of the extracellular matrix, for example collagen binding for myelin-associated glycoprotein, a neuron-expressed Ig family member. Signaling pathways activated by Ig family members include MAP kinase pathways (Isacke and Horton, 2000; Hubbard and Rothlein, 2000).

Syndecans

Syndecans (Elenius and Jalkanen, 1994; Tkachenko *et al.,* 2005) are a family of cell surface proteoglycans, varying in size from 20 to 45 kD. They are type I transmembrane proteins characterized by heparin sulfate and chondroitin sulfate attachment sequences on the extracellular N-terminus of their single polypeptide chain. There is little homology in the rest of their extracellular domain, but their single transmembrane domain and short cytoplasmic domains are highly conserved. The cytoplasmic domain contains three regions: C1, closest to the plasma membrane, and C2 at the C-terminal end are highly conserved in all syndecans, contain tyrosine residues that may be important in signaling events and both allow binding of intracellular proteins. C1 additionally appears to be important in dimerization. C1 and C2 enclose a variable region that differs extensively between the four mammalian syndecans. Syndecans are thought to function predominantly as coreceptors for other receptors such as integrins, members of the fibroblast growth factor family, vascular endothelial cell growth factor, and transforming growth factor β, which need heparin sulfate for signaling. In such situations the signaling is thought to occur via the cytoplasmic domains of associated receptors rather than the syndecan molecule itself, although more recently more direct involvement with signaling events, mediated via syndecan-associated cytoplasmic molecules has been suggested (see Tkachenko *et al.,* 2005).

There are four mammalian syndecans known, with syndecan-1 best studied so far. Syndecan-1 is the major syndecan of epithelia and can function as a cell-matrix receptor binding various matrix proteins (type I collagen, fibronectin, tenascin), and in addition can bind members of the FGF family. It appears that in different cell types syndecan-1 can have different patterns of glycosaminoglycans attached to its core protein and this influences the ligand-binding capabilities. Thus, where in one cell type syndecan-I may contain heparin sulfate as well as chondroitin sulfate side chains and bind collagen, this may not be the case in another cell type in which it has heparin sulfate side chains only. Syndecan-2 is the main form in mesenchymal cells and is present in neuronal tissues alongside syndecan-3. Syndecan-4 is present in many cells that form stable adhesions and *in vitro* is consistently present in focal adhesions (Fears and Woods, 2006). The functions of syndecans are varied with roles in cell–matrix interaction and cell proliferation. Signal transduction, in addition to associated tyrosine kinases, may also include cytoskeletal proteins through association with actin and tubulin.

CD44

CD44, also known as the hyaluronate receptor, is a family of transmembrane glycoproteins with molecular weights of 85 to 250 kD. They share an N-terminal region that is related to the cartilage proteoglycan core and link proteins. Alternative splicing of 10 exons and extensive post-translational modification such as glycosylation and addition of chondroitin sulfate produces the wide variety of CD44 proteins. Chondroitin sulfate containing variants can bind fibronectin, laminin, collagen, and osteopontin in addition to hyaluronate. Finally, CD44 may also bind homotypically. CD44 therefore functions in a variety of ways including in cell–cell interaction (homing of lymphocytes or cell clustering), but also in cell–matrix adhesion. Malignant transformation of cells leads to upregulation of CD44 expression and metastatic tumors often express an altered repertoire of CD44 variants. More recently evidence has pointed to a more complex role of CD44 than simply as a cell–cell or cell–matrix adhesion molecule. It is becoming apparent that CD44 can be enzymatically cleaved and that functional fragments from the cytoplasmic domain can act as transcriptional regulators, whereas functional fragments from the ectodomain circulate in body fluids or can become incorporated in the matrix and thereby modulate cellular behavior (Cichy and Pure, 2003).

Selectins

Selectins are a family of three closely related glycoproteins (P-selectin expressed on platelets and leukocytes, E-selectin expressed on endothelial cells, and L-selectin expressed on leukocytes, monocytes, neutrophils, and eosinophils). Their common structure consists of an N-terminal Ca^{2+}-dependent lectin type domain, an EGF domain and variable numbers of short repeats homologous to complement-binding sequences, a single transmembrane region, and a short cytoplasmic domain. Their molecular weights range from 74 to 240 kD, with differently glycosylated forms expressed in different cell types. In general, the function of selectins is in leukocyte trafficking. They are involved in the earliest stages of leukocyte extravasation where binding of the selectin ligand on the leukocyte to selectins expressed on the endothelial surface results in weak intercellular interactions and "rolling" of leukocytes over the endothelial surface. These functions have now been confirmed in selectin knockout mice for L- and P-selectin In a double knockout for E- and P-selectin, it appeared that E-selectin also contributes to leukocyte homeostasis (reviewed in Frenette and Wagner, 1997). Other functions for selectins are in β integrin activation and O_2^- production by leukocytes. Selectin ligands are specific oligosaccharide sequences in sialated and often in sulfated glycans, such as sialyl-Lewis^x. There remains some uncertainty about the natural ligands for selectins. Molecules that fulfill most of the criteria for true selectin ligands (i.e., with confirmation of a biological role), such as CD24 and P-selectin glycoprotein ligand-1 PSGL-1, can, when expressed on the neutrophil surface in a properly glycosylated and tyrosine sulphated form, bind to P-selectin in vascular endothelium and to L-selectin on other neutrophils to enable "rolling" over either surface. CD34, GlyCAM-1, and MAdCAM-1 are other candidate ligands for L-selectin and E-selectin ligand-1 for E-selectin (reviewed by Ehrhardt *et al.*, 2004; Buzás *et al.*, 2006). The signal transduction pathways linked to selectins are only partially elucidated (Crockett-Torabi, 1998). As with the other classes of adhesion molecules discussed earlier they include tyrosine phosphorylation cascades and increases in intracellular calcium.

DISTRIBUTION AND FUNCTION OF ADHESION RECEPTORS IN BONE

There is a recent and increasingly extensive literature on the expression of cell adhesion molecules by the stromal and matrix-forming components of the skeleton—osteoblasts, osteocytes, and chondrocytes (further discussed later). For each cell type, a number of receptors, including integrins, have been detected and experimental data supports a functional role for adhesion molecules in bone and cartilage homeostasis. In osteoclasts only five integrins have been described, and there is little evidence for expression of other adhesion proteins by mature osteoclasts other than CD44 (Athanasou and Quinn, 1990; Hughes *et al.*, 1994; Nakamura *et al.*, 1995) and possibly some cadherin family members (Mbalaviele *et al.*, 1995, 1998; Ilvesaro *et al.*, 1998; see Chapter 18). Moreover, there is strong functional information that demonstrates that antagonism of osteoclast integrins leads to a downregulation of osteoclastic bone resorption; this effect has clinical implications with drugs based upon this effect reaching the clinic.

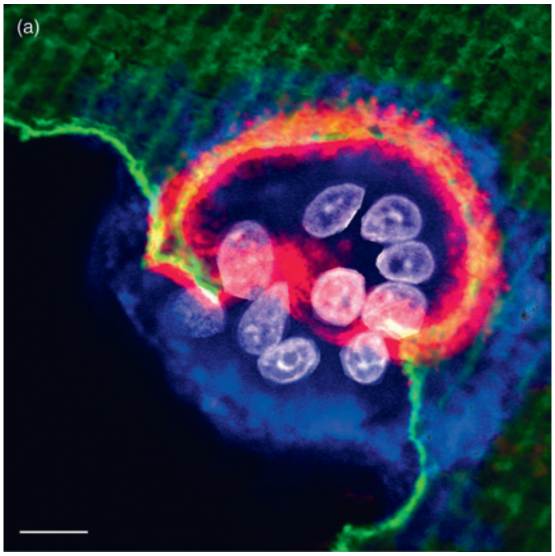
In the next sections we discuss adhesion molecule expression by osteoclasts, osteoblast lineage cells, and chondroblasts/chondrocytes separately, including evidence for their functional roles, and the targeting of adhesive interactions as therapeutic approaches in bone disease.

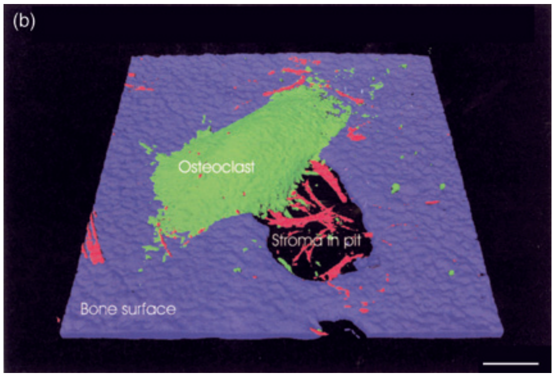
OSTEOCLASTS

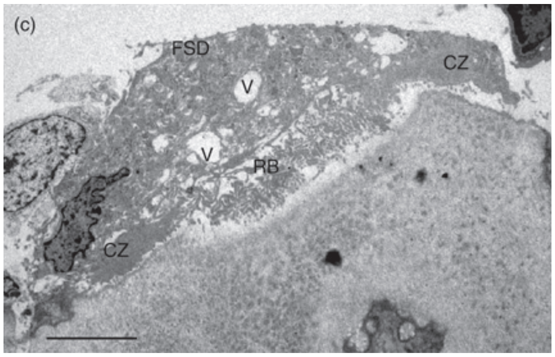
Expression of Integrins and Role in Osteoclastic Bone Resorption

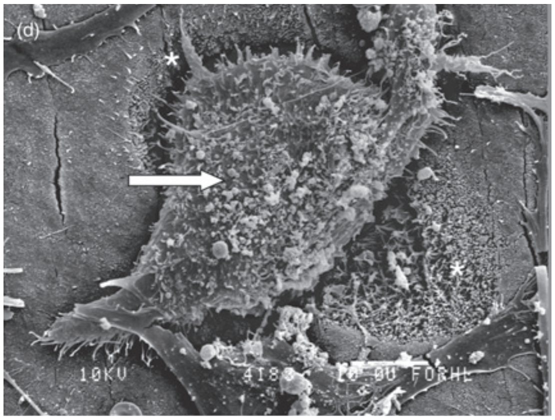
The first suggestion that adhesion receptors played a functional role in osteoclastic bone resorption was obtained when the monoclonal antibody 13C2 (Horton *et al.*, 1985) was found to inhibit bone resorption *in vitro* by human osteoclasts from the giant cell tumor of bone, osteoclastoma (Chambers *et al.*, 1986). It was later established that the inhibitory effect was mediated via the $\alpha_v\beta_3$ vitronectin receptor, a member of the integrin family of cell adhesion molecules (Davies *et al.*, 1989).

Subsequent detailed phenotypic (reviewed in Horton and Davies, 1989; Horton and Rodan, 1996; Helfrich and Horton, 2006) and biochemical analyses (Nesbitt *et al.*, 1993) demonstrated that mature mammalian osteoclasts express three integrin dimers: $\alpha_v\beta_3$, the "classical" vitronectin receptor; $\alpha_2\beta_1$, a collagen/laminin receptor; and $\alpha_v\beta_1$, a further "vitronectin receptor" (data summarized in Horton and Rodan, 1996; Helfrich and Horton, 2006; but first demonstrated for β_3 by Beckstead *et al.*, 1986 and Horton, 1986) (see Fig.1, see also color plate, and Table III). Mostly, the findings have been consistent among studies, and, where analysis has been possible, across species. More recently, a fourth integrin has been reported on mammalian osteoclasts, $\alpha_9\beta_1$, which is expressed from early osteoclast development stages through to mature osteoclasts (Rao *et al.*, 2006). A third "vitronectin receptor," $\alpha_v\beta_{5,}$ is expressed in osteoclast precursors (Inoue *et al.*, 1998, 2000), but levels in mature mammalian osteoclasts are low to undetectable (Shinar *et al.*, 1993; Nesbitt *et al.*, 1993). There have been reports that osteoclasts may express α_3 (Grano *et al.*, 1994) and α_5 (Steffensen *et al.*, 1992; Hughes *et al.*, 1993; Grano *et al.*, 1994), although this has not been a general finding. Though less extensively studied, some differences have been noted with avian osteoclasts, which additionally express β_2 integrins (Athanasou *et al.*, 1992), $\alpha_5\beta_1$, and, unlike in mammals,









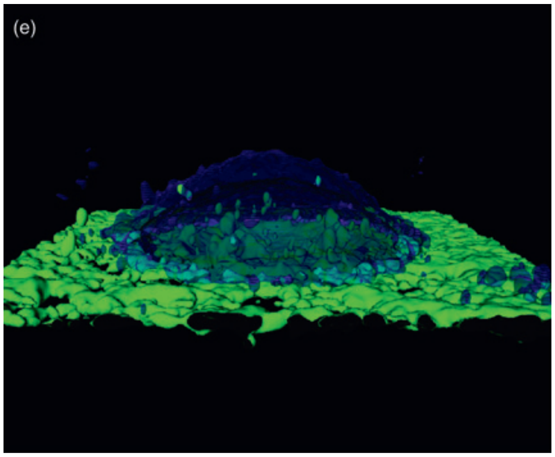


FIGURE 1 Five different microscopic views of osteoclasts to illustrate the different membrane areas involved in adhesion to matrix, bone resorption and transcytosis of resorbed matrix products (see also color plate). (a) A confocal microscopical image of a resorbing osteoclast on a piece of dentine *in vitro*. This image shows an optical slice through the area in direct contact with the matrix. The clear zone, rich in actin is shown in red (stained with phalloidin), the osteoclast membrane is stained by anti $\alpha_v\beta_3$ antibody 23C6 (blue), nuclei are stained by a DNA-binding dye (white) and the mineralized surface is stained with a fluorescent bisphosphonate (green). The resorbed area is contains no fluorescent material and shows in black. Scale bar is 10 μm. Image kindly provided by Dr. F. P. Coxon, University of Aberdeen. (b) A three-dimensional image of a site of osteoclastic bone resorption. The isosurface image of an *in vitro* site of osteoclastic resorption was constructed from a series of optical sections gathered by immunofluorescence confocal microscopy (Nesbitt *et al.*, 2000) using Bitplane Imaris software. Immunostaining shows the $\alpha_v\beta_3$ integrin in green, the matrix proteins at the bone surface in blue, and the cortical F-actin in the surrounding stromal cells in red. The osteoclast (predominantly stained green) is resorbing through bone (in blue), and a trail of resorption (in black) appears behind the osteoclast in which the stromal cells (which do not express $\alpha_v\beta_3$ and thus show as red) are seen to follow. Original magnification: ×630; scale bar 10 μm. (c) Transmission electron micrograph of an osteoclasts in a bone section illustrating the actin-rich clear zone (CZ) surrounding the ruffled border area (RB) and the basolateral surface (arrows) and the functional secretory domain (FSD) where transcytosed products are released. Osteoclasts are rich in a variety of vesicles (V), including small vesicles that only become clear at higher magnification, which transport enzymes, and protons to the RB and transcytose degraded collagens to the functional secretory domain on the opposite site of the cell. Currently little information is available about precise characterization of individual vesicles within the cells. Scale bar is 5 μm. (d) Scanning electron micrograph of an osteoclast on a slice of dentine *in vitro*. This image illustrates the adhesion of the cell in three dimensions. The functional secretory domain is rich in microvilli and is arrowed. Nonresorbing osteoclasts have a less elaborate surface structure. The resorption pit is indicated with an asterisk. Scale bar is 10 μm. Image kindly provided by Mr. K. S. Mackenzie, University of Aberdeen. (e) Computer-assisted three-dimensional reconstruction of a bone-resorbing osteoclast. The isosurface image of an *in vitro* site of osteoclastic resorption was constructed from a series of optical sections gathered by immunofluorescence confocal microscopy using Bitplane Imaris software (as described in Stenbeck and Horton, 2004). Rabbit osteoclasts plated on dentine were incubated for two minutes with low molecular weight fluorescently labeled dextran (green) before fixation and immunodecoration with antibodies directed against the $\alpha_v\beta_3$ integrin (blue). The negatively charged dextran binds to the dentine surface and accumulates in the resorption pit from where it is taken up by the cell in vesicular carriers. At early time points the dextran is found exclusively in close proximity to the bone surface and the pit. (See plate section)

TABLE III Integrin and Other Receptors Expressed by Mature Mammalian Osteoclasts[a]

	Receptor/integrin chain
Present[b,c]	$\alpha_v\beta_3$ ("vitronectin receptor") $\alpha_2\beta_1$ $\alpha_v\beta_1$ $\alpha_9\beta_1$ CD44H
"Not detected"	α_1, α_{3-9}, α_E β_2 and CD11, a, b, c, α_d β_{4-8} gpIIb (αIIb)

[a] *Data summarized from immunological analysis of human and rodent species, biochemistry of human giant cell tumor osteoclasts and gene deletion studies (as discussed in the main text). Some reports have suggested the presence of* $\alpha_5\beta_1$ (Grano et al., 1994; Hughes et al., 1993; Steffensen et al., 1992) *and* $\alpha_3\beta_1$ (Grano et al., 1994) *in osteoclasts. Some differences have been noted in avian osteoclasts* (Athanasou et al., 1992). *Expression of* α_{10} *and* α_{11} *has not been examined in osteoclasts. Aside from a publication describing expression of a truncated form of* β_3 (Kumar et al., 1997) *in osteoclasts, no detailed analysis of "splice variants" has been reported.*

[b] β_5 *is found on osteoclast precursors and replaced by* β_3 *in mature cells;* LFA-1 ($\alpha_L\beta_2$) *and* ICAM-1 *are expressed by osteoclast precursors only (see main text).*

[c] *There are limited data on expression of cadherins (E-cadherin) in osteoclasts* (Mbalaviele et al., 1995, 1998; Ilvesaro et al., 1998.

$\alpha_v\beta_5$ (Ross *et al.*, 1993); these latter integrins act as fibronectin receptors.

Adhesion of osteoclasts to the bone surface involves the interaction of osteoclast integrins with extracellular matrix proteins within the bone matrix (Table VII). There have been extensive reports on *in vitro* phenotypic analysis, and functional assays in several species. The $\alpha_v\beta_3$ vitronectin receptor mediates RGD peptide-dependent adhesion to a wide variety of proteins containing the RGD sequence, including bone sialoproteins and several extracellular matrix and plasma proteins, and osteoclast adhesion and resorption is inhibited by vitronectin receptor antibodies (Horton and Davies, 1989; Sato *et al.*, 1990, 1994; Horton *et al.*, 1991, 1993, 1995; Helfrich *et al.*, 1992a; Flores *et al.*, 1992, 1996; Hultenby *et al.*, 1993; Ross *et al.*, 1993; Ek-Rylander *et al.*, 1994; van der Pluijm *et al.*, 1994; Gronowicz and Derome, 1994). Which protein constitutes the natural ligand(s) of osteoclasts in bone remains to be determined. Co-localization studies have suggested that osteopontin is a candidate (Reinholt *et al.*, 1990), because it was found to be enriched underneath the clear zones of resorbing osteoclasts. However, because osteoclasts actively synthesize osteopontin (Dodds *et al.*, 1995) this finding should be interpreted with some reservation.

The function of $\alpha_2\beta_1$ in mammalian osteoclasts is predominantly as a receptor for type I collagen. In contrast to other cell types, adhesion of osteoclasts to collagen appears to be RGD-dependent (Helfrich *et al.*, 1996), although this could possibly be explained as a dominant-negative effect of RGD occupation of the abundant $\alpha_v\beta_3$ receptors on osteoclasts. Antibodies to α_2 and β_1 integrin inhibit resorption by human osteoclasts *in vitro*, but not to the same extent as anti-vitronectin receptor antibodies. Avian osteoclasts express abundant β_1 integrin, but do not adhere to collagen (Ross *et al.*, 1993) and probably use β_1 integrin predominantly in association with α_5 as a fibronectin-binding receptor. The role of $\alpha_v\beta_1$ on osteoclasts has not been explored in functional assays because no receptor complex-specific antibodies are available at present. This receptor is far less abundant than $\alpha_v\beta_3$ and $\alpha_2\beta_1$ in osteoclasts (Nesbitt *et al.*, 1993). By analogy with other cell types it is likely that $\alpha_v\beta_1$ functions as a receptor for collagen or fibronectin in osteoclasts. The integrin $\alpha_9\beta_1$ allows binding to the disintegrin domain of the autocrine factor ADAM8, which interestingly does not contain an RGD sequence, but instead a RX_6DLPEF sequence, which generally acts as a $\alpha_9\beta_1$ recognition sequence (Rao *et al.*, 2006). Antibodies to α_9 inhibit osteoclast formation and resorption *in vitro* (Rao *et al.*, 2006).

The demonstration that antibodies recognizing the vitronectin receptor block osteoclast adhesion, combined with the limited integrin repertoire of these cells, suggested that it may be possible to influence bone resorption *in vitro*, either by RGD-containing peptides or by function-blocking antibodies to osteoclast integrins (Chambers *et al.*, 1986; Horton *et al.*, 1991; van der Pluijm *et al.*, 1994; reviewed in Horton and Rodan, 1996; Helfrich and Horton, 2006). The observation that the RGD sequence containing snake venom protein, echistatin, blocked bone resorption supported this hypothesis (Sato *et al.*, 1990). Subsequently, these findings were confirmed using linear and cyclic RGD peptides, peptidomimetic agents (Engleman *et al.*, 1997), snake venom proteins, and antibodies to α_v and β_3 components of the vitronectin receptor and by the use of anti-sense oligodeoxynucleotides (Villanova *et al.*, 1999) in a variety of *in vitro* systems (reviewed in Horton and Rodan, 1996; Helfrich and Horton, 2006). Occupation of the vitronectin receptor by antibodies or RGD peptides *in vitro* causes osteoclasts to retract and detach from matrix, similar to the shape changes observed after administration of the potent anti-resorptive peptide hormone, calcitonin. In mammalian osteoclasts this effect is preceded by a rise in intracellular calcium localized predominantly to the nucleus (Shankar *et al.*, 1993; Zimolo *et al.*, 1994).

In vivo, echistatin and kistrin induce hypocalcemia in rats (Fisher *et al.*, 1993; King *et al.*, 1994): the former in the PTH-infused thyroparathyroidectomy model and the latter in parathyroid hormone-related protein (PTHrP)-induced hypercalcemia. Small cyclic RGD-containing peptides and peptidomimetics (Engleman *et al.*, 1997) also induce hypocalcemia in the former model. The inhibition seen *in vivo*, taken with the RGD sequence specificity observed with mutant (non-RGD sequence containing) echistatin (Fisher *et al.*, 1993; Sato *et al.*, 1994), suggests that integrins are mediating their hypocalcemic effect by inhibiting osteoclastic bone resorption. Direct action on an osteoclast integrin was demonstrated first by showing that a function-blocking antibody, F11 (Helfrich *et al.,* 1992b), to the rat β_3 chain of the osteoclast $\alpha_v\beta_3$ integrin is hypocalcemic in the rat thyroparathyroidectomy model (Crippes *et al.,* 1996). Second, infusion of echistatin or peptidomimetics totally blocked the acute loss of trabecular bone seen in secondary hyperparathyroidism (Masarachia *et al.,* 1998) and following ovariectomy in the mouse (Engleman *et al.,* 1997; Yamamoto *et al.,* 1998). This latter observation strongly suggests that the inhibitory effect of RGD occurs via a direct action on bone, most likely via the $\alpha_v\beta_3$ integrin on osteoclasts. The impressive effects of RGD peptide analogues developed by the pharmaceutical industry on bone resorption has led to their introduction into clinical trials for bone diseases associated with excessive bone resorption such as osteoporosis (discussed later in the chapter).

Integrin Gene Knockout Studies

Gene knockout studies where β_3 integrins have been deleted in the mouse have underscored the central role of $\alpha_v\beta_3$ integrin in osteoclast biology (McHugh *et al.,* 2000). β_3 null mice are relatively normal at birth but develop a mild but progressive osteosclerosis by adulthood; this is associated with *in vitro* evidence of abnormal osteoclast adhesion and bone resorption. They are protected against bone loss after ovariectomy (Zhao *et al.,* 2005a), a finding that, together with the reported downregulation of β_3 expression by estradiol in osteoclasts *in vitro* (Saintier *et al.,* 2004), suggests that the bone-sparing effects of estrogen may be in part through direct effects on osteoclasts. The critical role of β_3 integrin in osteoclasts is also underscored by its role in promoting osteoclast survival and in regulating apoptosis (Zhao *et al.,* 2005b). α_v knockout mice die perinatally because of vascular defects (Bader *et al.,* 1998) and osteoclast defects have therefore not been studied extensively. However, mice from both single chain deletions have shown normal skeletal development, contrary to the prediction that bone modeling would be significantly affected, suggesting that other integrins compensate in the process of bone recognition by osteoclasts. Indeed, this has been confirmed in a study with peripheral blood mononuclear cell cultures from patients with Glanzmann thrombasthenia who carry a β_3 integrin null mutation and, despite which, grow to skeletal maturity without apparent abnormality. In the face of absent $\alpha_v\beta_3$ there is an upregulation of $\alpha_2\beta_1$ collagen-binding receptor in osteoclasts generated in such cultures, which enables bone resorption to proceed, albeit to a reduced extent (Horton *et al.,* 2003).

A single knockout for β_5 and a double knockout for β_3/β_5 have been made (see Sheppard, 2000) and neither show apparent osteoclast malfunction *in vivo*. However, upon ovariectomy, the β_5 knockout loses dramatically more bone and osteoclast formation *in vivo* and *in vitro* is accelerated. This shows that the β_5 integrin has the opposite effect to β_3 in osteoclasts and acts as an inhibitor of osteoclast formation (Lane *et al.,* 2005).

Deletion of β_1 integrin leads to early embryonic death. There is no specific information about β_1 function in osteoclasts from conditional knockouts and transgenic models as studies have focused mainly on osteoblast function (see further later in chapter). It would be informative to examine bone cell function in tissue-specific conditional β_1 knockout mice; these have yet to be created with the appropriate targeting characteristics though the Fässler group have created hemopoietic lineage-specific knockouts (Nieswandt *et al.,* 2001), suggesting that it should be possible to generate osteoclasts from β_1 null myeloid precursors.

Mice with a deletion of α_9 (Huang *et al.,* 2000) show an osteopetrotic phenotype in keeping with the reduced activity of osteoclasts treated with α_9 function blocking antibody *in vitro* (Rao *et al.,* 2006).

A number of other knockouts, either natural or engineered, of adhesion molecules are relevant in this context, although some unexpectedly do not show a skeletal phenotype. Osteoclasts develop from "monocytic" precursor cells that express CD11/CD18 (LFA and β_2 integrins; Lader *et al.,* 2001). Interestingly, given the severe myeloid functional defects seen in knockout mice and patients with leukocyte adhesion deficiency (LAD I), no bone (osteoclast) functional or development defect has been reported in these patients. However, animal studies suggest further studies in this patient group are required, because Tani-Ishii and coworkers (2002) reported reduced osteoclast generation from LFA-1 deficient precursors *in vitro* at the level of interaction with the osteoblast counterreceptor, ICAM-1, whereas Miura and coworkers (2005) studied mice with a deletion of β_2 and reported an osteoporotic phenotype, caused by abnormalities in the bone marrow stromal stem cell population, leading to reduced osteogenic potential. Osteoclast formation and resorptive activity was not affected in the β_2 knockout mice, probably because increased leukocytosis is balanced by the reduction in osteoclast formation owing to absence of LFA-1.

Recently, two groups have described the phenotype of α_2 knockout mice (Holtkötter *et al.,* 2002; Chen *et al.,* 2002). As in the β_3 null mouse, the skeleton is grossly normal at birth, though a detailed analysis of changes on skeletal maturation or of osteoclast function has not yet been reported. Given our results with β_3 null GT patients, such studies should be undertaken to further elucidate the role of α_2 integrin in bone and, by inference, its interplay with $\alpha_v\beta_3$.

Non-Integrin Receptors in Osteoclasts

Early studies (Horton and Davies, 1989) were carried out to assess the expression of non-integrin adhesion receptors in osteoclasts. These suggested that a range of adhesion receptor families aside from integrins were absent from osteoclasts. More recently, data have been published indicating that indeed osteoclasts express some non-integrin adhesion proteins such as members of the cadherin family (see discussion later) and the 67-kDa laminin receptor, Mac-2 (Takahashi *et al.,* 1994).

Although some of these proteins are not major components of mature osteoclasts, or only present on a subpopulation of "immature" osteoclasts, it is possible that they could play a significant role in osteoclast development, fusion, or functional maturation from hemopoietic stem cells. They are discussed in more detail in the following section.

Cadherins

The first study on cadherin expression in mature osteoclasts (Mbalaviele *et al.,* 1995) reported expression of E-cadherin, and absence of P- and N-cadherin. This study also suggested that *in vitro* osteoclast development and fusion requires expression of E-cadherin, although it remains to be firmly established whether this is at the level of the osteoclast precursor, or whether E-cadherin expression is required in the accessory cells. In support of the former, a role for cadherin-mediated interactions in early hemopoietic development has been suggested (Puch *et al.,* 2001). In support of a role for cadherins in mature osteoclasts, it was shown that a cadherin dimer disrupting HAV peptide inhibited bone resorption (Ilvesaro *et al.,* 1998). Interestingly, pan-cadherin immunostaining in osteoclasts revealed prominent staining in the clear zone, suggesting a possible role for cadherin in creating a close contact with bone matrix (Ilvesaro *et al.,* 1998).

Immunoglobulin Family Members

There is good evidence for expression of receptors of the Ig family in osteoclasts precursors. Functional evidence implies ICAM-1 and VCAM-1 in osteoclast development *in vitro* (Feuerbach and Feyen, 1997; Harada *et al.,* 1998; Nakayamada *et al.,* 2003) and as discussed in the osteoblast section later in this chapter, osteoclast precursors, i.e., monocytes, not only express ICAM-1, but also its ligand LFA-1, allowing for cell–cell adhesion and possibly cell fusion (Harada *et al.,* 1998). Now that osteoclasts can be generated in absence of osteoblasts in defined cultures with RANKL and M-CSF, it will be possible to study in more detail the expression and functional role of this important class of adhesion molecules during osteoclast differentiation and in mature osteoclasts.

Syndecans

There is no published information about expression of syndecans in mature osteoclasts or for a role during osteoclast development.

CD44

CD44, for which several possible ligands are expressed in bone (Tables II and VII), is highly expressed in osteoclasts *in vivo* (Athanasou and Quinn, 1990; Hughes *et al.,* 1994; Nakamura *et al.,* 1995; Nakamura and Ozawa, 1996) and in osteoclasts generated *in vitro* (Flanagan *et al.,* 2000). Detailed studies of sites of expression have so far been confined to rodent osteoclasts where expression is at the basolateral membrane, rather than the clear zone or ruffled border of resorbing osteoclasts (Nakamura *et al.,* 1995). Kania and coworkers (1997) described that mouse osteoclast formation *in vitro* was inhibited by CD44 antibodies, whereas the resorptive capacity of mature osteoclasts was not affected and these *in vitro* data were confirmed when it was demonstrated that the interaction between CD44 and osteopontin is involved in osteoclast migration, fusion, and bone resorption (Suzuki *et al.,* 2002; Chellaiah *et al.,* 2003). However, two independently generated CD44 null mice show no differences in osteoclast formation, fusion, or bone resorption and only small differences in bone mass and anatomy (Cao *et al.,* 2005), suggesting that there is an increased level of complexity and redundancy in the actions of CD44 *in vivo*. This has also been highlighted by the finding that the role of CD44 in osteoclast formation *in vitro* is dependent on the culture substrate and that it plays no role when cells are grown on bone (de Vries *et al.,* 2004). In contrast, Hayer *et al.* (2005) have demonstrated a more complex relationship between CD44 and TNF-mediated inflammation; in their knockout model, increased osteoclast numbers and functional activation led to inflammatory bone loss. Because CD44 is expressed on circulating osteoclast precursors, such as CD14$^+$ cells (Kindle *et al.,* 2006), it is possible this receptor plays a role in homing to sites of future resorption (discussed later in the chapter).

Selectins

So far there are no reports describing expression of selectins on mature osteoclasts. Selectins are likely, however, to have a role during osteoclast development. Osteoclast precursors are present in the circulation and mature osteoclasts can be generated *in vitro* from mononuclear cells in blood. It remains unclear how osteoclast precursors *in vivo* reach the sites in bone where they differentiate into fully active osteoclasts, but this must at some point include extravasation, a process that, for all lymphoid cells at least, initially involves selectin-mediated interaction with endothelial cells, followed by integrin-mediated processes. Osteoclast-endothelial interactions are clearly important in osteoclast homing (McGowan *et al.,* 2001; Kindle *et al.,* 2006), and CD44 as well as ICAM-1/β_2 integrin interactions have been implicated in osteoclast precursor-endothelial adhesion and transmigration (Kindle *et al.,* 2006). The role of selectins in this setting remains unexplored. No obvious bone abnormalities have been reported in the selectin null mice. However, mice lacking E-selectin or E- and P-selectin show accelerated development of collagen-induced arthritis in mice, demonstrating a clear role for selectins in inflammation-induced joint disease (Ruth *et al.,* 2004) even though this seems largely mediated through enhanced lymphocyte recruitment to the site. The role of selectins in recruitment of osteoclast precursors deserves further detailed study as modulation of selectin-ligand binding might offer therapeutic potential in diseases where osteoclast formation is increased.

Adhesion Molecules and Osteoclast Development

The question of which adhesion receptors are expressed during the development of osteoclasts from stem cells to committed, mononuclear, postmitotic precursors has been difficult to address *in vivo*. This, in part, reflects the difficulty in isolating these cells from bone, prior to fusion, although they are identifiable within the periosteum of developing bone anlagen as TRAP-positive, calcitonin-binding mononuclear cells that express $\alpha_v\beta_3$. Evidence has been gained by using antibody or peptide inhibition in short-term murine and human peripheral blood or bone marrow cultures. Interpretation of such studies can prove problematic, as inhibitory effects can easily be indirect via other cell types critical for osteoclast differentiation, such as osteoblasts or marrow stromal cells. Nevertheless, rodent osteoclast development *in vitro* is inhibited by the RGD-containing snake venom protein echistatin (Nakamura *et al.,* 1998a), implying a role for the vitronectin receptor or other RGD-sensitive integrin receptors. In contrast, osteoclast size or numbers are not altered greatly in rodents treated chronically with $\alpha_v\beta_3$ antagonists, suggesting no major influence on osteoclast differentiation or fusion *in vivo* as does the fact that the *formation* of multinucleated osteoclasts, albeit not their resorptive function, proceeds normally in β_3 knockout mice (McHugh *et al.,* 2000). $\alpha_v\beta_5$ integrin is expressed in osteoclast precursors such as immature bone marrow macrophages (Inoue *et al.,* 1998, 2000), but no longer present at high levels in mature osteoclasts; again knockout studies imply a more subtle role for β_5 in osteoclast biology *in vivo*, as there is no overt bone phenotype of the β_5 knockout at birth (Sheppard, 2000). Studies with antibodies to $\alpha_2\beta_1$ (Helfrich *et al.,* 1996), presently limited to resorption and adhesion assays, suggest that a role in osteoclast fusion for this class of integrin is a distinct possibility. E- (but not P- or N-) cadherin has been reported to be expressed by human and rodent osteoclasts (Mbalaviele *et al.,* 1995, 1998). Function-blocking antibodies to E-cadherin and adhesion blocking "HAV peptide" inhibit osteoclast formation and fusion *in vitro*, as well as resorption by mature osteoclasts, supporting the view that this class of receptor may be active *in vivo* (Mbalaviele *et al.,* 1995; Ilvesaro *et al.,* 1998). However, because there is strong evidence for many cadherin types in osteoblasts (see later; Chapter 18), indirect effects may be more likely. There is also some initial evidence for the involvement of the β_2 integrins Mac-1 and LFA-1 and α_4 and their respective counterreceptors ICAM-1 and VCAM-1 (Kurachi *et al.,* 1993; Duong *et al.,* 1994, 1995; Tani-Ishii *et al.,* 2002). The expression and possible role of CD44 was discussed earlier and clearly this molecule is expressed during differentiation and may have a role *in vivo* in homing of osteoclast precursors. Osteoclast cultures from isolated monocytes ($CD14^+$) with synthetic RANKL and MCSF allow for more careful monitoring of adhesion receptor expression by PCR and/or immunostaining. In these cultures no contamination with other cell types occurs. There has not, as yet, been a comprehensive study focused on adhesion molecules in this setting. Better knowledge of the range of adhesion receptors involved in osteoclast maturation prior to terminal function is important, as is further knowledge of the receptors that allow transendothelial migration and access to resorption sites. Inappropriate levels of such receptors may lead to imbalances in precursor numbers and could lead to imbalance in bone resorption.

Adhesion Molecules and Function of the Osteoclast Clear Zone

Osteoclasts resorb bone after a series of cellular polarization events (Fig.1). These compartmentalize the cell and are essential for bone resorption to proceed. After osteoclast attachment to the bone surface, the cell initiates a cytoskeletal rearrangement and creates a zone that separates the dorsal (basolateral) and ventral plasma membranes (Fig. 1a and 1c). This clear, or "sealing," zone is "organelle free" (hence the term *clear zone*), rich in actin filaments and is closely apposed to the bone surface (Holtrop and King, 1977). There has been some controversy recently about the exact mechanism that leads to the formation of the sealing zone. Early work by Väänänen and coworkers suggested that the sealing zone is derived from the "fusion" of podosomes (Lakkakorpi and Väänänen, 1991, 1996). Podosomes are actin-rich structures of cell-matrix adhesion and matrix degradation that are mainly formed by cells of the monocyte lineage. They are enriched in integrins and have unique two-part architecture: a core of F-actin and actin-associated proteins that is surrounded by a ring of plaque proteins such as talin or vinculin (Linder and Aepfelbacher, 2003). This structure is very reminiscent of the sealing zone, which is formed by a circular band of actin (Fig. 1a) surrounded by a double ring of vinculin (Väänänen *et al.,* 2000 and Chapter 8). Osteoclasts plated on glass or plastic form podosomes belts, which are enriched in matrix metalloproteases (Sato *et al.,* 1997; Delaissé *et al.,* 2000). Work by Jurdic and coworkers has shown that in mature osteoclasts these podosome belts are only formed in cells plated on glass, whereas bone-resorbing osteoclasts form sealing zones (Saltel *et al.*, 2004). These results imply that the sealing zone is a unique structure that is related to, but not derived from, podosomes. However, more recent work using electron microscopy to analyze the sealing zone structure indicates that the structural units between individual podosome and sealing zone are very similar, the main difference being their density and interconnectivity (Luxenburg *et al.*, 2007).

The sealing zone of resorbing osteoclasts maintains close apposition to the bone surface and encloses the secretory ruffled border "isolating" the acidic microenvironment of the resorption lacuna (Fig. 1c and see Chapter 8). Protons and proteases cross the ruffled border and solubilize the adjacent bone matrix through demineralization and proteolytic activity. Subsequently, bone matrix, including calcium and type I collagen fragments, are liberated and a resorption compartment forms beneath the cell. Osteoclasts use transcytosis to remove degraded matrix from the active sites of bone resorption (Nesbitt and Horton, 1997; Salo *et al.*, 1997, Vääräniemi *et al.*, 2004), enabling the osteoclast to maintain the integrity of the enclosed resorption site and facilitate cell migration and penetration into bone. Degraded bone matrix is endocytosed along the ruffled border and transported through the osteoclast in a vesicular pathway toward the basolateral surface of the cell; finally it enters the extracellular space via a specialized exocytotic site located at the cell apex (Salo *et al.*, 1996).

The fact that the resorption area must maintain the microenvironment enabling bone resorption, i.e., acid pH and high concentration of proteases, leads to the suggestion that a specialized adhesion structure in the sealing zone mediates proton impermeable tight attachment to the bone surface. Over the years several adhesion molecules have been proposed as candidates (see also previous section on cadherins in osteoclasts). The finding that osteoclast attachment to matrix-coated glass or bone is interrupted by integrin inhibitors led to the suggestion that the osteoclast tight seal may be mediated by integrins. Some data has supported the view that $\alpha_v\beta_3$ is enriched in clear zones of resorbing osteoclasts (Reinholt *et al.*, 1990; Hultenby *et al.*, 1993; Nakamura *et al.*, 1996a), as well as podosomes of osteoclasts cultured on glass (Zambonin-Zallone *et al.*, 1989; reviewed in Aubin, 1992). Others, however, have been unable to confirm this finding, reporting that vitronectin receptor is undetectable in the sealing zone (Lakkakorpi *et al.*, 1991, 1993; Nakamura *et al.*, 1999; Duong *et al.*, 2000). We (Väänänen and Horton, 1995) have argued previously that the dimensions of the integrin molecule, when compared to a membrane to bone gap of 2 to 10 nm, precludes a direct involvement of integrins in the maintenance of a "tight seal" during resorption, as opposed to a role in initial osteoclast attachment and cell movement that is not in dispute. Additionally, the finding that formation of the sealing zone is dependent only on the presence of apatite crystals also points to an unknown adhesion molecule (Saltel *et al.*, 2004). The molecular mechanism of the *attachment* process in the established clear zone of a resorbing, nonmigratory osteoclast thus remains to be established (Väänänen and Horton, 1995; Saltel *et al.*, 2004) and is likely to involve both cell autonomous characteristics of the osteoclast in combination with chemical and physical features of the bone matrix (Nakamura *et al.*, 1996b), as described later.

Early work by Lucht suggested, using an *in vivo* model, that there is no tight sealing zone as the endocytic marker, horseradish peroxidase, could be detected in the ruffled border area as early as five minutes after injection into the animal (Lucht, 1972). However, the generation of a low pH zone underneath the actively resorbing osteoclast, against a substantially different surrounding media, requires the presence of a diffusion barrier. An alternate hypothesis is that the apposition of the osteoclast to the bone surface is not as tight as predicted, less than with high resistance, ion-impermeable epithelial tight junctions, and that the restriction of ionic movement from under the resorbing osteoclast is a combined feature of substrate (i.e., bone matrix) and osteoclast activity. Proton pumping by the osteoclast alters the properties of the bone surface (Delaissé *et al.*, 1987; Everts *et al.*, 1988), which one could envisage would result in swelling and physico-chemical modification of the extracellular matrix. In fact, incubation of type I collagen under mild acidic conditions *in vitro* leads to the formation of a gel (Chandrakasan *et al.*, 1976), a process that one could also envisage taking place *in vivo* within the resorption pit. The generation of a collagenous gel at the resorption site would have a triple effect: (1) acting as a diffusion barrier by increasing the viscosity of the medium in immediate apposition to the resorption site and this in turn would decrease the diffusion coefficient of all substances in this area; (2) acting as ion exchange matrix, as, because of the basic pI of collagen, the resorption pit would acquire an overall positive charge that could serve as a trap for negatively charged molecules; and (3) providing a matrix for hydrophobic interactions through the high content of nonpolar amino acid residues ($\sim$74%) in type I collagen. Released protons are likely to be tethered readily to the inorganic components of the bone matrix, which would reduce their free concentration and their mobility. As a consequence, a localized low pH zone would be established only at the site of proton release that could be sufficient to activate secreted lysosomal enzymes (Fig. 2). The presence of such a pH gradient has also been suggested to accommodate the different pH requirements of collagenase and lysosomal enzymes in the resorption area (Delaissé *et al.*, 1993). During the resorption process, the low pH zone, followed by the collagenous gel zone, would advance further into the bone matrix thus providing a localized microenvironment for bone resorption without the need for a static, lateral tight seal. An efficient osteoclast endocytotic mechanism would be responsible for the removal of the reaction products before they leave the resorption area by diffusion (Fig. 2). In keeping with our model, negatively charged molecules with *Mw* up to 10,000 rapidly accumulate underneath actively resorbing osteoclasts. Live cell imaging shows that accumulation underneath the osteoclasts occurs as early as 30 sec after the addition of the low molecular weight markers (Stenbeck and Horton, 2000). As a consequence, in bone-resorbing osteoclasts externally added fluid phase markers are only endocytosed if they have access to the resorption area (Stenbeck and Horton, 2004). We could show that after internalization, these fluid phase markers follow a similar intracellular route as the digested bone matrix and we therefore used them to establish the kinetics of trafficking events originating at the ruffled border. Endocytosis and transcytosis from the ruffled border are fast processes, with a half-life of the endocytosed material inside the cells of 22 minutes (Stenbeck and Horton, 2004), providing further evidence for the dynamic model of the sealing zone (summarized in Fig. 2).

This dynamic model also accounts for the apparently contrary role of integrins in osteoclast resorption and fits well with structural features of the sealing zone (Nakamura *et al.,* 1996a; Luxenburg *et al.,* 2007). Without the need for a sterically close membrane-matrix contact zone, integrins could well be involved in the establishment and functioning of the sealing zone, in addition to their role in initial cell attachment and migration (Väänänen and Horton, 1995). Additionally, an attractive possibility is that integrins function as endocytic matrix receptors. Integrins, like the receptor $\alpha_v\beta_3$, are constantly endocytosed and recycled back to the plasma membrane making them ideal candidates for this function (Bretscher, 1996; Lawson and Maxfield, 1995). This cycling process is thought to promote cell motility by providing "fresh" adhesion receptors at the leading edge of the cell (Pellinen and Ivaska, 2006). However, several integrins have also been shown to regulate matrix turnover by endocytosis of bound ligand, which is then targeted for degradation (Ng *et al.,* 1999). This pathway is also used by several pathogens to gain access to host cells; and in an epithelial cell model transport of adenovirus across cells was increased by RGD peptides (Ivanenkov and Menon, 2000). If osteoclasts use a similar RGD-dependent mechanism in transcytosis, then candidate receptors involved in the uptake of bone matrix at the ruffled border would include $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrins that, respectively, bind native and denatured collagens (Nesbitt *et al.,* 1993; Helfrich *et al.,* 1996). Proteolysis of collagenous matrix exposes cryptic RGD sites (Holliday *et al.,* 1997) during bone resorption, and engagement with $\alpha_v\beta_3$ integrin could initiate endocytosis and subsequent transcytosis of denatured collagenous matrix. Conversely, higher concentrations of RGD peptides, produced after extensive matrix proteolysis, could inactivate matrix transcytosis and, thus, lead to cessation of resorption.

Another group of collagen-binding proteins in osteoclasts (Nesbitt *et al.,* 1994) are the annexins, a family of calcium-dependent phospholipid-binding proteins that exhibit a wide tissue distribution (reviewed by Raynal and Pollard, 1994). Annexins have been shown to participate in endocytosis, transcytosis, and exocytosis in several polarized cells (Creutz, 1992; Burgoynee, 1994; Futter and White, 2007), in addition to their role in a number of other cellular processes (Gerke *et al.,* 2005). Evidence shows that annexin II participates in matrix transcytosis during bone resorption (Nesbitt and Horton, 1999). It is found at the cell surface of resorbing osteoclasts and co-localizes with degraded bone matrix in the resorption pit; it is also highly expressed within the basolateral cell body and at apical exocytotic sites. Furthermore, the addition of exogenous annexin II to resorption cultures increases transcytosis of bone matrix and bone resorption by osteoclasts (Nesbitt and Horton, 1999). The ability of annexins to associate with membrane phospholipids in a calcium dependent

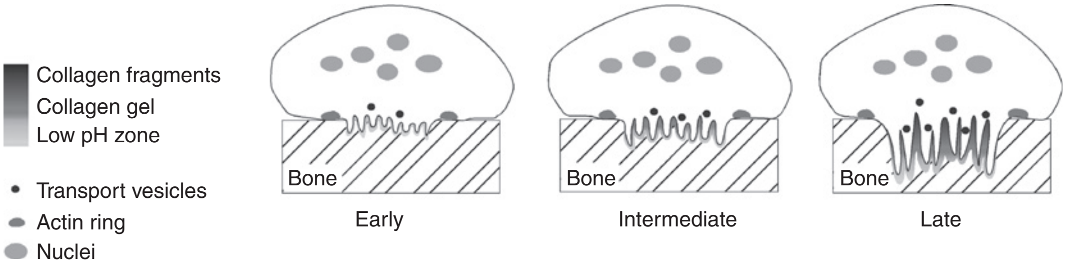


FIGURE 2 Model of the osteoclast sealing zone. During the early stages of resorption, the proton pump (VATPase) is inserted into the osteoclast plasma membrane that is enclosed by the actin ring (green). Proton extrusion by the VATPase leads to the formation of a localized low pH zone that dissolves the mineral content of the bone (yellow area). Proton movement is restricted by binding to hydroxyapatite. Lysosomal enzymes, secreted during the intermediate stages of resorption, digest the organic content of the bone in the low pH zone (red area). In the later stages of resorption, the ruffled border expands deep into the bone matrix by fusion of transport vesicles with the plasma membrane. The low pH zone moves with expansion of the ruffled border further into the bone matrix. The area behind the low pH zone consists of a collagenous "gel" that is endocytosed by the osteoclast (dark brown). This further restricts solute diffusion from below the osteoclast. However, because the net concentration of secreted and resorbed components is a balance between generation rate and limited diffusion rather than the presence of an impermeable barrier, externally added small molecules have access to the resorption area. Modified from Stenbeck and Horton (2000), with permission.

manner enables them to functionally organize membrane domains and recruit interacting proteins to these domains (Gerke *et al.*, 2005). It is thus tempting to speculate that annexins might be involved in formation and maintenance of the sealing zone by binding to hydroxyapatite and/or organization of the actin cytoskeleton (Hayes *et al.*, 2004), in addition to their function in osteoclast transcytosis. However, a detailed analysis of the role of annexins and integrins in matrix transcytosis and sealing zone maintenance has to await the possibility of performing live cell imaging of integrin turnover and annexin trafficking in bone-resorbing osteoclasts.

$\alpha_v\beta_3$-Mediated Signal Transduction

Signal transduction through osteoclast integrins is complex and discussed in detail in Chapter 9. Here we only introduce the key players involved in $\alpha_v\beta_3$ mediated signaling pathways associated with osteoclast polarization and rearrangement of the actin cytoskeleton that, as discussed before, is essential for bone resorption. A number of candidate molecules, such as c-Src, phosphatidylinositol 3-kinase (PI-3 kinase), FAK, and Pyk2, are expressed at high levels in osteoclasts (reviewed in Duong *et al.*, 2000; Golden and Insogna, 2004). c-Src knockout mice have severe osteopetrosis owing to lack of ruffled border formation in osteoclasts, clearly implicating this molecule in signaling cascade leading to polarization of osteoclasts (Soriano *et al.*, 1991). PI-3 kinase has been shown to be associated with the cytoskeleton in osteoclasts when attached to bone matrix and is associated with the β_3 subunit in bone-adherent osteoclasts (Lakkakorpi *et al.*, 1997). The importance of FAK in osteoclast function has been queried because no osteoclast abnormalities are seen in the FAK knockout mouse (Ilic *et al.*, 1995). However, Pyk2, another molecule of the FAK family with high homology to FAK and a similar role as adaptor protein orchestrating cytoskeletal architecture (Xiong and Feng, 2003), is highly expressed in osteoclasts (Tanaka *et al.*, 1995a) and seems to fulfill the role that FAK plays in less motile cell types (Duong *et al.*, 1998). Pyk2 has been shown to interact with gelsolin, an actin-binding protein involved in the formation of adhesive structures in osteoclasts and in bone resorption (Wang *et al.*, 2003; Chellaiah *et al.*, 2000). Following engagement of $\alpha_v\beta_3$ with ligand, clustering of the receptor occurs and association of its cytoplasmic domain with a complex containing Pyk2 and p130^{CAS} (Lakkakorpi *et al.*, 1991). Autophosphorylation of Pyk2 then leads to recruitment of c-Src and ultimately to cytoskeletal reorganization and bone resorption (Duong *et al.*, 2000; Lakkakorpi *et al.*, 2003). Activated c-Src also recruits and phosphorylates c-Cbl, which binds to the SH3 domain of c-Src and negatively regulates Src kinase activity (Tanaka *et al.*, 1996). It is suggested that this may enable transient adhesion and allow for cell migration, a process critically dependent on turnover of adhesive structures (Sanjay *et al.*, 2001). In addition, the Src-dependent phosphorylation of c-Cbl has been shown to be critical for osteoclastic resorption (Miyazaki *et al.*, 2004). In c-Src null osteoclasts, Pyk2 has been shown to associate with PLC-γ, a process that can be induced not only by adhesion, but also by M-CSF, and can lead to recruitment of integrin to adhesion contacts and cytoskeletal reorganization (Nakamura *et al.*, 2001). More recently, a number of additional molecules in this signaling cascade have been identified, largely through loss of function studies. The tyrosine kinase Syk and the Rac-specific guanidine exchange factor Vav3 were identified as key molecules downstream of $\alpha_v\beta_3$ in osteoclasts (Miyazaki *et al.*, 2004; Faccio *et al.*, 2005). The Syk knockout mouse displays a similar osteoclast phenotype to the Src knockout with absence of polarization *in vitro* and loss of osteoclast adhesion to bone *in vivo*. The Vav3 knockout is also osteopetrotic and, similar to the $\alpha_v\beta_3$ knockout mouse, protected from bone loss induced by systemic pro-resorptive stimuli. Zou and coauthors (2007) recently showed that activated $\alpha_v\beta_3$ recruits Syk, which is then phosphorylated by c-Src, mediated by two other crucial receptors, Dap 12 and FcRγ, and that this leads to cytoskeletal reorganization mediated by Vav3. Clearly, $\alpha_v\beta_3$ signaling is complex, but careful dissection of the pathways has already pointed to crucial nonredundant mediators of polarization and hence ability to resorb, and such molecules are clear candidate targets for antiresorptive therapies (see also Teitelbaum, 2007).

$\alpha_v\beta_3$ Integrin as a Therapeutic Target for Bone Disease

Early Studies and Rationale

Osteoporosis places a large and growing medical and financial burden on health services in developed countries; however, it remains a clinical area where, despite recent advances in therapy and diagnosis, there are still unmet needs. Although potent drugs have been developed, e.g., bisphosphonates (see Chapter 78), the pharmaceutical industry is still developing novel antiresorptive agents. The $\alpha_v\beta_3$ vitronectin receptor presents a key step in the process on bone resorption (as discussed earlier), which is being exploited by the pharmaceutical industry. In part this optimism is underscored by the finding that polymorphisms in the β_3 gene confer an increase in hip fracture (Tofteng *et al.*, 2007).

The development (for reviews, see Horton and Rodan, 1996; Hartman and Duggan, 2000; Miller *et al.*, 2000; Meyer *et al.*, 2006; Tucker, 2006) of a number of orally active, nonpeptidic integrin antagonists, particularly based on modification of the RGD peptide motif identified in fibronectin by Pierschbacher and Ruoslahti in 1984, suggests that treatment of a range of bone diseases may be susceptible to strategies that involve the blockade of integrin function or modulation of their expression. The development of $\alpha_v\beta_3$ antagonist drugs has been aided considerably by the prior existence of an analogous set of agents that have been developed for use in thrombosis (see references in Hartman and Duggan, 2000; Miller *et al.*, 2000); here, the platelet integrin fibrinogen receptor, gpIIbIIIa/$\alpha_{IIb}\beta_3$, which is structurally related to the $\alpha_v\beta_3$ integrin on osteoclasts and shares the same β chain, is targeted. These were the first of the integrin antagonist "drugs" that have been approved for clinical use (Coller, 1997; Phillips and Scarborough, 1997; Theroux, 1998) and they form the paradigm for potential application to bone disease.

The functional role of $\alpha_v\beta_3$ in osteoclast biology, first examined by Horton and colleagues in antibody studies over a decade ago, has been confirmed in a large battery of *in vitro* systems and *in vivo* proof of concept studies (as discussed earlier). Target specificity is aided by the *in vivo* distribution of $\alpha_v\beta_3$, which is expressed at high levels in osteoclasts (Horton, 1997). Much lower levels are found in platelets and megakaryocytes, kidney, vascular smooth muscle, some endothelia, and placenta (Horton, 1997). Thus, the therapeutic drug levels that would influence osteoclastic bone resorption are less likely to modify $\alpha_v\beta_3$ function at other sites. In certain pathological situations, though, tissue levels of $\alpha_v\beta_3$ are increased; for example, tumor microvessels show increased levels of $\alpha_v\beta_3$, as do melanoma cells when they metastasize (Horton, 1997), and these features are being exploited.

Strategies for Therapeutic Modification of Integrin Function

From basic principles, there are two main strategies for inhibiting cell adhesion molecule function therapeutically (Table IV). First, a direct approach: competitive antagonists of the receptor–ligand interaction can be developed, and this has been the usual pharmaceutical approach with the aim of producing orally active, synthetic nonpeptide mimetic agents. They have been identified by a variety of standard industry techniques, as summarized in Table IV

TABLE IV Strategies for Therapeutic Modification of Integrin Adhesion Receptor Function *in Vivo*

Direct approaches
Naturally occurring protein inhibitors and their engineered derivatives (e.g., RGD-containing snake venoms and proteins from ticks, leeches, etc.)[a]
Blocking antibodies, and their engineered derivatives, to adhesion molecules[b]
Arg-Gly-Asp (RGD) peptides and their chemical derivatives (e.g., designed to improve specificity and stability)[c]
Nonpeptidic mimetics,[d] produced via different compound selection strategies[e]
Indirect approaches
Altered receptor synthesis via use of antisense oligonucleotides[f]
Inhibition of adhesion receptor expression via regulatory cytokines and their receptors
Modification of integrin receptor function via regulatory integrin-associated proteins
Modulation of integrin receptor affinity (i.e., activation) for ligands
Modification of downstream receptor-associated signaling (e.g., c-Src and other kinases, adhesion-associated apoptosis genes)

[a] *Echistatin has been used as a proof of concept inhibitor of* $\alpha_v\beta_3$ *in bone disease studies* (Fisher *et al.*, 1993; Yamamoto *et al.*, 1998). *Barbourin snake venom protein contains* KGD *instead of* RGD *and is the basis of selective inhibitors of platelet gpIIbIIIa* (Phillips and Scarborough, 1997).

[b] *Antibodies to gpIIbIIIa (i.e.,* 7E3, ReoPro, Centocor Inc*) formed the first cell adhesion receptor inhibitor licensed for clinical use (in the various vascular/thrombotic condition, see* Tcheng, 1996; Coller, 1997). *A humanized* $\alpha_v\beta_3$ *antibody (Abegrin, clone* LM609; Vitaxin^R*) has been tested in clinical trials for cancer acting via induction of apoptosis in tumor vessels* (Mulgrew *et al.*, 2006; Gramound *et al.*, 2007).

[c] Integrilin (Cor Therapeutics Inc), *a cyclic* KGD*-containing peptide gpIIbIIIa inhibitor, is in clinical trial* (Phillips and Scarborough, 1997; Coller, 1997), *as are RGD-derived cyclic peptides with selectivity for* $\alpha_v\beta_3$ [*cyclic* RGDfVA, E. Merck (Haubner *et al.*, 1996; Smith, 2003)].

[d] *A number of companies have intravenous and orally active nonpeptidic gpIIbIIIa antagonists in clinical trial for platelet-related disorders* (Phillips and Scarborough, 1997; Coller, 1997; Theroux, 1998). *Analogous mimetics are in late preclinical development for inhibition of* $\alpha_v\beta_3$ (Horton and Rodan, 1996; Hartman and Duggan 2000; Miller *et al.*, 2000) *in bone disease and cancer and one non-peptidic agent has been tested in patients with osteoporosis* (Murphy *et al.*, 2005).

[e] *Structure–function, combinatorial chemistry, phage display, compound/natural product library screening, etc.* (Lazarus *et al.*, 1993; Pasqualini *et al.*, 1995; Corbett *et al.*, 1997; Hoekstra and Poulter, 1998).

[f] *Antisense therapeutics directed against adhesion receptors are in clinical trials; antisense oligonucleotides to* α_v *block bone resorption in vitro* (Villanova *et al.*, 1999).

(e.g., see Ferguson and Zaqqa, 1999; Wang *et al.,* 2000). Other approaches, such as using receptor-specific antibodies, peptides, and naturally occurring protein antagonists, together with molecular engineering, have generally been used in proof of principle experiments rather than as clinical drug candidates, although there are some notable examples of protein therapeutics in the field (for examples, see Table IV). Directly acting antagonists have entered clinical trial to modify activation-dependent platelet aggregation in thrombotic conditions via the integrin platelet fibrinogen receptor, gpIIbIIIa/$\alpha_{IIb}\beta_3$. Thus, groundbreaking trials [EPIC, EPILOG, etc. (Tcheng, 1996)] have demonstrated efficacy of the humanized anti-gpIIIa monoclonal antibody 7E3 (ReoPro) in various ischemic heart conditions (Coller, 1997). Results from trials with RGD mimetics [e.g., lamifiban, tirofiban (Ferguson and Zaqqa, 1999; Wang *et al.,* 2000)] and the cyclic KGD peptide integrilin have, though, been less impressive (Theroux, 1998). As with gpIIbIIIa-specific agents, the possibility of developing osteoclast $\alpha_v\beta_3$ (vitronectin receptor) antagonists as resorption inhibitors in bone disease was initially demonstrated *in vitro* using a variety of techniques to disrupt receptor function, and small molecule inhibitors of $\alpha_v\beta_3$ are now at the late stage of preclinical development or entering the early stages of clinical trial evaluation (Hartman and Duggan, 2000; Miller *et al.,* 2000; Meyer *et al.,* 2006). Thus, general principles for the use of adhesion receptor antagonists in disease have been established, and useful drugs are thus likely to be available for a wide variety of indications in the future.

The second approach is indirect, with the aim of modifying expression or intracellular function (such as signal transduction) of cell adhesion molecules, especially integrins. Some examples of such strategies are given in Table IV. The furthest advanced are the use of antisense oligonucleotide inhibitors of receptor protein synthesis with the more recent RNA interference approach to knocking down α_v expression offering promise (Graef *et al.,* 2005). Because inhibitors of ICAM-1 expression are finding promise in the treatment of various inflammatory diseases, such as of the bowel or eye, then modulation of α_v expression by an antisense approach (Villanova *et al.,* 1999) could be a fruitful strategy. Likewise, a number of agents to block the function of c-Src, a cellular kinase that acts downstream in the signaling pathway of integrin receptors in bone cells, are being developed for the treatment of osteoporosis, based on the earlier finding in knockout mice that c-Src plays a central role in osteoclastic bone resorption (Soriano *et al.,* 1991).

Current Drug Development Status of $\alpha_v\beta_3$ Antagonists for Use in Bone and Other Diseases

The action in bone models of several candidate mimetic $\alpha_v\beta_3$ antagonists has been reported by a number of companies, and their evolution has been reviewed (Hartman and Duggan 2000; Miller *et al.,* 2000; Meyer *et al.,* 2006); as yet, these are not drugs but still agents used for proof of concept and pharmaceutical experiments. Compounds based on a variety of proprietary scaffolds, which have all shown varying efficacy and specificity for $\alpha_v\beta_3$ in the number of *in vitro* screening assays, have inhibitory effects on the calcemic response in thyroparathyroidectomized rodents and bone-sparing responses in ovariectomy and other rodent models of increased bone turnover. Positive findings in proof of concept studies using small molecule mimetics in models of bone metabolism underline $\alpha_v\beta_3$ antagonists as promising candidates for a new class of bone disease therapeutics, although they still require optimization. Furthermore, the expression, albeit at lower levels, of $\alpha_v\beta_3$ in other tissues suggests that their inhibition could produce unwanted side effects: for example, will they, on chronic administration, interfere with wound healing, the function of the related α_v integrins in respiratory tract or intestinal epithelium, or platelet ($\alpha_{IIb}\beta_3$-mediated) aggregation?

Humanized complex-specific monoclonals antibodies to $\alpha_v\beta_3$ have been developed and tested in cancer models and patients with solid tumors and bone metastasis (Mulgrew *et al.,* 2006; Gramoun *et al.,* 2007) where there is upregulated $\alpha_v\beta_3$ but not to the levels seen in osteoclasts. These antibodies are known to block bone resorption *in vitro* but they have not been developed in bone resorption models or patients.

Finally, although $\alpha_v\beta_3$ antagonists have been developed for use in bone diseases, other clinical targets also show promise (such as rheumatoid arthritis, angiogenesis in eye diseases and cancer, vascular restenosis following coronary angioplasty, and direct targeting of tumors expressing $\alpha_v\beta_3$). These are all being investigated for possible new applications of $\alpha_v\beta_3$ antagonist drugs.

Drug candidates with optimized pharmacokinetics/dynamics have entered clinical trial for bone disease and for other indications where $\alpha_v\beta_3$ is involved in disease pathogenesis. The Merck compound L-000845704 has been tested in a multicenter trial and efficacy at the spine and hip was demonstrated indicating that $\alpha_v\beta_3$ antagonists could be developed for the treatment of osteoporosis (Murphy *et al.,* 2005).

$\alpha_v\beta_3$-Based Clinical Imaging Tools

The high expression of $\alpha_v\beta_3$ by osteoclasts together with selective interaction to its ligands, such as RGD and peptidomimetics, have been used to develop “integrin-specific” imaging probes for use in a variety of diseases, including various forms of cancer, angiogenesis, and bone metastasis (reviewed in Lim *et al.,* 2005). These include ^{64}Cu labeled cyclic RGD peptides (Sprague *et al.,* 2007), ^{18}F-cycRGD SPECT/PET radiotracers (Sprague *et al.,* 2007), ^{111}In labeled peptidomimetic (Harris *et al.,* 2007, Jang *et al.,* 2007), and $\alpha_v\beta_3$ complex-specific antibodies (Cai *et al.,* 2006).

OSTEOBLASTS

Expression and Role of Adhesion Molecules in Bone formation

Osteoblasts are uniquely involved with the synthesis and maintenance of the bone matrix and lie in direct contact with the specialized extracellular matrix of bone. They also form cell–cell adhesive contacts (see Chapter 18) with other osteoblasts, but also with osteocytes embedded within the bone matrix. In their terminally differentiated form as osteocytes, they are completely surrounded by extracellular matrix and respond to mechanical forces exerted on the skeleton, suggested to be transmitted via adhesive interactions with matrix and/or between osteocytes within their vast network (see Chapter 6 and discussions by Wang and Ingber, 1994; Ruoslahti, 1997). There is also some data on the matrix in the osteocyte lacunae, including adhesion proteins synthesized by osteocytes themselves (Aarden *et al.,* 1996a and references therein).

It is increasingly clear that cell adhesion receptors play important roles in the function of cells in the osteoblast lineage. Some of the possible roles of adhesion receptors in osteoblasts are listed in Table I. Later, we discuss the expression (Table V) and known function of integrins and the other classes of adhesion molecules in osteoblasts and osteocytes and, where known, in their precursor, the mesenchymal stem cell.

TABLE V Integrin and Other Receptors Expressed by Mature Mammalian Osteoblasts[a]

	Receptor/integrin chain
Present[b,c,d]	$\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$ ("vitronectin receptors") α_{1-5} associated with β_1 α_{10}–a_{11} associated with β_1 E-Cadherin, Cadherin 4 and 11 and N-cadherin ICAM-1, ICAM-2, V-CAM, LFA3, and N-CAM Syndecan 1, 2, 3, and 4 CD44H
"Not detected"[e]	β_2 and CD11, a, b, c, α_d β_4

[a] *Data summarized from immunological and biochemical analysis of human and rodent species and gene deletion studies (as discussed in the main text). Data focus on osteoblasts; there are no comprehensive studies in mammalian osteocytes.*

[b] *Definitive proof of the association of individual integrin subunits in dimers is not always available.* $\alpha_5\beta_1$ *is the most abundant integrin expressed in osteoblasts and osteocytes.* $\alpha_v\beta_5$ *is the most important vitronectin receptor on osteoblasts. There is no definitive proof for expression of the other vitronectin receptors in vivo.*

[c] *In vivo* ICAM-1 *appears confined to bone lining cells* (Everts *et al.*, 2002).

[d] *Osteocytes express high levels of* CD44H.

[e] *A subpopulation of bone marrow stromal cells has recently been shown to express functional* β_2 *integrin* (Miura *et al.*, 2005).

Integrins

A diverse range of integrins have been shown to be expressed by osteoblasts (Table V), especially β_1 and β_5 integrins (Horton and Davies, 1989; Brighton and Albelda, 1992; Clover and Gowen, 1992; Clover *et al.,* 1992; Hughes *et al.,* 1993; Majeska *et al.,* 1993; Grzesik and Gehron Robey, 1994; Pistone *et al.,* 1996; Ganta *et al.,* 1997; Gronthos *et al.,* 1997; reviewed by Bennett *et al.,* 2001a). β_2 and β_4 integrins have not been reported in osteoblastic cells. Cultured human primary osteoblasts also express $\alpha_v\beta_6$ and $\alpha_v\beta_8$ integrin (Lai and Cheng, 2005). There remains some contradiction between different studies as to the specific β_1 heterodimers expressed. This may reflect the heterogeneity of osteoblast-like populations (see Chapter 4) and includes the possibility that cells at successive stages of osteoblast differentiation, or from fetal or adult bone, or from different anatomical sites, or actively synthesizing osteoblasts, versus quiescent cells, show different patterns of integrin expression. In addition it has been clearly demonstrated that *in vitro*, the substrate on which cells are cultured directly influences the pattern of integrin expression by osteoblasts (Sinha and Tuan, 1996; Gronowicz and McCarthy, 1996). Generally, cultured osteoblasts express a wider integrin repertoire than osteoblasts *in situ* (Clover *et al.,* 1992; Grzesik and Robey, 1994) and in particular increased expression of the vitronectin receptor $\alpha_v\beta_3$ is seen. A new class of integrin ligands, the CCN proteins (for review see Leask and Abraham, 2006), have been found to be important in bone and cartilage formation and during fracture repair. These proteins can bind a range of integrins including $\alpha_v\beta_3$ and $\alpha_5\beta_1$ and are expressed by osteoblasts and chondroblasts (Schütze *et al.,* 2005). The six known CCN proteins are called matricellular proteins as they combine up to four modules allowing roles in attachment to matrix, migration, chondrogenesis, angiogenesis, proliferation, and others. In terms of adhesion, there is evidence that Cyr61 (CCN1) is involved in early osteoblast differentiation from mesenchymal cells (Si *et al.,* 2006).

It is yet unclear whether altered expression of integrins has an equivalent in bone pathology, because osteoblast integrin expression in bone disease is relatively unexplored. However, bone diseases such as Paget's disease and osteopetrosis (Helfrich, 2003) are accompanied by major changes in the numbers of "real" osteoblasts, i.e., osteoid synthesizing cells and hence differences in integrin expression in the overall osteoblastic population are likely. In addition, recent studies are beginning to examine the effects of mechanical stimuli on integrin expression and suggest that external stimuli may influence the integrin expression in the osteoblastic population. Expression profiling of osteoblasts during defined differentiation in culture is now possible, but no specific reports on adhesion molecule profiles have as yet been published.

Integrin expression in osteocytes has not been studied extensively, but there is some data from immunocytochemical analysis of human bone sections and from functional studies in the chick, where isolation procedures for osteocytes exist. Chick osteocytes and osteoblasts bind a comprehensive range of extracellular matrix proteins *in vitro* in a β_1- and partially RGD-dependent way (Aarden *et al.*, 1996b). The exact receptor involved was not determined, because of lack of α chain specific antibodies for avian integrins. Equally, isolated rat osteocytes have been reported to adhere to a wide range of matrix proteins, including, in an RGD-dependent way, to fibronectin, vitronectin, and osteopontin (Miyauchi *et al.*, 2006), although again the receptor involved was not formally identified. Expression of β_1 integrin has been confirmed in mammalian osteocytes (Hughes *et al.*, 1993; Gohel *et al.*, 1995), including the osteocytic cell line MLO-Y4 (Helfrich *et al.*, 2001) and $\alpha_v\beta_3$ has been reported specifically on mandibular osteocytes (Bennett *et al.*, 2001b). However, there are few definitive reports where integrin expression has been followed throughout osteoblastic differentiation to osteocytes within the same species. Difficulties in interpretation of immunocytochemical staining in bone sections, in particular for cells embedded within matrix, have been reported by many authors and this may well have contributed to the continuing controversies in integrin phenotype of osteoblasts/osteocytes. From the limited reports on isolated cells at different stages of differentiation, it has become clear that, at least in the mouse, $\alpha_5\beta_1$ is the most abundantly expressed integrin throughout osteoblastic differentiation and that, may be surprisingly, $\alpha_2\beta_1$ is expressed at much lower levels, in particular in more differentiated cells (Helfrich *et al.*, 2001). Expression of a wide range of β_1 and β_3 integrins has also been reported in mesenchymal stem cells, including β_4 integrin (Bruder *et al.*, 1998; Kuznetsov *et al.*, 2001; Majumdar *et al.*, 2003) and Stewart and coworkers (2003) recently showed that selection of α_1 integrin positive bone marrow cells markedly enriched the population of CFU-F, clonogenic precursors with osteogenic potential, confirming the importance of α_1 early in the osteoblast lineage. Current interest in differentiation of osteogenic cells from stem cells may reveal additional information on expression of integrins and other cell adhesion molecules during differentiation, an area that is still poorly understood. For example, treatment of α_7-positive myoblasts with BMP-2 resulted in differentiation along the osteogenic lineage with loss of α_7 but gain of strong expression of α_2 integrin, associated with increased binding to collagen (Ozeki *et al.*, 2006). Understanding integrin expression during osteogenic differentiation will require comprehensive studies in well-defined osteoblastic populations (both in developmental stage and in synthetic activity) combining immunocytochemical, biochemical, and molecular techniques.

Clearly β_1 and β_5 integrins are the classes of integrin receptors with the major functional role in cells of the osteoblast lineage. In keeping with this, osteoblasts adhere to osteopontin, bone sialoprotein, vitronectin, and fibronectin in an RGD-dependent way (with fibronectin requiring much higher peptide concentrations for inhibition), whereas binding to type I collagen and thrombospondin is less inhibited by RGD peptides (Puleo and Bizios, 1991; Majeska *et al.*, 1993; Grzesik and Robey, 1994). Next we discuss the role of osteoblast integrins as receptors for specific matrix proteins.

Function of Collagen Receptors

The $\alpha_1\beta_1$ and $\alpha_2\beta_1$, integrins reported to be expressed by osteoblasts are collagen binding receptors, whereas $\alpha_3\beta_1$ is a laminin receptor (Gullberg *et al.*, 1992). Collagen type 1 is the main protein of the bone matrix, and interactions involving collagen receptors are therefore strong candidates for a role in regulating osteoblast behavior. There is now abundant evidence this is indeed the case. In organ cultures of mineralizing fetal rat parietal bone RGD peptides decreased bone formation accompanied by a decrease in α_2 and β_1 expression and disruption of the organization in the osteoblast layer (Gronowicz and DeRome, 1994). Downregulation of α_2 and β_1 integrin expression in osteoblasts by glucocorticoids has also been noted, accompanied by a similar disruption in osteoblast organization, whereas IGF-1 increased β_1 expression in osteoblasts and increased calcified bone formation (Gohel *et al.*, 1995; Doherty *et al.*, 1995). It has become clear that the role of osteoblast integrins extends beyond adhesion. Although the evidence for a role of fibronectin in osteoblast differentiation is strongest (see later in chapter), collagen is also implicated in osteoblast differentiation. Ascorbic acid deficiency, which leads to underhydroxylation of type I collagen, resulted in downregulation of $\alpha_2\beta_1$ in osteoblasts and dysregulation of differentiation and mineralization in cultures of rat calvaria (Ganta *et al.*, 1997). In addition, α_1 and α_2 integrins mediate differentiation signals, such as from BMP-2, in early osteoblastic cells (Jikko *et al.*, 1999); a role for $\alpha_2\beta_1$ and type I collagen in osteoblast differentiation from early progenitors was demonstrated (Mizuno *et al.*, 2000) and $\alpha_2\beta_1$ ligand binding was shown to lead to expression or upregulation of markers of osteoblastic differentiation, such as Runx2, alkaline phosphatase, and osteocalcin (Xiao *et al.,* 1998, 2002), while blocking antibodies to α_2 modulate $\alpha_2\beta_1$-dependent expression of osteoblast markers (Takeuchi *et al.,* 1997), cell motility, and contraction of collagen gels *in vitro* (Riikonen *et al.,* 1995). These data correlated well with the higher levels of α_1 and α_2 collagen binding integrins in early stages of the lineage and reduced expression in more differentiated cells as discussed earlier.

Because β_1 integrins have a critical role in embryogenesis, not much information has been gained from studies of β_1 knockout mice, which are embryonic lethal at a time well before skeletal development begins (Fässler and Meyer, 1995; Stephens *et al.,* 1995). A transgenic mouse in which a dominant negative β_1 integrin is expressed under the control of the osteoblast specific osteocalcin promoter has, however, been very informative (Zimmerman *et al.,* 2000). *In vitro,* osteoblasts from these animals do not properly adhere to matrix, whereas *in vivo,* osteoblast and osteocyte morphology, polarity, and matrix secretion is severely affected, resulting in decreased bone mass, especially in females. Anatomical differences are found in older animals (Globus *et al.,* 2005). The tibia of the transgenic animals are straighter, consistent with the possibility that in absence of functional β_1 integrin, osteoblastic cells are not able to sense and/or respond adequately to load bearing, a stimulus that in wild-type animals results in the characteristic curvature of this long bone. Viable knockouts have been generated for α_1 and α_2 integrins. Surprisingly, neither has a prominent bone phenotype (Chen *et al.,* 2002; Holtkötter *et al.,* 2002; Ekholm *et al.,* 2002), although detailed bone histology has not been reported. It is possible that compensation occurs in these single knockouts for collagen binding integrins, because overall the absence of gross abnormalities in *any* tissue in the α_2 null was largely unexpected (Mercurio, 2002). In specific disease models, however, loss of α_1 or α_2 has revealed important functions in kidney, vascular, and inflammatory conditions (see Popova *et al.,* 2007). Given the important roles of α_2 integrin in osteoblasts and osteoclasts (Helfrich *et al.,* 1996; Horton *et al.,* 2003), more detailed studies should be undertaken to elucidate further the role of α_2 integrin in the α_2 knockout mouse, using skeletal disease models. The α_1 knockout mouse does show abnormalities during fracture healing, with reduced callus formation compatible with an effect on mesenchymal precursors of chondrocytes and osteoblasts, which express high levels of this integrin (Ekholm *et al.,* 2002).

Single knockouts for β_3 and β_5 and α_v and a double knockout for β_3/β_5 have been made (see Sheppard, 2000) and none of these appear to have osteoblast malfunction. The relatively subtle defects seen in the skeleton of the β_3 knockout are caused by osteoclast malfunction and have been discussed earlier.

A number of other integrin gene deletions have demonstrated skeletal phenotypes that are likely to be caused by effects on cells of the osteoblast lineage (Brouvard *et al.,* 2001). The knockout of β_2, described earlier in the osteoblast section, unexpectedly showed that this myeloid integrin is indeed expressed in a stromal cell population and absence leads to abnormalities in cell adhesion, growth, and osteogenic differentiation, ultimately leading to an osteoporotic phenotype. Constitutive expression of β_2 on the contrary led to enhanced bone formation (Miura *et al.,* 2005). The α_4 knockout mouse (Yang *et al.,* 1995) exhibits defective closure of cranial sutures, although it is unclear whether this is because of a local osteoblast or other cell type defect.

Combined deletion of the laminin-binding integrins α_3 and α_6 (De Arcangelis *et al.,* 1999) results in severe skeletal abnormalities involving both the axial and peripheral skeleton that are not seen in single gene deletion animals; this phenotype is reminiscent of that seen on deletion of laminin α_5 gene.

Function of Fibronectin Receptors

Of the integrins that are fibronectin receptors, $\alpha_4\beta_1$, $\alpha_5\beta_1$, and α_v heterodimers are reported to be expressed in osteoblasts. Most are capable of binding fibronectin, but in addition a range of other extracellular matrix proteins, using both RGD-dependent and independent mechanisms (see Table VII). Here we focus on evidence for a functional role as fibronectin receptors.

The critical role of fibronectin in osteoblast differentiation was first demonstrated in the *in vitro* rat osteoblast nodule formation assay (Moursi *et al.,* 1996) and the importance of the central cell-binding domain of fibronectin in this effect suggested that the selective fibronectin receptor, $\alpha_5\beta_1$, was implicated. Later studies by this group also suggested a role for $\alpha_3\beta_1$ in osteoblast differentiation (Moursi *et al.,* 1997). In mature cells, $\alpha_5\beta_1$–ligand binding appears to be necessary for cell survival and receptor blockade leads to osteoblast apoptosis (Globus *et al.,* 1998).

Fibronectin is a normal constituent of human bone, but data on its distribution within the bone matrix are sparse, and it has been reported absent from mature lamellar bone (Carter *et al.,* 1991). Supporting evidence from rodent tissues suggests that fibronectin synthesis and expression are restricted to developing or immature bone (Weiss and Reddi, 1980; Cowles *et al.,* 1998). It is, therefore, possible that $\alpha_5\beta_1$–ligand interaction is a feature of bone formation during development or repair and may not play a prominent role in the turnover and maintenance of mature lamellar bone. There is, however, also some evidence for the involvement of the $\alpha_5\beta_1$ integrin in mechanical sensing by osteoblasts, at least *in vitro* (Salter *et al.,* 1997). Given this fact, the role of fibronectin and fibronectin receptors deserves further study in mature matrix synthesizing osteoblasts.

Unsurprisingly there is great interest in the orthopedics/biomaterials field in identification of bioactive surface coatings to enhance osteoblast attachment and matrix (reviewed by Siebers *et al.*, 2005). Coating of biomaterials with fibronectin has been shown to give the best adhesion of osteoblastic cells, which is completely in agreement with all data presented here. Many studies integrating adhesive proteins with micro- and nano-topography on biomaterials are underway and will increase our understanding of integrin-matrix interactions and applications in tissue engineering.

α_v Integrins

Several studies report expression of α_v in cells of the osteoblast lineage. However, the published literature varies with regard to which β subunit is utilized (the balance of data favoring $\alpha_v\beta_5$ expression), and staining appears more prominent in osteoblasts than osteocytes (Hughes *et al.*, 1993; Grzesik and Robey, 1994). Long-term glucocorticoid exposure has been shown to lead, *in vitro* at least, to downregulation of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins in human osteoblasts, a phenomenon that together with the negative effect on β_1 integrin mentioned earlier, may help to explain the bone loss associated with long-term glucocorticoid usage (Cheng *et al.*, 2000). More recently it has become clear that the role of osteoblast integrins extends beyond adhesion. BMP-2, a potent stimulator of bone formation increases expression of the whole repertoire of osteoblast α_v integrins and the BMP-2 receptor co-localizes with integrins. The finding that blocking of the function of α_v inhibited the BMP-2 effects in osteoblast indicates that integrins may regulate BMP-2 effects in bone (Lai and Cheng, 2005) and suggests that there is potentially a wider role for integrins in facilitation growth factor signaling in bone.

Role of Integrins in Mechanosensing by Osteocytes

An area of increasing interest is that of the possible role of adhesion molecules, especially integrins, in mechanosensing in bone. Osteocytes and osteoblasts are known to be highly responsive to mechanical effects on bone. In other cell systems it has been demonstrated that twisting or turning of integrin molecules directly affects gene transcription (Wang and Ingber, 1994). Stretching of cells, i.e., change of cell shape and dimension, has also been shown to affect gene transcription and cell survival (Ruoslahti, 1997) and cell shape is largely controlled by extracellular matrix (Chen *et al.*, 1997). It has therefore been speculated that positive mechanical effects on bone cells resulting in bone formation may be mediated indirectly via adhesion receptors and/or cellular shape changes resulting from the bone deformation. In contrast, absence of mechanical stimuli that leads to a decrease in bone mass may be the result of lack of "cellular stretch" in particular in osteocytes, which may lead to apoptotic cell death (Noble and Reeve, 2000), a cellular phenomenon associated with induction of bone resorption (Bonewald, 2004 and Chapter 6). Despite these plausible hypotheses, the mechanisms whereby bone cells sense and respond to mechanical strain remain largely unresolved, as it remains technically challenging to subject bone cells, in particular osteocytes to defined levels of strain and measure single cell responses. A further complication is that osteocytes/osteoblasts *in vivo* exist as a syncytium and these cell–cell interactions are not readily reestablished *in vitro*. Despite these difficulties some progress has been made recently. Charras and Horton used atomic force microscopy to estimate the cellular strain necessary to elicit cellular responses in bone cells (Charras and Horton, 2002a) and then, using finite element modeling, determined the strain exerted on individual cells when stimulated by a number of commonly used methods *in vitro*, such as the magnetic bead twisting and stretch experiment described earlier (Charras and Horton, 2002b). Interestingly, fluid shear stress, the main mechanism by which osteocytes are thought to detect strain in bone (see Chapter 6) was found to give rise to the lowest cellular strain (deformation) leading to the hypothesis that cells may have different mechanisms for detecting different magnitudes of strain (Charras and Horton, 2002b). Using paramagnetic beads coated with specific integrin antibodies, it was demonstrated that drag forces (shear) applied to α_2 and β_1 integrin subunits resulted in a cellular response, in this case intracellular calcium increase (Pommerenke *et al.*, 2002). Focal adhesions in osteoblasts were found to promote mechanosensing and RGD peptides could inhibit either, again suggesting a direct requirement of adhesion mechanisms for mechanosensing (Ponik and Pavalko, 2004). *In vivo* studies supporting a role for integrins in mechanosensing are still scarce. Skeletal unloading in rats leads to an increase in osteoblast and osteocyte apoptosis, an effect shown to be directly preceded by $\alpha_5\beta_1$ downregulation (Dufour *et al.*, 2007). This effect is most likely mediated via the α_5 subunit as the effect persisted in animals with disrupted β_1 (Iwaniec *et al.*, 2005). There is also some ultrastructural evidence for the presence *in vivo* of adhesive interactions, "tethering elements," between osteocytes processes and the walls of their lacunae, although the molecular nature of this adhesive interaction remains to be identified (You *et al.*, 2004). Other studies (further discussed later) indicate that cadherins, and therefore cell–cell interactions, are also directly involved in the response to strain (Norvell *et al.*, 2004) and there is strong evidence for integrin-mediated mechanosensing in chondrocytes (see Millward-Sadler and Salter, 2004, and further discussion later). Although further information is clearly needed, especially in osteocytes, the principle that integrins and other classes of adhesion molecules are involved in mechanosensing appears well established.

Non-Integrin Cell Adhesion Molecules in Osteoblasts

Cadherins

Osteoblasts and bone-lining cells form gap and adherens type cell junctions with each other and with the osteocytes (Palumbo *et al.,* 1990; Doty, 1981). Cadherins are among the best characterized cell–cell adhesion molecules (Isacke and Horton, 2000), localizing to sites of intercellular attachment. The expression pattern and function of cadherins in osteoblasts are covered in depth in Chapter 18 and are only reviewed briefly herein.

Cells of the osteoblast lineage express a limited repertoire of cadherins, E-cadherin, cadherin-4, cadherin-11 (OB-cadherin), and N-cadherin (Okazaki *et al.,* 1994; Cheng *et al.,* 1998; Hay *et al.,* 2000). Some differences have been found between different species, skeletal site and differentiation stage of the osteoblasts, but it is clear from a range of immunocytochemical and molecular studies that N-cadherin and cadherin-11 are abundantly expressed in osteoblastic cells in many mammalian species (reviewed in Marie, 2002, and Chapter 18).

Cadherin expression during the differentiation from early mesenchymal cells into mature osteogenic cells has been studied both *in vivo* and *in vitro*. N-cadherin appears to be expressed widely in all mesenchymal lineage cells and remains expressed at all stages of bone formation with especially high levels of expression at late stages of differentiation (Ferrari *et al.,* 2000). Cadherin-11 seems to be more specifically associated with the osteoblast lineage (Kawaguchi *et al.,* 2001). Cadherin expression is reported to be lost in osteocytes (Kawaguchi *et al.,* 2001; Monaghan *et al.,* 2001). It has been suggested that this loss is linked to osteoblast apoptosis at the end of a formation cycle and is necessary to allow a proportion of mature osteoblasts to enter into the osteoid and become osteocytes. However, it is somewhat surprising that cadherins might not contribute to maintaining the cellular contacts between osteocytes and surface osteoblasts. In fact, recent data from our lab (Huesa and Helfrich, unpublished) shows high levels of β-catenin in cell–cell junctions between MLO-Y4 cells or primary osteocytes *in vitro*, strongly suggesting that in contact sites cadherins are expressed on the osteocyte cell surface and that difficulties in detection of cadherin expression *in vivo* may be related to the difficulties in visualizing such sites in whole bone. Clearly more information is required on the molecules that form the cell–cell contacts in the osteocyte/osteoblast network.

There is good functional evidence that cadherins are important for bone cell function. HAV peptides inhibit cell–cell contacts in osteoblast cultures and matrix formation *in vitro* suggesting a role for class I cadherins in osteoblast synthetic activity (Cheng *et al.,* 1998; Ferrari *et al.,* 2000). Likewise, antibodies to E-cadherin inhibit cell–cell adhesion (Babich and Foti, 1994). Expression of a dominant negative N-cadherin *in vitro* in committed preosteoblastic cells as well as more differentiated osteoblasts inhibited expression of genes associated with bone formation and mineralization (Cheng *et al.,* 2000; Ferrari *et al.,* 2000). Recently, cadherins were implicated in the response to mechanical stimulation in cultured osteoblasts by the finding that fluid shear stress increased translocation of β-catenin to the nucleus and regulated COX-2 expression in osteoblasts, while reducing recruitment of β-catenin by N-cadherin on the cell surface (Norvell, 2004).

In mice in which the gene for cadherin-11 is deleted, a reduction in bone density is seen, strongly implying cadherin-11 in osteogenesis (Kawaguchi *et al.,* 2001). This has been further confirmed in *in vitro* studies demonstrating that cadherin-11 directly regulates differentiation of mesenchymal cell lines into osteogenic and chondrogenic lineages (Kii *et al.,* 2004). Cadherin-11 has recently been shown to be critically important in synovial lining formation in the joint and plays an essential role in the orchestration of the tissue response to inflammation during rheumatoid arthritis (Lee *et al.,* 2007), making it a strong therapeutic target in this disorder.

At present there is little *in vivo* information about the bone phenotype in absence of N-cadherin because the N-cadherin null mice die at day E10, before mature osteoblasts are present (Radice *et al.,* 1997). However a dominant negative approach (Castro *et al.,* 2004) showed that a reduction in functional N-cadherin in osteoblasts *in vivo* leads to a reduction in osteoblast number and thus in peak bone mass, while increasing the number of adipocytes suggesting a role of N-cadherin in mesenchymal cell lineage commitment. In addition, dominant negative N-cadherin expression in stromal ST2 cells *in vitro* strongly reduced RANKL expression independent of cell–cell adhesion (which was unaffected) illustrating that cadherin signaling can affect both osteoblast and osteoclast formation (Shin *et al.,* 2005). Most recently, Lai and coworkers (2006) found that haploinsufficiency of N-cadherin in heterozygous N-cadherin knockout mice leads to reduced cell–cell adhesion and osteoblast function, illustrated by increased bone loss after ovariectomy. Overall, these data provide strong evidence for an important role of N-cadherin in osteoblast lineage commitment and osteoblast function.

Osteoblast cadherins are upregulated, through as yet unknown mechanisms, by a variety of cytokines and hormones including BMP-2, FGF-2, PTH, and downregulated by IL-1 and TNFα (reviewed in Marie, 2002; and

Chapter 18) and it is plausible that some of the well-known effects of these hormones are mediated in part through modulation of cadherin function. Application of stretch to osteoblasts *in vitro* selectively upregulated expression of N-cadherin (Di Palma *et al.*, 2004), leading to increased cell–cell adhesion. The signaling pathways controlling cell–cell adhesion through osteoblast cadherins and the ways they regulate osteoblastic gene expression remain as yet largely unclear.

There is a role for this class of adhesion molecules in bone pathology. Osteoblast cadherins are implicated in malignancy: N-cadherin expression is reduced and cadherin-11 is not normally displayed on the cell surface in osteosarcoma (Kashima *et al.*, 1999) and β-catenin mutations are found in malignant bone tumors (Iwao *et al.*, 1999). Upregulation of E- and N-cadherin is found in Apert syndrome (discussed in Marie, 2002). Finally, because cadherin expression is differentially regulated in osteoblastic versus adipocytic cell populations (Shin *et al.*, 2000; Castro *et al.*, 2004), cadherins may play a role in development of osteoporosis and osteoarthritis, diseases associated with changes in the differentiation of mesenchymal cells to osteogenic versus adipocytic cells.

CD44

Immunohistochemical studies of human tissues showed CD44 expression in osteocytes, but not osteoblasts or bone-lining cells (Hughes *et al.*, 1994). This is consistent with other mammalian models in which strong expression has been observed in osteocytes, with weaker staining in cells earlier in the osteoblast lineage (Jamal and Aubin, 1996; Nakamura and Ozawa, 1996; Noonan *et al.*, 1996; Cao *et al.*, 2005). However, in a recent study expression of CD44 in immature periosteal cells was reported (Park *et al.*, 2007), suggesting that this molecule is expressed throughout mesenchymal-osteoblastic differentiation, but with highest levels in the late differentiation stages. Detailed localization studies have shown that CD44 expression in mature osteoblasts is confined to cytoplasmic processes only (Nakamura *et al.*, 1995). The functional significance of CD44 expression in the osteoblastic lineage is not fully understood as yet. *In vitro*, osteoblastic cells have been shown to bind to and degrade hyaluronate in the transition zone from cartilage to bone in the growth plate and utilize a CD44-dependent mechanism (Pavasant *et al.*, 1994). There are a variety of other known ligands for CD44, e.g., type I collagen, fibronectin, laminin, and osteopontin, and these are also produced by both osteoblasts and osteocytes (Aarden *et al.*, 1996a) and co-localize with CD44, indicating that a much wider range of functions for this molecule may exist. Cross-linking of CD44 on osteoblasts leads to upregulation of ICAM-1 and VCAM-1 (Fujii *et al.*, 2003), which in turn can lead to increased osteoclast formation (Harada *et al.*, 1998; Okada *et al.*, 2002). In addition, CD44 is involved in the upregulation of RANKL expression by hyaluronate in bone marrow stromal cell cultures (Cao *et al.*, 2005). However, the bone phenotype of the CD44 null mouse is very mild, suggesting that *in vivo* such processes may be finely balanced.

Ig Family Members

A number of Ig family members are expressed in osteoblasts. During skeletal development transient expression of NCAM is seen (Lee and Chuong, 1992; Lackie *et al.*, 1994; Chimal-Monroy and Diaz, 1999). In mature osteoblasts expression of ICAM-1 and ICAM-2, VCAM-1, and LFA-3 have been reported (Lee *et al.*, 1992; Kurachi *et al.*, 1993; Tanaka *et al.*, 1995b and 2000; Harada *et al.*, 1998; Okada *et al.*, 2002; Reyes-Botella *et al.*, 2002; Fujii *et al.*, 2003; Nakayamada *et al.*, 2003) and VCAM-1 is also seen in early osteogenic cells (Kuznetsov *et al.*, 2001). There is circumstantial evidence for expression of NCAM in human osteoblasts from studies in multiple myeloma, where NCAM antibodies blocked IL-6 production in osteoblasts-myeloma co-cultures (Barillé *et al.*, 1995). Similarly, cross-linking of ICAM-1 or VCAM-1 resulted in the production of bone resorbing cytokines by osteoblasts (Tanaka *et al.*, 1995b), suggesting that in general expression of Ig family members by osteoblasts may lead to direct cellular interaction with immune cells, which express their ligands (Table VII) and subsequently to activation of production of bone resorbing cytokines.

In addition, Ig family adhesion molecules expressed on osteoblasts are firmly implicated in osteoclast formation. VCAM-1 has been shown to be involved in the development of osteoclasts *in vitro*, an effect that could be mediated via osteoblastic cells (Feuerbach and Feyen, 1997). Further studies on involvement of ICAM-1 and its ligand LFA-1 in osteoclastogenesis suggested a role not only for osteoblast–osteoclast interaction, but also for osteoclast precursor fusion, enabled by transient expression of LFA-1 in pre-osteoclasts (Harada *et al.*, 1998). The role of ICAM-1 has also been studied in more detail. Tanaka and coworkers (2000) found that ICAM-1 expression characterized a population of osteoblasts, induced by proinflammatory cytokines that have arrested in G_0/G_1 and are uniquely equipped to sustain osteoclast differentiation. Interestingly, Everts and coworkers describe that the only cells positive for ICAM-1 on the bone surface in resorbing mouse calvaria are bone-lining cells (Everts *et al.*, 2002). Linking roles for integrins and Ig family members, it was reported how, following β_1-dependent osteoblast adhesion, signaling through FAK results in upregulation of ICAM-1 and RANKL leading to osteoclast formation (Nakayamada *et al.*, 2003). Further molecules that have been found to induce ICAM-1 expression in rodent osteoblasts *in vitro*, leading to increased osteoclast formation, are PTH, IL1, TNFα, and 1,25D$_3$ (Kurokouchi *et al.,* 1998; Okada *et al.,* 2002). The same stimuli also independently induce expression of RANKL and it is becoming clear that efficient stimulation of osteoclast precursors by RANKL is dependent upon high affinity adhesion between osteoblast and osteoclast precursors, a process coined as "juxtacrine stimulation" (Okada *et al.,* 2002).

ICAM-1 expression on osteoblasts *in vivo* in pathologies has yet to be studied. Expression of human primary osteoblasts *in vitro* is highly variable, but has been associated with pathological status: increased expression was seen in cells derived from patients with osteoporosis and to a lesser extent patients with osteoarthritis, compared to normal individuals and increased expression was related in particular to the presence of elevated levels of IL6 and PGE$_2$ (Lavigne *et al.,* 2004). These data fit well with the established role of ICAM-1 in inflammatory diseases in general and especially in rheumatoid synovium, where levels are considerably increased during active periods of disease and where anti-ICAM-1 therapy has been considered (Kavanaugh *et al.,* 1996, 1997). Taken together with the experimental data it seems likely that high osteoblastic ICAM-1 levels are involved in the mechanism of diseases associated with bone loss by increasing osteoclast recruitment and formation.

Selectins

There is as yet no published data on expression of selectins in osteoblasts. As discussed for osteoclasts earlier, they may well play a role in extravasation of the mesenchymal precursors and in support of this L-selectin, but not E- or P-selectin, has been found expressed in human mesenchymal stem cells (Bruder *et al.,* 1998).

Syndecans

The expression of syndecans in bone is best studied during differentiation. Syndecan-3 (also known as N-syndecan) expression is found in cartilage (see next section) and periosteum during endochondral bone formation, where it interacts with tenascin to set boundaries in tissues (Hall and Miyake, 2002), but low levels are still seen in osteoblasts and osteocytes close to periosteal surfaces (Koyama *et al.,* 1996) and higher levels are found in osteoblasts and precursors in areas of bone regeneration after damage (Imai *et al.,* 1998). Messenger RNA for the three cloned human forms of syndecan syndecan-1, -2, and -4 are found in primary human osteoblast cultures and a number of osteoblast cell lines (Birch and Skerry, 1999; Worapamorn *et al.,* 2002), where expression levels are regulated by cytokines implicated in tissue regeneration (Worapamorn *et al.,* 2002).

Culture of osteoblastic cells under conditions that induce differentiation results in a decrease of syndecan-1 levels (Birch and Skerry, 1999). Syndecan-2 has been implicated in the mitogenic effect of GM-CSF in osteoblasts (Modrowski *et al.,* 2000). A role of syndecans in presentation of growth factors to their receptors has been suggested as their most prominent function in bone. In addition, syndecan-1 could also be an adhesion receptor regulating interaction of osteoblastic cells with the extracellular matrix. The heparin-binding growth associated molecule (HB-GAM, or pleiotrophin) that is expressed by osteoblasts and osteocytes (Imai *et al.,* 1998; Liedert *et al.,* 2004) indeed seems to act in such a way by attracting N-syndecan expressing osteoblasts resulting in enhanced bone deposition (Imai *et al.,* 1998), a process that may be important both during development as well as in response to bone injury.

ADHESION RECEPTORS IN CARTILAGE

The role of cell adhesion molecules in cartilage is beginning to be elucidated and some of their putative functions are summarized in Table I. These include roles in chondrocyte proliferation and cartilage differentiation during fetal development (see Chapter 3); responses to mechanical forces (e.g., in articular cartilage or menisci); maintenance of tissue architecture and integrity, including matrix synthesis and assembly; or cell adhesion, regulation of chondrocyte gene expression, and cell survival. Additionally, there is likely to be a role for cell adhesion molecules in the response in cartilage to injury and disease (Forster *et al.,* 1996; Lapadula *et al.,* 1997; Millward-Sadler *et al.,* 2000; Ostergaard *et al.,* 1998). The differing distribution of both integrin and matrix proteins (Salter *et al.,* 1995) in the zones of cartilage suggests a role in chondrocyte differentiation from mesenchymal precursors (Hirsch and Svoboda, 1996; Tavella *et al.,* 1997; Shakibaei *et al.,* 1995) and/or interaction with matrix, or a specialized function such as response to mechanical stresses. Next we discuss the different classes of adhesion receptors reported in chondroblasts and chondrocytes, some functional data, and information on knockout animals where relevant and available.

Integrins in Chondrocytes

As with the osteoblast lineage, the reported integrin phenotype of chondrocytes is complex, with additional inconsistency between publications (Durr *et al.,* 1993; Enomoto *et al.,* 1993; Loeser *et al.,* 1995; Salter *et al.,* 1992, 1995; Woods *et al.,* 1994; Ostergaard *et al.,* 1998; most recently reviewed in Woods *et al.,* 2007). Differences in reported integrin expression patterns could well relate, in part, to a variation in sampling site, use of fetal versus adult material, species differences, artifacts induced in culture, or influences of disease on phenotypes; indeed the first possibility is born out by the study of Salter *et al.* (1995) where the distribution of integrin clearly differs

TABLE VI Integrin and Other Receptors Expressed by Mammalian Chondroblasts/Chondrocytes[a]

	Receptor/integrin chain
Present[b,c,d]	α_{1-3} and α_{5-6} and α_{10}–$\alpha_{11,}$ all associated with $\beta_{1,}$ $\alpha_v\beta_{3,}$ $\alpha_v\beta_5$
	Cadherin 4 and 11 and N-cadherin ICAM-1, V-CAM, and N-CAM Syndecan 3 CD44H
"Not detected"[e]	β_2 and CD11, a, b, c, α_d β_4 β_{7-9}

[a] *Data summarized from immunological and biochemical analysis of human and rodent species and gene deletion studies (as discussed in the main text).*

[b] *Definitive proof of the association of individual integrin subunits in dimers is not always available.*

[c] *$\alpha_5\beta_1$ is the most abundant integrin expressed in chondrocytes.*

[d] *Osteocytes express high levels of CD44H, cadherin expression in osteocytes is as yet unclear.*

[e] *α_4 and β_2 have been reported in osteoarthritic cartilage (Ostergaard et al., 1998). β_7 has recently for the first time been reported in differentiating chondrocytes (Djouad et al., 2007).*

by site (human articular, epiphyseal, and growth plate chondrocytes were studied) and variation in expression has been observed in cartilage when comparing fetal and adult samples, but not during the endocrine-driven pubertal growth (Hausler *et al.,* 2002). Likewise, changes have been reported in *in vitro*-cultured chondrocytes (Loeser *et al.,* 1995; Shakibaei, 1995, Shakibaei *et al.,* 1993) and differences were observed in osteoarthritic versus normal cartilage (Millward-Sadler *et al.,* 2000). A synthesis of the literature suggests that human chondrocytes express the β_1 integrins α_1, α_2, α_3, α_5, and α_6, but not α_4, although a recent miroarray study of differentiating chondrocytes from mesenchymal cells did find α_4 as well as α_7 (Djouad *et al.,* 2007). β_2, β_4, and β_6 are absent, and analysis of β_{7-9} and CD11 has not been reported (Table VI). Some studies have shown high expression of α_v integrin; as in osteoblasts, this is mainly as $\alpha_v\beta_5$ and not the $\alpha_v\beta_3$ dimer seen in osteoclasts, although a subpopulation of superficial articular chondrocytes was found to be $\alpha_v\beta_3$ positive (Woods *et al.,* 1994).

Two more recently discovered integrins, α_{10} and α_{11} (Camper *et al.,* 1998; Tiger *et al.,* 2001), are collagen receptors. They show expression in or near cartilage and are also expressed in fibroblasts and they have different, in many cases complementary, roles. α_{10} is highly expressed during cartilage development, whereas α_{11} is expressed in perichondral cells and mesenchymal cells in areas of interstitial collagen formation (see Popova *et al.,* 2007a). Whereas α_{10} integrin appear to be expressed in areas with high levels of type II collagen, binding studies have shown this integrin prefers types IV and VI collagens (Tulla *et al.,* 2001); α_{11} is not really expressed in cartilage itself, but in perichondral areas and bind preferably to type I collagen. It is possible however, that expression of this integrin is influenced by its proximity to developing cartilage. The phenotypes of the respective knockout animals have been described. The α_{10} knockout mouse, in keeping with its prominent role in cartilage formation, shows growth plate abnormalities (albeit these are rather mild) owing to abnormal shape of chondrocytes and increased apoptosis, resulting in growth retardation (Bengtsson *et al.,* 2005). Mice with a deletion of α_{11} are also much reduced in size, although through a different mechanism. They do not show any gross abnormalities in skeletal structures, or in proliferation of chondrocytes. Instead, they display a tooth phenotype with abnormal incisors because of abnormal periodontal ligament cell function (Popova *et al.,* 2007b). This tooth defect leads to malnutrition and dwarfism. Knockouts of the other two collagen-binding integrins show different skeletal phenotypes: α_1 knockout mice develop osteoarthritis but no growth plate abnormalities (Zemmyo *et al.,* 2003) and the phenotype of the α_2 knockout mouse with delayed callus formation and paucity of cartilage in callus suggests mainly a problem at the level of mesenchymal precursors (Ekholm *et al.,* 2002). Loss of collagen-binding integrins is accompanied by abnormalities in collagen fibril formation, illustrating the interdependence of matrix proteins and their cellular receptors (discussed in Popova *et al.,* 2007a). Clearly, deletion of individual collagen-binding integrins does not lead to profound skeletal phenotypes in unchallenged animals. Further studies with combined deletions of integrins, or under skeletal stress conditions, may reveal additional phenotypes and help understand the nature of the redundancy between the receptors.

The most abundant integrin in cartilage is $\alpha_5\beta_1$. Many studies have been performed to address the role of $\alpha_5\beta_1$ in chondrocyte interactions with fibronectin (Durr *et al.,* 1993; Enomoto *et al.,* 1993; Loeser, 1993; Xie and Homandberg, 1993; Homandberg and Hui, 1994; Enomoto-Iwamoto *et al.,* 1997; Clancy *et al.,* 1997; Shimizu *et al.,* 1997). Function-blocking antibodies and RGD peptides have been shown to inhibit cell adhesion to fibronectin and its fragments, thus modifying chondrocyte behavior and cartilage function. In keeping with this, plating of mesenchymal stem cells on scaffolds coated with RGD peptides blocks chondrogenesis (Connelly *et al.,* 2007). Chondrocyte recognition of collagen (including types I, II, and VI collagen) has likewise been extensively studied *in vitro* (Durr *et al.,* 1993; Enomoto *et al.,* 1993; Loeser, 1993; Holmvall *et al.,* 1995; Shimizu *et al.,* 1997; Enomoto-Iwamoto *et al.,* 1997; Camper *et al.,* 1997) and shown to be mediated via β_1 integrins: $\alpha_1\beta_1$ for adhesion to type I collagen (Shakibaei, 1995; Loeser, 1997) and type VI collagen (Loeser, 1997) and $\alpha_3\beta_1$ (Shakibaei *et al.,* 1993; Shakibaei, 1995, Loeser, 1997) or $\alpha_2\beta_1$ (Holmvall *et al.,* 1995) for adhesion to type II collagen. Blocking antibodies to these integrins directly interfere with chondrocyte survival (Hirsch *et al.,* 1997).

The important role of $\alpha_5\beta_1$ in developing cartilaginous structures is underscored by studies showing that inhibition of $\alpha_5\beta_1$ *in vivo* inhibits prehypertrophic chondrocyte differentiation and results in ectopic joint formation, whereas misexpression of this integrin leads to joint fusion by interfering with chondrocyte hypertrophy (Garciadiego-Cázares *et al.,* 2004). Given the large number of β_1 integrins with a role in chondrocyte differentiation and cartilage function, it is not surprising that a cartilage-specific knockout of β_1 integrin shows a profound cartilage phenotype with severe disruption of the growth plate through inability of cells to adhere to fibronectin and progress through mitosis (Aszódi *et al.,* 2003).

There is abundant evidence for a functional interaction between integrins and mechanical strain in cartilage, although the mechanisms differ from those seen in osteoblasts and osteocytes, and different types of strain seem to lead to different responses (Wright *et al.,* 1997; Millward-Sadler *et al.,* 1999; Lee *et al.,* 2000; Lucchinetti *et al.,* 2004 and reviewed in Millward-Sadler and Salter, 2004). Extensive studies with monolayers of isolated articular chondrocytes show that integrins, especially $\alpha_5\beta_1$ interacting with fibronectin, are critical in the hyperpolarization response to mechanical load. Strain also induces a variety of downstream signaling events and cytokine secretion (Chowdhury *et al.,* 2004, 2006). Adhesion via integrins leads to formation of focal adhesions in chondrocytes and, following mechanical stimulus, phosphorylation of focal adhesion components, including signaling molecules in the MAP kinase pathway, are seen. Synthesis of autocrine/paracrine factors known to be involved in transducing the biological effect from mechanosensor to effector cells (similar to osteocyte–osteoblast interactions described earlier) is stimulated by mechanical load on chondrocytes. In this context, IL-4 and substance P are especially important, in addition to nitric oxide and prostaglandins (Millward-Sadler *et al.,* 1999, 2003, reviewed in Millward-Sadler and Salter, 2004). Interestingly tension was recently shown to *inhibit* chondrogenesis via signaling through β_1, α_2, and α_5 integrin and FAK (Takahashi *et al.,* 2003; Onodera *et al.,* 2005). Clearly the complex forces exerted on cartilage have the potential to lead to different responses and these are currently not understood in full.

Chondrocyte adhesion to extracellular matrix proteins, especially fibronectin, is connected with chondrocyte–synovial cell interaction (Ramachandrula *et al.,* 1992), chondrocyte cell signaling (Loeser, 2002), and chondrocyte survival (Hirsch *et al.,* 1997; Cao *et al.,* 1999; Lucchinetti *et al.,* 2004, and reviewed in Millward-Sadler and Salter, 2004). Regulation of integrin expression and function by cytokines such as IL1, TGFβ and IGF1 has been linked to the release of matrix metalloproteinases (Arner *et al.,* 1995) and hence cartilage breakdown (Xie and Homandberg, 1993; Loeser, 1993, 1994; Yonezawa *et al.,* 1996; Clancy *et al.,* 1997). Such events are likely to be involved in the pathogenesis of cartilage destruction seen in osteoarthritis and rheumatoid arthritis. In addition, upregulation of α_2, α_4, and β_2 subunit expression were reported by Ostergaard *et al.* (1998) in osteoarthritic versus normal cartilage and altered responses to mechanical stress mediated by $\alpha_5\beta_1$ (Millward Sadler *et al.,* 2000). Sandya and coworkers (2007) recently reported increased levels of $\alpha_5\beta_1$ in articular cartilage in experimental arthritis and, as already indicated earlier, age-dependent cartilage degradation and synovial hyperplasia is found in α_1 knockout mice (Zemmyo *et al.,* 2003), indicating an important functional role for this integrin in cartilage homeostasis and suggesting that α_1 might be a potential drug target for osteoarthritis, a disease in urgent need of mechanism-specific therapies.

The key role for downstream signaling following integrin interaction with matrix ligands is underscored by the phenotype of integrin-linked kinase (ILK) knockout mice. ILK acts as a pleiotropic adapter interfacing β integrin cytoplasmic domains into the PKB/Akt signaling pathway. Mice with ILK deletion in the chondrocyte lineage have chondrodysplasia and show dwarfism. Isolated chondrocytes from such mice have matrix adhesion defects, similar to those seen in the cartilage-specific β_1 knockout described earlier (Grasshoff *et al.,* 2003, Terpstra *et al.,* 2003).

As discussed earlier for osteogenic cells, there is an increasing interest in articular cartilage tissue engineering. Adhesion processes, especially integrin-matrix interactions, will need to be considered when designing appropriate scaffolds, which, as the earlier discussion suggests, must in addition be able to transduce the correct types of strain to the cells for optimal proliferation and survival (van der Kraan *et al.,* 2002; Hunziker *et al.,* 2007).

Non-Integrin Cell Adhesion Molecules of Cartilage

Cadherins

Cadherin-11 is expressed in mesenchymal cells migrating from the neuroectodermal ridge that form presumptive cartilage in the developing mouse (Simonneau *et al.,* 1995). Likewise, N-cadherin is expressed in prechondrocytic cells in avian and mammalian limb buds, but not mature cartilage (Oberlender and Tuann, 1994; Tavella *et al.,* 1994; DeLise *et al.,* 2002a). There is some evidence for differential expression of cadherins (N-cadherin and cadherin-11) in prechondrocytic cells of developing limb primordia with cadherin-11 being expressed in growth plate but not articular chondrocytes (Matsusaki *et al.,* 2006). *In vitro* culture of mesenchymal cells is inhibited by neutralizing

N-cadherin antibody, suggesting a functional role for N-cadherin in early chondrogenesis (Oberlender and Tuann, 1994; Tavella *et al.,* 1994; DeLise *et al.,* 2002b) and that its function may be regulated by calciotropic factors such as $1{,}25D_3$ and TGFβ (Tsonis *et al.,* 1994). Unexpectedly however, the N-cadherin knockout mouse has no skeletal phenotype, suggesting that other family members, presumably cadherin-11, may compensate for loss of N-cadherin (Luo *et al.,* 2005). The cadherin-11 knockout mouse has a defect in synovial lining formation (Lee *et al.,* 2007), and defects in bone formation at specific sites (Kawaguchi *et al.,* 2001), but no direct effects in cartilaginous structures have been reported.

CD44

CD44 is expressed by cartilage and has been studied for a variety of sites and species (Hughes *et al.,* 1994; Noonan *et al.,* 1996; Stevens *et al.,* 1996, reviewed in Knudson and Loeser, 2002). The predominant isoform detected is the standard CD44H variant (Salter *et al.,* 1996). There is some evidence from the use of function-blocking antibodies showing that CD44 is involved in chondrocyte pericellular matrix assembly (Knudson, 1993; Knudson *et al.,* 1996). The full range of extracellular matrix molecules recognized by CD44 in cartilage is unclear, but interaction with hyaluronan clearly occurs (Knudson and Knudson, 2004). CD44 is upregulated during cartilage catabolism, (for example on IL1α treatment of bovine articular cartilage (Chow *et al.,* 1995) and chondrocytes have been shown to actively take up hyaluronan via CD44-mediated endocytosis (Hua *et al.,* 1993). Thus, it is reasonable to speculate that this molecule plays a regulatory role in cartilage matrix turnover in health and disease (Neidhart *et al.,* 2000; Takagi *et al.,* 2001). More recent evidence points to the presence of soluble CD44 in many tissues and especially in serum. Although mainly studied in the context of tumor biology (Hill *et al.,* 2006), there is evidence for elevated levels of soluble CD44 in inflammation such as in rheumatoid arthritis (Cichy and Pure, 2003).

Ig Family Members

NCAM is similarly distributed to N-cadherin in early cartilage development (Hitselberger Kanitz *et al.,* 1993; Tavella *et al.,* 1994), with N-cadherin expression temporally preceding N-CAM. It is therefore suggested that the role of NCAM is to stabilize cell–cell adhesions formed initially by N-cadherin (Tavella *et al.,* 1994). Primary human articular chondrocytes constitutively express VCAM-1 and ICAM-1 and this expression is increased by cytokines such as IL1β and TNFα and reduced by TGFβ and γ interferon (Davies *et al.,* 1991; Bujia *et al.,* 1996; Kienzle *et al.,* 1998). *In vitro* VCAM-1 and ICAM-1 contribute to T-cell adhesive processes suggesting that *in vivo* these molecules may be important players in mediating T cell–chondrocyte interactions at sites of inflammatory joint destruction (Horner *et al.,* 1995; Seidel *et al.,* 1997; Kienzle *et al.,* 1998).

Syndecans

Syndecan-3 is highly expressed in proliferating chondrocytes, below the tenascin-C-rich layer of articular chondrocytes; decreased levels are found in hypertrophic cartilage (Shimazu *et al.,* 1996). High levels are also found in forming perichondrium (and later in periosteum) in the developing avian limb (Seghatoleslami *et al.,* 1996) and it has been suggested that syndecan-3 is involved with tenascin-C in establishing, or maintaining, boundaries during skeletogenesis (Koyama *et al.,* 1995). Other data supports a role for syndecan-3 in the regulation of chondrocyte proliferation (Pfander *et al.,* 2001; Kirsch *et al.,* 2002).

Selectins

There is no information on selectin expression in chondrocytes.

CONCLUDING REMARKS: MODULATION OF INTEGRIN FUNCTION IN BONE — NEW THERAPEUTIC POSSIBILITIES FOR BONE DISEASE

Bone and cartilage cells express a wide variety of adhesion molecules (summarized with their known and potential functions in Table VII). Integrin expression has been studied extensively, but, generally, there is less information on expression of other adhesion molecule family members. Furthermore, there is also relatively little information on the expression and function of adhesion molecules of all classes during skeletal cell development, largely because we currently lack adequate markers to identify immature bone cells, though the phenotypes of knockout mice are increasingly informative. Adhesion receptors fulfill many functions in the skeleton, and these are frequently linked to a variety of intracellular signaling pathways, leading to a central regulatory role for this class of molecules in bone metabolism. Knowledge of their role in bone resorption and cartilage integrity is extensive, although a function for cell adhesion receptors in bone formation has only been defined recently. Although no unique osteoblast, osteoclast, or chondrocyte adhesion molecule has been identified to date, therapeutic strategies based on selectively inhibiting highly expressed receptors, such as the $\alpha_v\beta_3$ integrin in osteoclasts, have proved to be successful in regulating excessive bone resorption. Better knowledge of the expression of adhesion molecules in bone and cartilage pathology is required, and elucidation of the role of cell–matrix interactions in the etiology of skeletal disease will, therefore, remain a research challenge for the foreseeable future.

TABLE VII Key Functional Roles for Cell Adhesion Molecule Interactions in Bone

Receptor	Cell type	Ligand(s) bound[a]	Known/potential functions
Integrins			
$\alpha_v\beta_3$	Osteoclast	Vitronectin, osteopontin, bone sialoprotein, fibronectin, fibrinogen, denatured collagen, etc.	Matrix adhesion Signal transduction Osteoclast polarization through role in organization of the cytoskeleton Role in osteoclast motility ? Cessation of resorption
$\alpha_v\beta_5$	Osteoclast	As for $\alpha_v\beta_3$	Regulation of osteoclast formation
$\alpha_9\beta_1$	Osteoclast	ADAM8	Osteoclast formation and resorption
$\alpha_1\beta_1$	Osteoblast/Mesenchymal cell	Collagen	Osteoblast lineage differentiation
$\alpha_1\beta_1$	Cartilage cells/ Mesenchymal cell	Collagen	Cartilage lineage differentiation
$\alpha_2\beta_1$	Osteoclast	Native collagens	Matrix adhesion
	Osteoblast	Native collagens	Matrix adhesion Osteoblast differentiation
	Chondrocyte	Type II collagen	Matrix adhesion, orchestration of matrix deposition
$\alpha_{10}\beta_1$	Chondrocyte	Types II, IV, and VI collagens, ? type IX collagen	Matrix adhesion, orchestration of matrix deposition
$\alpha_{11}\beta_1$	Perichondral cells, Mesenchymal cells	Type I collagen, ? type XIII collagen	Organization of collagen bundles
$\alpha_5\beta_1$	Osteoblast	Fibronectin (RGD)	Osteoblast differentiation and survival Mechanosensing
	Chondrocyte	Fibronectin (RGD)	Mechanosensing Cartilage breakdown
$\alpha_6\beta_1$	Chondrocyte	Laminin	Matrix adhesion
Cadherins			
N-cadherin	Osteoblast	N-cadherin	Osteoblast development, mineralization ? Mechanosensing
	Chondrocyte	N-cadherin	Cartilage development/mesenchymal condensation
Cadherin-11	Osteoblasts/ Mesenchymal cells	Cadherin	Osteogenesis, mesenchymal fate decisions
E-cadherin	Osteoclast	E-cadherin	? Osteoclast differentiation
Ig superfamily			
ICAM-1	Osteoclast	LFA-1 on leukocytes	Osteoclast differentiation
	Osteoblast	LFA-1 on leukocytes	Osteoblast differentiation Production of cytokines
	Chondrocyte	LFA-1 on leukocytes	Cartilage breakdown
VCAM-1	Osteoblast	α_4 integrins on leukocytes	Osteoblast differentiation Production of cytokines
Cell surface proteoglycans			
Syndecan-1	Osteoblast, Osteocyte	Type I collagen, tenascin-C	? Matrix adhesion ? Osteoblast differentiation ? Role in mechanosensing
Syndecan-3	Chondrocyte	Tenascin-C	Cartilage development
CD44	Osteoclast	Hyaluronate, osteopontin, ? type I collagen, ? fibronectin	Osteoclast formation Osteoclast migration ? Osteoclast-osteoblast interaction
	Osteoblast	Hyaluronate ?	Hyaluronate degradation
	Osteocyte	Hyaluronate, ? osteopontin, ? type I collagen, ? fibronectin	? Matrix adhesion ? Role in mechanosensing ? Osteocyte-osteoblast interaction
	Chondrocyte	Hyaluronate	Pericellular matrix assembly

[a] *There is no definitive information on the natural ligands in bone or cartilage for these molecules. The range of ligands demonstrated to be bound in in vitro adhesion assays is shown. ?indicates where the function is highly likely, but there is no definite evidence.*

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Chapter 21

Intercellular Junctions and Cell–Cell Communication in the Skeletal System

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INTRODUCTION

The organization of cells in tissues and organs is controlled by molecular programs that afford cells the ability to recognize other cells and the extracellular matrix and to communicate with their neighbors. Adhesive interactions are essential not only in embryonic development, but also in a variety of other biologic processes, including the differentiation and maintenance of tissue architecture and cell polarity, the immune response and the inflammatory process, cell division and death, tumor progression and metastases (Goodenough *et al.*, 1996; Simon and Goodenough, 1998; Vleminckx and Kemler, 1999; Zbar *et al.*, 2004). Cell–cell and cell–matrix adhesion are mediated by four major groups of molecules: cadherins, immunoglobulin-like molecules, integrins, and selectins. Cadherins are an integral part of *adherens junctions*, which along with tight junctions and desmosomes, constitute the so-called anchoring junctions, which join cells by anchorage through their cytoskeletons (Halbleib and Nelson, 2006). A different type of intercellular junction is the *gap junction*, which does not provide cell anchorage but allows direct communication via specialized intercellular channels (Goodenough *et al.*, 1996). In addition to cell–cell adhesion and gap junctional communication, cell-to-cell propagation of locally generated signals, such as mechanically induced "calcium waves" can occur via short-range intercellular signaling systems that require either gap junctions or extracellular release of nucleotides and activation of purinergic receptors (Jørgensen *et al.*, 1997).

In the adult skeleton, bone remodeling occurs via repeated sequences of bone resorptive and formative cycles, which require continuous recruitment and differentiation of bone marrow precursors. The cooperative nature of bone remodeling requires efficient means of intercellular recognition and communication that allow cells to sort and migrate, synchronize their activity, equalize hormonal responses, and diffuse locally generated signals. Likewise, cell–cell interactions are critical for aggregation and condensation of immature chondro-osteoprogenitor cells and mesenchymal precursors during skeletal development (bone modeling). This chapter reviews current knowledge about the role of direct cell–cell interactions in the development and remodeling of the skeletal tissue, focusing on cell–cell adhesion via cadherins, cell–cell communication via gap junctions, and short-range calcium signals, or calcium waves, via extracellular nucleotides and purinergic receptors.

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CADHERINS AND CELL–CELL ADHESION

Adherens Junctions and the Cadherin Superfamily of Cell Adhesion Molecules

Cadherins owe their name to their main function in mediating calcium-dependent cell–cell adhesion. As many as 80 members of this large superfamily of cell adhesion molecules have been cloned, and most tissues express more than one cadherin (Gumbiner, 1996; Yagi and Takeichi, 2000). These single chain integral membrane glycoproteins have a molecular mass of about 120 kDa and their structure includes a long extracellular domain (EC), a single transmembrane-spanning domain, and a relatively small intracellular (IC) C-terminus tail (Halbleib and Nelson, 2006). The EC domain, composed of five repeats (EC1 through EC5) contains calcium-binding sites and allows binding to the same cadherin on neighboring cells (Fig. 1). Structural differences allow classification of cadherins into two main subfamilies, type I, which mediate strong cell–cell adhesion via a putative His-Ala-Val motif in EC1, and include, among others, N-, E-, M-cadherin, and cadherin-4 (the human ortholog of mouse R-cadherin); and type II, comprising cadherin-5 through -12 as well as VE-cadherin, which do not have the His-Ala-Val motif. An additional group of cadherins includes those lacking the intracellular tail, i.e., T-cadherin and cadherin-13, and whose function in cell–cell adhesion remains to be determined. Close relatives of this superfamily of "classical" cadherins are protocadherins, cadherin-related neuronal receptor and other structurally related molecules present primarily in the central nervous system (Suzuki, 1996;Yagi and Takeichi, 2000), as well as desmocollins and desmogleins, which differ from the typical cadherins in their cytoplasmic domain (Buxton and Magee, 1992).

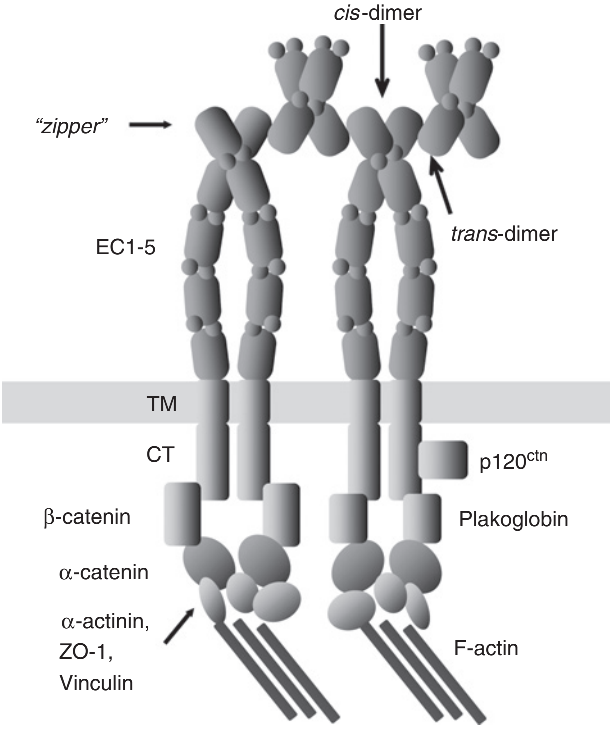

FIGURE 1 Schematic representation of the cadherin adhesion complex. Each cadherin is shown with its five extracellular domains (EC1–5), as well as their transmembrane (TM) and cytoplasmic (CT) domains. Small circles between the EC domains symbolize calcium ions. Two complete cadherin *cis* dimers are shown side by side. These engage in binding with similar complexes (only the first 2 EC domains represented) from an apposing cell, thus forming *trans* dimers. The alignment of EC1 domains forms the so-called "zipper" structure of the adhesion complex. Through their CT domain, cadherins bind to β-catenin and plakoglobin, which in turn tether the complex to the actin cytoskeleton via α-catenin and other interacting proteins, including α-actinin, ZO-1, and vinculin. Binding to $p120^{ctn}$ also contributes to stabilizing the cadherin-based adhesion structure.

Crystallographic analysis of E-cadherin ectodomains has demonstrated that cadherin-mediated cell–cell adhesion occurs via the formation of cis-homodimers upon Ca^{2+} binding (Fig. 1). As extracellular Ca^{2+} concentration increases to more than 1mM, the two cadherins participating in the dimer become rigid and form an X-shaped assembly, interfacing through their EC1 and EC2 domains (Pertz *et al.,* 1999; Shapiro *et al.,* 1995). Higher Ca^{2+} concentration allows Trp2 to dock into a hydrophobic pocket in EC1 of an apposing cadherin, thus forming a trans-homodimer and generating a "zipper" structure of multiple cis-dimers on apposing membranes (Patel *et al.,* 2006; Troyanovsky, 1999). Stabilization of cadherin dimers occurs by lateral clustering and linkage to the cytoskeleton via the highly conserved cytoplasmic tail. The IC domain binds to β-catenin and plakoglobin, which connect cadherins to the actin cytoskeleton via α-catenin, either directly or via binding to other proteins, including actinin, vinculin, or ZO-1 (Yamada and Geiger, 1997). Recent data indicate that α-catenin binding to either the cadherin/β-catenin complex or to actin filaments is mutually exclusive (Drees *et al.,* 2005), implying that the connection between the adhesion structure and the cytoskeleton is dynamic rather than static (Yamada *et al.,* 2005). Adhesion is controlled by cadherin binding to other proteins, most importantly $p120^{ctn}$, also a member of the catenin family, and IQGAP1, which contribute to stabilize cadherin-β-catenin binding and mediates regulatory effects of Rho family members, particularly RhoA, Rac1, and Cdc42 (Kaibuchi *et al.,* 1999; Reynolds and Carnahan, 2004). The assembly of cadherins and their associated cytoskeletal elements form the junctional structures known as adherens junctions, which provide anchorage between two adjoining cells (Foty and Steinberg, 2005; Gumbiner, 2005).

The catenins (α-catenin, β-catenin, plakoglobin, and $p120^{ctn}$) serve at least three distinct functions; they provide a link between cadherins and the cytoskeleton, they control the adhesive state of cadherin dimer complexes, and they interact with components of signaling pathways (Halbleib and Nelson, 2006). The most relevant example is β-catenin, which is an integral component of Wnt signaling (reviewed in detail under Chapter 6), and therefore it directly links adhesion and signaling (Gottardi and Gumbiner, 2001; Nelson and Nusse, 2004). The most widely held belief is that by keeping β-catenin bound at adhesion structures on the cell surface, cadherins sequester β-catenin from its transcriptionally active pools (Fagotto *et al.,* 1996; Sadot *et al.,* 1998), thus potentially attenuating Wnt signaling (Ciruna and Rossant, 2001). By contrast, reduced abundance of cadherins on the cell surface would make more β-catenin available for transcriptional regulation, at the expense of destabilizing the adhesion complex with loss of cell–cell adhesion and increased cell movement (Nelson and Nusse, 2004). Thus, an inverse relationship exists between adhesion and signaling: Wnt signals induce conformational changes of β-catenin that favor the formation of transcriptional complexes, whereas β-catenin association with α-catenin favors cadherin binding and adhesion (Brembeck *et al.,* 2004; Gottardi and Gumbiner, 2004). An alternative, hypothetical view is that β-catenin bound to cadherins on the cell surface might provide a rapidly available source of this signaling molecule for transcriptional activation in response to continuous Wnt signaling. Thus, a lower level of membrane-bound β-catenin might actually attenuate signaling because less β-catenin is available for mobilization into active pools upon Wnt activation. In any case, intersection of cadherin-mediated adhesion and Wnt signaling via β-catenin represents a flexible mechanism of cell regulation (Gottardi and Gumbiner, 2001; Nelson and Nusse, 2004; Tuan, 2003), a mechanism of particular importance in the skeleton, considering that β-catenin activation is now recognized as a necessary step in osteogenic differentiation (Day *et al.,* 2005; Hu *et al.,* 2005). Recent data suggest that α-catenin and p120^{ctn} also have their own roles in signaling, independent of the Wnt pathway. Both α-catenin and p120^{ctn} have been shown to interact with RAS-MAPK, NFκB, hedgehog (Lien *et al.,* 2006; Vasioukhin *et al.,* 2001), and Rac and Cdc42 (Ciesiolka *et al.,* 2004; Elia *et al.,* 2006), respectively. Thus, interactions with Rho family members provide an inside-out signaling mechanism that regulates cadherin assembly and adhesion properties.

Cadherins in Skeletal Development, Growth, and Maintenance

Cells of the osteoblastic lineage express two major cadherins, N-cadherin (gene: *Cdh2*) and cadherin-11 (gene: *Cdh11*) (Cheng *et al.,* 1998; Ferrari *et al.,* 2000; Okazaki *et al.,* 1994). E-cadherin immunoreactivity has been observed in UMR 106–01 cells (Babich and Foti, 1994), and mRNA for other cadherins, including P-cadherin, VE-cadherin, cadherin-8, and cadherin-6 have been detected in primary mouse calvaria cultures and cell lines (Kawaguchi *et al.,* 2001b; Mbalaviele *et al.,* 1998). However, the abundance of these cadherins is low, and screening for all cadherin family members expressed by human trabecular bone cells using degenerate PCR primers yielded only *Cdh2* and *Cdh11* mRNA transcripts (Cheng *et al.,* 1998). A splice variant of *Cdh11* lacking the IC domain has also been described on the surface of osteoblasts, but its function remains uncertain (Kawaguchi *et al.,* 1999). In embryogenesis, N-cadherin is abundant in mesenchymal cells undergoing cartilage nodule condensation (Tsonis *et al.,* 1994); and while not present in mature cartilage, *Cdh2* expression clearly persists in mature and adult bone (Ferrari *et al.,* 2000; Hoffmann and Balling, 1995; Simonneau *et al.,* 1995). Earlier work in limb bud micromass cultures showed that although dominant negative interference with N-cadherin negatively affects cell aggregation and condensation (Oberlender and Tuan, 1994a; Tavella *et al.,* 1994), persistent expression of *Cdh2* prevents full cartilage differentiation (Cho *et al.,* 2003; DeLise and Tuan, 2002). However, a recent *in vivo* study elegantly demonstrated that *ex vivo* limb bud cultures from *Cdh2* null embryos partially rescued by transgenic expression of *E-cadherin* were able to undergo cartilage condensation and develop into structured limbs in the absence of *Cdh2* (Luo *et al.,* 2004). Therefore, N-cadherin is dispensable for early phases of embryonic skeletogenesis.

Cadherin-11 is transiently expressed in the cephalic mesoderm and then in the paraxial mesoderm of the trunk during early development, particularly in the head, somites, and limb buds (Hoffmann and Balling, 1995; Kimura *et al.,* 1995; Simonneau *et al.,* 1995). However, at later developmental stages, a wide variety of mesenchymal tissues in both mesodermal and neural crest derivatives express *Cdh11* (Simonneau *et al.,* 1995). Thus, both *Cdh2* and *Cdh11* are present in mesenchymal cells, but with distinct expression patterns; *Cdh2* is less abundant than *Cdh11* in the head and it is absent in branchial arches, in sharp contrast with the abundant presence of *Cdh11* (Kimura *et al.,* 1995). Furthermore, while *Cdh2* is present in the perichondrium (Kimura *et al.,* 1995; Oberlender and Tuan, 1994b), *Cdh11* appears only in the primary spongiosa but not in condensing or proliferating chondrocytes of the growth plate, where *Cdh2* is abundant. Nonetheless, mice with homozygous *Cdh11* null mutation have no skeletal malformations (Kawaguchi *et al.,* 2001a). Hence, absence of either *Cdh2* or *Cdh11* can be tolerated in skeletal development, and *Cdh11* may compensate for lack of *Cdh2*, as suggested by the more severe disruption of somite structure in double *Cdh2*;*Cdh11* null embryos relative to single cadherin gene deletion (Horikawa *et al.,* 1999), and by strong *Cdh11* expression at sites of chondrogenesis in *Cdh2* null mice (Luo *et al.,* 2004).

As just noted, mice genetically deficient of *Cdh11* have no major skeletal malformations, but display minor calcification defects of the cranial sutures and become osteopenic by three months of age, an abnormality seen primarily in the trabecular bone and linked to a cell autonomous functional defect of bone forming cells (Kawaguchi *et al.,* 2001a). Homozygous *Cdh2* null mutation is embryonically lethal (Radice *et al.,* 1997), but *Cdh2* haploinsufficient mice exhibit an accentuated bone loss after ovariectomy relative to wild type mice (Fang *et al.,* 2006), demonstrating that while a single *Cdh2* allele is sufficient to maintain bone mass in resting conditions, it does hinder adaptive responses to an acute stimulation of bone remodeling. As an approach to overcome cadherin redundancy in bone, a dominant negative *Cdh2* mutant, *NCadΔC* was expressed *in vivo* selectively in bone forming cells using the mouse *osteocalcin 2* (*Og2*) promoter (*Og2- NCadΔC*). Peak bone mass acquisition is delayed in these transgenic animals, resulting in osteopenia up to four months of age. This is the consequence of decreased osteoblast number and impaired osteogenic differentiation, defects associated with both decreased cell–cell adhesion and interference with β-catenin function (Castro *et al.,* 2004). Because the inhibitory action of the mutant molecule is not restricted to a specific cadherin, the more pronounced skeletal phenotype of *Og2- NCadΔC* mice relative to *Cdh11* null mice is consistent with the concept that *Cdh2* may partially compensate for lack of *Cdh11*. The partial redundancy of these two cadherins in bone formation is further underscored by preliminary reports of dramatically decreased bone mass with microarchitectural defects of trabecular and cortical bone in mice with *Cdh2* haploinsufficiency in a *Cdh11* null background (Donsante *et al.,* 2005).

Cadherins in Commitment and Differentiation of Bone Forming Cells

In uncommitted mesenchymal cells, such as the embryonic mouse cell line C3H10T1/2, expression of *Cdh2* is increased by bone morphogenetic protein-2 (BMP-2), presumably reflecting the transition to a chondro-osteogenic phenotype (Shin *et al.,* 2000). However, N-cadherin abundance decreases in calvaria cell as they progress through their *in vitro* differentiation program (Fang *et al.,* 2006). Likewise, *Cdh2* mRNA is sharply downregulated by dexamethasone (Lecanda *et al.,* 2000a) and by IL-1 or TNF-α (Tsutsumimoto *et al.,* 1999), whereas it is upregulated by a constitutively active, mutated FGFR-2 receptor (Lemonnier *et al.,* 1998). On the other hand, *Cdh11* is upregulated in immature mesenchymal cells under stimulation by BMP-2 and other osteogenic factors, whereas it is downregulated when cells undergo adipogenic or myogenic differentiation and disappears in adipocytes (Kawaguchi *et al.,* 2001b; Shin *et al.,* 2000). Similarly, the uncommitted C2C12 cells lose *Cdh4* upon osteogenic differentiation, whereas cadherin-11 abundance increases. These cells also express M-cadherin, indicative of their myogenic potential, but transdifferentiation from myogenic to osteogenic cell phenotypes is associated with M-cadherin to cadherin-11 transition (Kawaguchi *et al.,* 2001b). Furthermore, teratomas overexpressing *Cdh11* preferentially form bone and cartilage tissue (Kii *et al.,* 2004). Therefore, it is conceivable that coexpression of *Cdh11* and *Cdh2* may allow sorting and segregation of mesenchymal progenitors committing to osteogenic differentiation from those entering the adipogenic pathway. However, as differentiation progresses, *Cdh2* is downregulated whereas *Cdh11* is present throughout the osteoblast differentiation program. In fact, in cells already committed to the osteogenic pathway, *Cdh11* expression does not change substantially with differentiation (Cheng *et al.,* 1998; Kawaguchi *et al.,* 2001b), though it is modestly downregulated by dexamethasone (Lecanda *et al.,* 2000a). Interestingly, cadherin-11 abundance decreases with aging in rat bone marrow stromal cells (Goomer *et al.,* 1998), raising the possibility that decreased cadherin-11 may contribute to the reduced osteogenic potential in the aging skeleton, an hypothesis consistent with the impaired osteoblast function of *Cdh11* null cells (Kawaguchi *et al.,* 2001a). Whether the cadherin repertoire changes with terminal differentiation into osteocytes has not been thoroughly investigated, although neither *Cdh11* nor *Cdh2* have been detected in the osteocyte-like cell line MLO-Y4 (Kawaguchi *et al.,* 2001b).

A number of *in vitro* studies consistently demonstrated that disruption of cadherin function using peptide inhibitors (Cheng *et al.,* 1998; Ferrari *et al.,* 2000), function blocking antibodies (Hay *et al.,* 2000), antisense RNA (Lemonnier *et al.,* 1998), or overexpression of dominant negative mutants (Cheng *et al.,* 1998; Ferrari *et al.,* 2000) impairs osteoblast differentiation and/or differentiated function in cell lines and in primary osteoblast cultures. Intriguingly in *Og2- NCadΔC* mice, a lower than normal bone mass was associated with a concomitant increase in percent body fat and number of bone marrow adipogenic precursors, apparently at the expense of osteogenic precursors (Castro *et al.,* 2004). This osteogenic to adipogenic shift in *Og2- NCadΔC* transgenic mice was in part consequent to reduced β-catenin signaling, because this abnormality was rescued by expression of an active β-catenin mutant (Castro *et al.,* 2004). Indeed, the dominant negative *NCadΔC* mutant sequesters β-catenin on the cell surface (Castro *et al.,* 2004), and inhibits β-catenin dependent transactivation (Shin *et al.,* 2005). These findings demonstrate that osteoblast cadherins are involved in bone marrow stromal cell lineage allocation, and support the notion that cadherins influence Wnt/β-catenin signaling. Furthermore, as in the *Og2- NCadΔC* mice the dominant negative mutant is expressed only in differentiated osteoblasts, the osteogenic to adipogenic shift in bone marrow precursors also suggests that osteoblasts may control the fate of their own precursors in a cadherin-dependent fashion. Whether this intriguing new regulatory mechanism involves cell–cell contact or paracrine mechanisms remains to be determined.

Even though the experimental evidence discussed earlier strongly points to some degree of redundancy between *Cdh2* and *Cdh11*, new and in part still preliminary *in vivo* data are uncovering specific roles of N-cadherin and cadherin-11 at different steps of osteogenesis. In particular, N-cadherin and cadherin-11 segregate away from each other when coexpressed in the same cells, and impart different spatial organization to the cultures when expressed separately (Kii *et al.,* 2004). As noted, N-cadherin is relatively abundant compared to other cadherins in undifferentiated cells (Kawaguchi *et al.,* 2001b; Shin *et al.,* 2000), but it is downregulated during *in vitro* differentiation (Fang *et al.,* 2006), and overexpression of *Cdh2* prevents full chondrocyte differentiation (Cho *et al.,* 2003; DeLise and Tuan, 2002). These data suggest that N-cadherin may have a "braking" effect on mesenchymal/stromal cell differentiation, a notion supported by the finding of a decreased number of osteoprogenitors in bone marrow of *Cdh2* haploinsufficient mice (Fang *et al.,* 2006), and by a preliminary report showing osteopenia and altered osteoblast differentiation in mice overexpressing full length *Cdh2* in osteoblasts (Hay *et al.,* 2006).Thus, the primary function of N-cadherin may be to keep uncommitted progenitors in an undifferentiated state, perhaps favoring aggregation of bone marrow stromal (skeletal) stem cell in a niche, an organization similar to hematopoietic stem cell niches (Gregory *et al.,* 2005). In fact, stromal and hematopoietic stem cells may even coexist in the same microanatomical niches (Baksh *et al.,* 2004). In this scenario, differentiated osteoblasts and/or bone lining cells may help support and stabilize the stem cell niche via N-cadherin (and possibly cadherin-11) mediated cell–cell adhesion. Commitment to osteogenesis is associated with upregulation of *Cdh11*, whereas downregulation of *Cdh11* favors adipogenesis (Kawaguchi *et al.,* 2001b; Kii *et al.,* 2004; Shin *et al.,* 2000). As osteoblast differentiation progresses, *Cdh2* is progressively downregulated, thus allowing committed cells to escape from the niche, and *Cdh11* remains as the major osteoblast cadherin (Mbalaviele *et al.,* 2006) (Fig. 2). Although a few steps of this model remain to be demonstrated experimentally, cadherins can provide the means for cell–cell aggregation and migration of committed cells to the areas of active bone remodeling. Thus, by allowing direct interactions among cells of different lineages and by modulating β-catenin signaling, cadherins represent fundamental players in coordinating the activity of cells in the bone marrow microenvironment.

Other Cell Adhesion Molecules in Bone and Cartilage

Neural cell adhesion molecule (N-CAM), a member of the immunoglobulin superfamily, is present in chick limb buds before mesenchymal cell condensation and its abundance increases during cell aggregation in a pattern similar to that of N-cadherin (Tavella *et al.,* 1994). Both adhesion molecules are undetectable in hypertrophic chondrocytes, but are reexpressed in preosteoblastic cells (Lee and Chuong, 1992; Tavella *et al.,* 1994). Although N-CAM and N-cadherin are present during chondrogenesis, subtle differences in the timing of expression suggest that N-cadherin may initiate cell–cell aggregation while N-CAM stabilizes the aggregates, although such hypothesis has not been proven in a more mechanistic fashion. Although disappearing in differentiated cartilage, N-CAM persists in the perichondrium of long bones and sclerotomes, it is present in the calvarium during mesenchymal cell aggregation differentiation (Lee and Chuong, 1992), but is not regulated by BMP-2 (Hay *et al.,* 2000).

Cell Adhesion Molecules in Osteoclast Differentiation and Hematopoiesis

Two critical steps of osteoclastogenesis, i.e., heterotypic interactions between hematopoietic osteoclast precursors and stromal/osteoblastic cells and osteoclast precursor fusion are both dependent on cell–cell adhesion. Osteoblast/stromal cell support of osteoclastogenesis is mediated by the interaction of RANKL on the surface of osteoblasts and stromal cells and its receptor, RANK, present on osteoclast precursors. Although soluble RANKL is sufficient to induce osteoclastogenesis *in vitro*, close proximity between osteoclast precursors and their

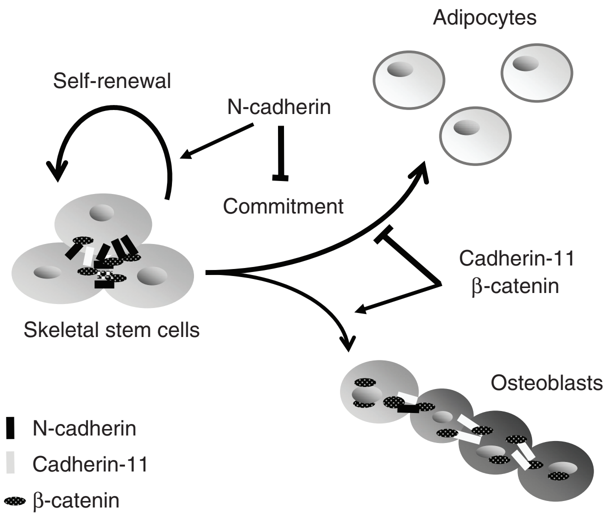

FIGURE 2 Cadherins in skeletal stem cell (SSC) maintenance and lineage allocation. In their undifferentiated stage, SSC express a relatively wide repertoire of cadherins, including *N-cadherin* (*Cdh2*), *cadherin-11* (*Cdh11*), and *R-cadherin* (*Cdh4*), with the former being the most abundant. SSC are maintained by self-renewal, i.e., production of daughter cells with conserved multipotential capacity, and are organized in microanatomical niches. The stem cell niche is held together by N-cadherin mediated cell–cell adhesion. SSC commitment to osteogenesis is associated with upregulation of *Cdh11*, whereas downregulation of *Cdh11* favors adipogenesis. As differentiation progresses, *Cdh2* is downregulated, thus allowing cells to escape from the niche and reach the areas of active bone remodeling. This switch in cadherin expression may allow more β-catenin to become available for transcriptional regulation, thus contributing to osteogenesis and anti-adipogenesis. Reproduced with modifications from Mbalaviele *et al., J. Bone Miner. Res.* 2006; **21**,1821–1827 with permission of the American Society for Bone and Mineral Research.

supporting cells is likely to be required *in vivo*, as both receptor and ligand are membrane bound (Suda *et al.*, 1999). However, it is unlikely that RANKL–RANK interaction by itself provides cell–cell adhesion, as anti-RANKL antibodies do not affect osteoblast adhesion to peripheral monocytes, whereas antibodies against vascular adhesion molecule VCAM-1 or against intercellular adhesion molecule ICAM-1 do (Tanaka *et al.*, 1995; Tanaka *et al.*, 2000). It is more likely that osteoclast precursors anchor to their supporting cells to allow stable RANK-RANKL engagement and osteoclastogenic signaling. Earlier work convincingly demonstrated that such anchorage may be mediated by cadherin-6 (*Cdh6*), the murine homologue of human K-cadherin, and its splice variant, cadherin-6/2, as expression of a dominant negative, adhesion defective *Cdh6* mutant prevented stromal cell support of osteoclastogenesis (Mbalaviele *et al.*, 1998). More recent data suggest that cadherins expressed by osteogenic cells can also control osteoclastogenesis by regulating the expression of RANKL via a β-catenin dependent mechanism (Shin *et al.*, 2005). In contrast, E-cadherin seems to be important in the fusion of mononuclear precursors, because interference with E-cadherin adhesion prevents the formation of multinucleated bone-resorbing osteoclasts (Mbalaviele *et al.*, 1995). In addition, co-localization studies have demonstrated that cadherins may be involved in the formation or maintenance of the actin ring in the osteoclast sealing zone and thus, they may control osteoclast attachment to the matrix, at least in an indirect manner (Ilvesaro *et al.*, 1998). Based on these observations, it seems plausible that interference with cadherin mediated cell–cell interactions may be a reasonable target for inhibition of osteoclast differentiation.

Growing evidence suggests that cadherin mediated heterotypic cell–cell interactions extend to hematopoiesis. Hematopoietic stem cell niches have been found in contact with cells lining the bone surface and both cell types express *Cdh2* and *β-catenin in vivo* (Zhang *et al.*, 2003; Zhu and Emerson, 2004). Furthermore, stimulation of osteoblast activity by either expression of a constitutively active PTH/PTHrP receptor, or by exogenous administration of PTH increases the number of hematopoietic stem cells via modulation of Notch signaling (Calvi *et al.*, 2003). Because Notch signaling is also based on membrane-bound ligand and receptors, close cell–cell contact is essential in this interaction, as it is for the RANK-RANKL system. Recent studies also show that the balance between self-renewal and differentiation of hematopoietic stem cells is controlled by *c-Myc* in a N-cadherin dependent fashion: *c-Myc* stimulation of differentiation and escape from the niche is associated with downregulation of *Cdh2* (Wilson *et al.*, 2004). These findings reinforce the idea that N-cadherin contributes to maintaining cells in an undifferentiated state and that its persistence prevents differentiation, as noted earlier for the stromal cell compartment. Thus, osteoblast/stromal cell support of osteoclastogenesis can be seen as a particular case of a more general mechanism of osteoblast-hematopoietic cell interactions, in which cadherins play a prominent role.

CONNEXINS AND GAP JUNCTIONAL COMMUNICATION

Connexins, Gap Junctions, and "Hemichannels"

One mechanism by which cells can rapidly and efficiently coordinate function is through gap junction-mediated intercellular communication. Gap junctions are transcellular channels that permit the diffusion of small molecules (~1.2 kDa or less), including second messengers, metabolites, and ions among coupled cells. The biologic relevance of direct intercellular communication in multicellular organisms is underscored by the evolution of two gene families, connexins (Goodenough *et al.*, 1996) and pannexins (Bruzzone *et al.*, 2003), which serve similar functions.

A connexin is the monomeric unit of a gap junction. More than 20 connexin genes have been identified in the mouse and human genomes, and most cells express more than one connexin (Goodenough *et al.*, 1996; Sohl and Willecke, 2003). Connexins are integral membrane proteins with four transmembrane domains, two small extracellular loops, a cytoplasmic loop, and cytoplasmic N- and C-terminal tails (Fig. 3A). The cytoplasmic loop and the long C-terminal tail differ widely between various connexins in both amino acid sequence and length. Connexins assemble into a hexamer in the plasma membrane, forming a unit referred to as a connexon, or "hemichannel" (Fig. 3B). When two connexons on appositional cell membranes dock to each other, a gap junction channel is formed (Fig. 3C). Docking is stabilized by disulfide linkage that forms via conserved cysteine residues in the two extracellular loops of the apposing connexins (Goodenough *et al.*, 1996; Kumar and Gilula, 1996). Growing evidence suggests that connexons may also function as hemichannels without docking to another connexon, and allow exchange of solutes and ions between the cytoplasm and the extracellular space, in a similar fashion as a membrane channel (Goodenough and Paul, 2003).

Although in most circumstances connexons formed by a single connexin (homomeric connexons) pair with like connexons on the apposing cell, heterotypic gap junction channels are possible, depending on the compatibility of the extracellular loops (White *et al.*, 1994). Likewise, co-oligomerization into heteromeric connexons may occur when more than one connexin is present in the same cell (Fig. 3D), resulting in heterotypic gap junctions with unique biophysical properties (Kumar and Gilula, 1996). The phosphorylation state of connexins controls the assembly and degradation of the protein, as well as the

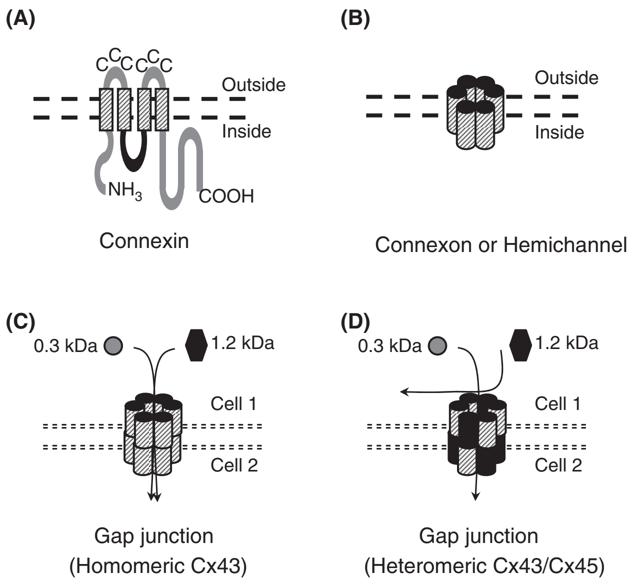

FIGURE 3 Structure and different conformations of gap junction channels. (**A**) A connexin is the monomeric unit of gap junctions and hemichannels. The cysteine residues in the extracellular loops permit docking to apposed connexins in an adjacent cell. The intracellular loop (in black) and the C-terminus tail are thought to be involved in size permeability and in opening and closing of the channel, whereas the transmembrane domains (shaded blocks) confer charge selectivity to the channel. The C-terminus also serves as a docking platform for numerous signal complexes. (**B**) Connexin monomers oligomerize into a hexameric structure, termed a connexon. A connexon is also called "hemichannel," to indicate that under certain circumstances it can function as membrane channel, thus opening a pore into the extracellular space. (**C**) When two connexons on apposed plasma membranes dock they form a gap junction channel, a transcellular pore that provides aqueous continuity between two cytoplasms and permits the diffusion of signaling molecules, ions, and nutrients among cells. In the example, homomeric Cx43 connexons pair with a similar connexon to form a homotypic gap junction that allows molecules as large as 1.2 kDa to pass from cell to cell. (**D**) Heteromeric connexons may also form when multiple connexins are expressed in the same cell. These may form heteromeric, heterotypic gap junctions, whose biophysical properties are different from those of homomeric connexons. In the example, a heteromeric Cx43 (shaded blocks)/Cx45 (black blocks) gap junction allows molecules not larger than 0.3 kDa to pass through the channel, as in such a mixed connexin environment Cx45 is functionally dominant. Homomeric heterotypic gap junctions can also form but they are usually nonfunctional.

functionality of the gap junction pore (Herve *et al.*, 2004; Laird *et al.*, 1995). Three-dimensional maps of a recombinant gap junction formed by a truncated Cx43 offer a spectacular confirmation of the predicted hexameric structure of the gap junction channel, with rings of α helices delimiting the pore crossing two plasma membranes and the intercellular gap (Unger *et al.*, 1997; Unger *et al.*, 1999).

The size and charge permeability of the aqueous pore is dictated by the specific connexins that form the gap junction (Elfgang *et al.*, 1995; White *et al.*, 1995). For example, connexin43 (Cx43; gene: *Gja1*) and connexin45 (Cx45; gene: *Gjc1*) have distinct biophysical properties. Cx43 permits the passage of molecules ~1.2 kDa or smaller and has a preference for passing negatively charged molecules. In contrast, Cx45 forms a much smaller pore, permitting the passage of molecules of ~0.3 kDa or less with preference for positively charged molecules (Elfgang *et al.*, 1995; Steinberg *et al.*, 1994; Veenstra *et al.*, 1994). As connexins may form heteromeric connexons, a wide range of molecular permeability and junctional conductance can be found in cells that express more than one connexin. For example, when Cx43 and Cx45 participate to heteromeric, heterotypic gap junctions, the permeability and conductance of the pore are closer to those of Cx45 than Cx43 (Koval *et al.*, 1995; Martinez *et al.*, 2002); and in general, the ratio between the connexins contributing to the heteromeric connexon dictates the permeability of the resulting gap junction (Cottrell *et al.*, 2002; Koval *et al.*, 1995; Martinez *et al.*, 2002). In addition to their classic function as conduits of ions and small molecules, gap junctions may serve as docking platforms for components of intracellular signaling systems, including β-catenin, c-src, extracellular signal regulated kinase (ERK), protein kinase C α and ε, phosphatidylinositol-3-kinase and p38 mitogen-activated protein kinase (Giepmans, 2004; Herve *et al.*, 2004; Saez *et al.*, 2003). As many of these interactions are connexin specific, by gating the signals passing through the gap junction pore, connexins may specify and regulate the repertoire of signaling complexes assembling at the gap junction.

Connexin Expression and Distribution in Bone and Cartilage

Gap junction plaques among adjacent osteoblasts, osteocytes, and periosteal fibroblasts were first identified in bone by electron microscopy (Doty and Schofield, 1972; Stanka, 1975), and by ultrastructural studies in histological sections of bone (Jones *et al.*, 1993; Palumbo *et al.*, 1990; Shapiro, 1997). Numerous *in vitro* studies have demonstrated the presence of functional gap junctions formed primarily by Cx43 among calvaria osteoblasts (Jeasonne *et al.*, 1979), odontoblasts (Ushiyama, 1989), primary human trabecular bone cells (Civitelli *et al.*, 1993) and a variety of osteoblastic (Donahue *et al.*, 1995b; Schiller *et al.*, 1992; Yamaguchi *et al.*, 1994), and osteocytic cell lines (Cheng *et al.*, 2001b; Yellowley *et al.*, 2000). Cx43 has also been shown to assemble in hemichannels in osteocytes (Cherian *et al.*, 2005; Genetos *et al.*, 2007), and it is likely that both gap junctions and hemichannels contribute to osteoblast–osteocyte communication. Cx43 has also been detected in osteoclasts at sites of active bone resorption, primarily at contact sites between osteoclasts and overlying marrow mononuclear cells (Ilvesaro *et al.*, 2000; Jones *et al.*, 1993; Su *et al.*, 1997), although the biological role of connexins in osteoclasts remains controversial.

Cartilage may seem an unlikely tissue for gap junctions as mature chondrocytes are isolated and embedded in the extracellular matrix. However, both articular and growth plate chondrocytes may express Cx43 when grown in culture (D'Andrea and Vittur, 1996; Donahue *et al.,* 1995a; Schwab *et al.,* 1998), and formation of functional gap junctions in the superficial zone of articular chondrocytes has been documented *in vivo* (Chi *et al.,* 2004). Although the functionality of Cx43 in growth plate chondrocytes *in vivo* has not yet been proven, Cx43 is functional among adult bovine articular chondrocytes in culture (Donahue *et al.,* 1995a), suggesting that gap junctional communication may be reestablished in mature cartilage in conditions that lead to cell proliferation and tissue repair.

Bone forming cells are intimately associated with the vasculature and hematopoietic cells. *In vitro* work has shown that vascular endothelial cells can form functional gap junctions with bone marrow stromal cells in co-culture (Villars *et al.,* 2002); and the formation of gap junctional communication among vascular endothelial cells and bone marrow stromal cells supports differentiation of the latter toward the osteogenic lineage. Conversely, inhibition of intercellular communication among these two cell types can attenuate osteogenic potential (Guillotin *et al.,* 2004; Villars *et al.,* 2002).

Although less abundant than Cx43, other connexins are expressed by bone cells, but their biological role in the skeleton remains unknown. As noted, Cx45 is present at appositional membranes in osteoblast cultures (Civitelli *et al.,* 1993; Donahue *et al.,* 2000), and forms functional gap junctions of different molecular permeability than those formed by Cx43 (Koval *et al.,* 1995; Martinez *et al.,* 2002). Cx46 is also present in osteoblastic cells, but it is never found on the cell surface, it is retained in trans-Golgi compartments, and does not oligomerize to form gap junctions in these cells (Koval *et al.,* 1997). Connexin40 (Cx40; gene: *Gja3*) is detected in the embryonic skeleton, primarily in developing limbs, ribs, and sternum (Pizard *et al.,* 2005), but there is no evidence that *Gja3* is also expressed in the adult skeleton.

Regulation of Connexin Expression and Function in Skeletal Cells

Osteoblasts and Osteocytes

A number of *in vitro* studies have highlighted the critical role of Cx43 for the differentiation and function of bone forming cells. *Gja1* expression and intercellular communication increase upon osteoblast differentiation, whereas *Gjc1* expression does not change (Donahue *et al.,* 2000; Schiller *et al.,* 2001a). Interference with Cx43 function (Lecanda *et al.,* 1998; Stains *et al.,* 2003) or *Gja1* expression (Li *et al.,* 2006) alters the expression of osteoblast gene products critical for bone formation, including alkaline phosphatase, bone sialoprotein, and osteocalcin. Furthermore, chemical inhibition of gap junctional communication leads to delayed bone nodule formation and disruption of osteoblast gene expression in human murine osteoblasts (Donahue *et al.,* 2000; Schiller *et al.,* 2001a). Accordingly, primary osteoblast cell cultures isolated from *Gja1* null mice exhibit defective expression of osteoblast genes and delayed ability to form mineralized nodules *in vitro* (Lecanda *et al.,* 2000b). Closely similar defects are also seen in mice with an osteoblast specific deletion of *Gja1* (Chung *et al.,* 2006).

The presence of Cx43 gap junctions is also permissive for normal cell responsiveness to hormonal and physical stimuli. Osteoblastic cells rendered communication deficient by expression of a Cx43 antisense construct display a reduced cAMP response to parathyroid hormone (Van der Molen *et al.,* 1996), impaired contraction of osteoblast populated collagen lattices (Bowman *et al.,* 1998), and reduced alkaline phosphatase induction in response to electromagnetic fields (Van der Molen *et al.,* 2000). The reduced hormonal response of Cx43 deficient cells occurs despite a normal adenylate cyclase system, indicating that Cx43 gap junctions amplify the signals generated by local receptor activation, perhaps by allowing diffusion of signaling molecules or ions from responsive to nonresponsive cells, thus equalizing differences in receptor distribution and hormonal responses in osteoblastic populations (Civitelli *et al.,* 1992; Civitelli *et al.,* 1994). Emerging *in vitro* data have led to hypothesize that the pharmacologic action of the bisphosphonate, alendronate, a strong inhibitor of bone resorption, also include an anti-apoptotic effect on osteoblasts and osteocytes, an effect dependent on Cx43 (Plotkin *et al.,* 2002). This action is mediated by alendronate-induced opening of Cx43 hemichannels, in a src-ERK dependent fashion (Plotkin *et al.,* 2005). *In vivo* testing of this intriguing hypothesis should be forthcoming.

Several osteoblast regulatory factors, including BMP-2 and TGF-β can enhance gap junctional communication, and the former increases Cx43 abundance (Rudkin *et al.,* 1996). In contrast, retinoic acid (Chiba *et al.,* 1994) and cytoplasmic acidification (Yamaguchi *et al.,* 1995) decrease gap junctional communication and Cx43 expression. The effects of prostaglandin E_2 (PGE_2) and PTH have been studied more in depth for different reasons. PGE_2 enhances gap junctional communication in murine osteoblastic cells (Shen *et al.,* 1986) via increased Cx43 assembly into gap junction channels (Civitelli *et al.,* 1998). PTH also stimulates gap junctional communication among osteoblasts and upregulates *Gja1* mRNA in a cAMP dependent fashion (Civitelli *et al.,* 1998; Donahue *et al.,* 1995b; Schiller *et al.,* 1992). Both PGE_2 and PTH effects result in feed-forward mechanisms, as upregulation of *Gja1* expression enhances a cell's ability to respond to PTH. Likewise, PGE_2, which upregulates Cx43 abundance is released by osteoblasts and osteocytes in a Cx43 dependent fashion, as discussed later.

The number of gap junctions declines in weightlessness conditions (Doty and Morey-Holton, 1982), and application of intermittent compressive load increases the number of gap junctions in osteocytes (Lozupone *et al.,* 1996). Furthermore, *Gja1* expression is increased in periodontal ligament after experimental tooth movement and in osteocytes after tooth extraction (Gluhak-Heinrich *et al.,* 2006; Su *et al.,* 1997). At the cellular level, application of cyclical equibiaxial strain by deformation of the tissue culture substrate leads to a rapid increase of Cx43 abundance at cell–cell contact sites and intercellular communication among osteoblastic cells (Ziambaras *et al.,* 1998), and to upregulation of *Gja1* expression in tendon cells (Wall and Banes, 2005). Fluid flow also increases *Gja1* expression and function in osteocytic cells (Alford *et al.,* 2003; Cheng *et al.,* 2001b; Cheng *et al.,* 2001a; Cherian *et al.,* 2003), although opposite effects have also been reported (Thi *et al.,* 2003). Nonetheless, gap junction-deficient cells are dramatically less responsive to fluid flow (Saunders *et al.,* 2001; Saunders *et al.,* 2003) and electric fields (Van der Molen *et al.,* 2000) than are normal osteoblastic cells. Thus, as in the case of PTH (Chung *et al.,* 2006; Van der Molen *et al.,* 1996), GJIC increases the sensitivity of bone cells to mechanical signals (Donahue, 2000).

Chondrocytes

In vitro studies have demonstrated that articular chondrocytes are not only capable of forming communicative gap junctions, but mechanical perturbation can amplify chondrocyte response by permitting diffusion of second messengers through the gap junction channel (Capozzi *et al.,* 1999; D'Andrea *et al.,* 1998; D'Andrea *et al.,* 2000). Although the role of Cx43 in chondrocytes has not been extensively studied, Cx43 is not absolutely required in the growth plate for bone growth during embryogenesis, as long bones of *Gja1* null mice at birth are of normal length and size (Lecanda *et al.,* 2000b). Nevertheless, micromass cultures of chondrocytes have demonstrated that gap junctions affect chondrocyte differentiation, as inhibition of gap junctional communication reduces production of proteoglycans and type II collagen (Zhang *et al.,* 2002). Other connexins may contribute to chondrocyte function and cartilage formation. For example, *Gja3* is highly expressed in the periarticular perichondrium, and *Gja3* null mice exhibit malformations and decreased length of limb bones (Pizard *et al.,* 2005). Moreover, Connexin29 (Cx29; gene: *Gje1*) promoter activity has recently been detected in chondrocytes *in vivo* (Eiberger *et al.,* 2006), although expression of *Gje1* has yet to be confirmed.

Osteoclasts

Although no osteoclast abnormalities have been reported thus far in different *Gja1* mutant mouse models (see later section), *in vitro* work has implicated Cx43 in the process of monocyte precursor fusion into osteoclasts and in osteoclast activity. Treatment of osteoclasts with pharmacologic gap junction inhibitors reduces the number of multinucleated osteoclasts and the ability to form resorption pits (Ilvesaro *et al.,* 2000; Ilvesaro *et al.,* 1998; Ransjo *et al.,* 2003). Similarly, the ability of PTH and vitamin D_3 to stimulate osteoclast activity is markedly inhibited by interference with gap junction function (Ransjo *et al.,* 2003), an effect that may be independent of the RANK/RANKL/OPG axis (Matemba *et al.,* 2006).

Connexins in Mechanotransduction

Intercellular communication is important for mechanotransduction, not only because mechanical and physical factors can modulate gap junctional communication, but also because gap junctions may provide the means by which mechanical signals can be received and propagated (Donahue, 2000). As discussed in the next section, gap junctions among cultured chondrocytes and osteoblastic cells are involved in propagation of certain types of intercellular Ca^{2+} waves produced by plasma membrane deformation (D'Andrea and Vittur, 1996; Donahue *et al.,* 1995a; Jørgensen *et al.,* 1997; Jørgensen *et al.,* 2000). Participation of Cx43 in Ca^{2+} wave propagation is not limited to providing transcellular conduits through which second messengers can flow from cell to cell, but it may also allow ATP to flow in the extracellular space through gap junction hemichannels, thus contributing to Ca^{2+} wave diffusion (see later in this chapter). Although functional hemichannels present in osteoblastic cells (Jørgensen *et al.,* 2003) do not seem to be involved in ATP release (Romanello *et al.,* 2001), application of fluid flow or mechanical perturbation of the plasma membrane can induce PGE_2 release via opening of Cx43 hemichannels in osteoblast and osteocytes (Cherian *et al.,* 2005). Studies in a novel co-culture system where osteocytic and osteoblastic cells were physically separated but could establish gap junctional communication, showed that application of fluid flow to the osteocytic cells results in increased alkaline phosphatase in the osteoblasts, whereas direct mechanical stimulation of osteoblasts does not (Taylor *et al.,* 2007). These experiments provide initial proof to the concept that gap junctions may convey mechanically generated signals from osteocytes to osteoblasts.

Modulation of Cell Signaling by Connexins

Recent work has begun to unravel the molecular details of how connexins can affect gene expression and ultimately, osteoblast function. As noted, osteoblasts isolated from *Gja1*$^{-/-}$ and conditional osteoblast *Gja1* ablated mice express lower levels of many markers of osteogenic differentiation (Chung *et al.,* 2006; Lecanda *et al.,* 2000b). Furthermore, interference with Cx43-dependent gap junctional communication by either overexpression of chick *Gjc1* or pharmacologic inhibitors results in transcriptional downregulation of osteoblast genes, primarily *osteocalcin*, and α_1*(I) collagen* (Lecanda *et al.,* 1998; Schiller *et al.,* 2001b; Upham *et al.,* 2003). Conversely, expression of *Gja1* in poorly coupled cells upregulates osteoblast gene transcription (Gramsch *et al.,* 2001; Lecanda *et al.,* 1998). Moreover, using *Gjc1* overexpression to modulate Cx43 channels, a DNA element binding Sp1/Sp3 transcription factors was identified in the rat *osteocalcin* promoter. This element, named connexin response element (CxRE), is necessary and sufficient to confer connexin sensitivity to both the *osteocalcin* and α_1*(I) collagen* promoters (Stains *et al.,* 2003). Thus, the permeability properties of homomeric Cx43 junctional channels interact with signaling pathways involved in regulation of gene activity.

Further studies demonstrated that either *Gja1* deficiency or interference with Cx43 function alters ERK signaling, and this in turn modulates gene transcription from several osteoblast gene promoters (Stains *et al.,* 2003; Stains and Civitelli, 2005b). Downstream effects of gap junction modulation of ERK signaling converge upon the CxRE in the *osteocalcin* and α_1*(I) collagen* promoters. When Cx43 is abundant, both the activator Sp1 and the repressor Sp3 can occupy the promoter, with prevalence of Sp1; resulting in high transcriptional activity. When Cx43 function is inhibited, Sp3 almost exclusively occupies the CxRE, resulting in low transcriptional activity. Thus, ERK cascade dependent phosphorylation of Sp1 mediates preferential recruitment of Sp1 over Sp3 in well coupled cells, and loss of Sp1 phosphorylation results in the preferential recruitment of Sp3 (Stains and Civitelli, 2005b). Interestingly, osteocyte stimulation by fluid flow is also dependent upon ERK activation (Genetos *et al.,* 2007), thus suggesting that the ERK pathway may be involved in transmission of mechanically induced signals from osteocytes to osteoblasts, resulting in transcriptional upregulation of gene involved in bone formation. The current knowledge on connexin modulation of gene expression can be integrated into a model predicting that activation of signaling cascades by extracellular cues triggers a "primary response," which depends upon ligand availability and receptor abundance. Signals generated by this "primary" response, for example, cAMP, inositol-trisphosphate (IP$_3$) or cADP-ribose, are propagated to adjacent cells through gap junction channels formed by Cx43, thus resulting in a "secondary response," which potentiates the "primary" response and equalizes cell responses throughout a communicating network (Stains and Civitelli, 2005a; Stains and Civitelli, 2005b) (Fig. 4).

Connexins in Normal Skeletal Development

Earlier immunohistochemical studies in chick embryos demonstrated the presence of connexins in developing tooth germs of neonatal rats (Pinero *et al.,* 1994) and on mesenchymal cells at early stages of intramembranous bone formation in chick mandible, preceding the appearance of osteogenic cells (Minkoff *et al.,* 1994). Underscoring the importance of Cx43 in the heart, germline *Gja1* ablation causes severe malformations of the outflow tract incompatible with postnatal life (Reaume *et al.,* 1995). The skeleton of *Gja1* null ($Gja1^{-/-}$) mutants at birth reveals delayed intramembranous and endochondral ossification and skull abnormalities, with brittle, misshapen ribs and hypoplastic skull. The delayed development and hypomineralization of all the cranial vault elements results in a flattened skull and open parietal foramen (Lecanda *et al.,* 2000b). Most facial and axial skeletal elements are hypomineralized at E15.5, indicating approximately a two-day delay in skeletal ossification (Fig. 5A). Such phenotype is associated with an osteoblast autonomous cell defect, leading to defective production of mineralized bone matrix (Lecanda *et al.,* 2000b).

Malformations of facial bone development leading to aberrant maxillary and mandibular primordium and nasal pit defects have been produced in chick embryos after treatment with *Gja1* antisense oligonucleotides (Becker *et al.,* 1999; McGonnell *et al.,* 2001a). These abnormalities are associated with downregulation of the homeobox transcription factor Msx1 (McGonnell *et al.,* 2001b), a critical modulator of cranial development and patterning (Alappat *et al.,* 2003; Cohen, Jr., 2000). Additional evidence of the

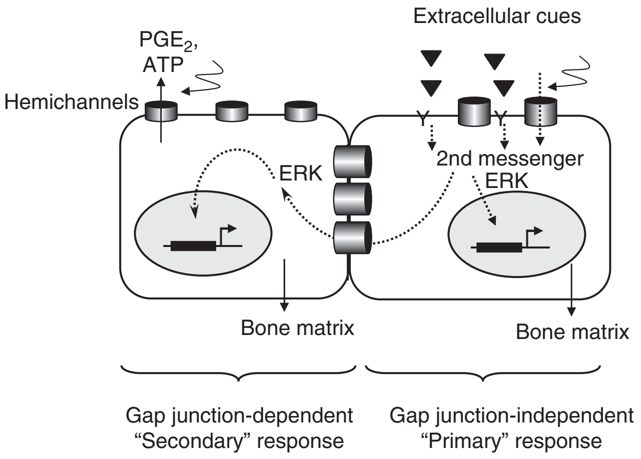

FIGURE 4 Gap junctions coordinate and amplify cellular activities by transmitting signals among a coupled cell network. Extracellular cues in the form of hormonal, paracrine (black triangles), or mechanical signals (jagged arrow) activate signaling cascades ("primary" response) generating second messengers that may be directly transmitted from cell to cell via gap junctions. Many of these signals regulate activation of ERK signaling and converge upon specific regulatory elements that modulate osteoblast gene transcription. By amplifying the signal or by diffusing the signal response to cells that might not be autonomously responsive to a certain stimulus, gap junctions contribute to generate a more coordinated, "secondary" response at the tissue level. In addition, hemichannels may contribute to cell responses by allowing release of factors (i.e., ATP, PGE$_2$ after mechanical stimulation) that in turn may act as autocrine signals for response amplification.

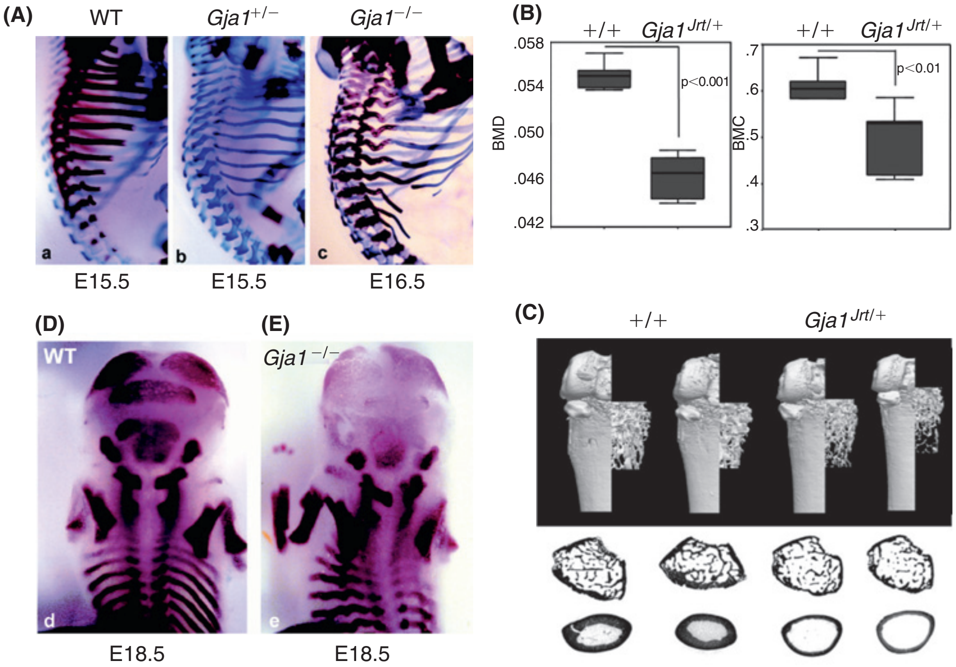

FIGURE 5 Skeletal consequences of *Gja1* mutations. (**A**) Genetic ablation of *Gja1* in mice leads to a developmental delay in ossification of the ribs (**A–C**), vertebrae and the skull (**D**,**E**), as seen in these alizarin red/alcian blue stained whole mount embryos. (**B**) Similarly, adult $Gja1^{jrt/+}$ mutant mice, which reproduce the human disease oculodentodigital dysplasia, exhibit decreased whole body bone mineral density (BMD) and bone mineral content (BMC). (**C**) MicroCT scanning of the femoral distal metaphysis shows rarefied trabecular structure and thinner cortical bone in $\text{Gja1}^{jrt/+}$ mutant mice relative to wild type mice, on 2D cross-sections. Reproduced from Lecanda *et al.,* (2000) *J. Cell Biol.*, **151**, 931–943. Copyright 2000 Rockefeller University Press (panel **A**); and from Flenniken *et al.* (2005) (panels **B** and **C**). (See plate section)

role of Cx43 in skeletal development across different species has emerged from work in zebrafish. The length of the zebrafish fin skeleton is determined by the number and size of fin bony segments (Iovine and Johnson, 2000). The short fin (*sof*) mutation, which was mapped to the *Gja1* locus, results in segments that are approximately one-third the length of wild type bony segments. These zebrafish *Gja1* mutations appear to cause defects of cell proliferation and osteogenic differentiation (Iovine *et al.,* 2005).

Considering the evidence for a role of Cx43 in skeletal growth and patterning, it may seem surprising that *Gja1* ablation does not cause a more severe skeletal phenotype. One possible explanation is the presence of compensatory mechanisms, as other connexins are present in bone cells. The obvious candidate is Cx45, but *Gjc1* null mutants die very early in embryogenesis, before condensation of skeletal elements (Kumai *et al.,* 2000), thus the role of Cx45 remains to be determined. Cx40 may provide another compensatory mechanism in early skeletogenesis, as demonstrated by studies in a mouse model of Holt-Oram syndrome, a human disorder caused by haploinsufficiency of T-box transcription factor, Tbx5, and characterized by limb malformations, shortened arms, abnormal sternum, and heart disease (Basson *et al.,* 1997). Tbx5 regulates expression of *Gja5*, and many of the skeletal abnormalities present in $Tbx5^{+/-}$ mice are shared by $Gja5^{+/-}$ and $Gja5^{-/-}$ mice (Pizard *et al.,* 2005). Interestingly, although dysmorphisms of phalanges, carpal bones, and sternum are common to $Tbx5^{+/-}$ and *Gja5* mutant mice, other features, such as rib and hindlimb defects are observed only in $Gja5^{-/-}$ mice, strongly suggesting that Cx40 is involved in skeletal patterning during development.

Connexin43 in the Postnatal Skeleton

The biologic role of Cx43 in the postnatal skeleton has only begun to emerge with the recent development of conditional *Gja1* deletion in mice (Castro *et al.,* 2003; Chung *et al.,* 2006). Not surprisingly, *Gja1* ablation using the Cre/*loxP* system, in which Cre recombinase is expressed by a 2.3 kb fragment of the α_1*(I) collagen* promoter, active in differentiated osteoblasts, does not cause skeletal malformations, nor the ossification defects seen in germline $Gja1^{-/-}$ mutants. Lack of dysmorphisms in these mice most likely reflects the timing of conditional gene inactivation, which occurs around birth, therefore after most of the skeleton has been modeled. However, conditional *Gja1* ablation in osteoblasts results in low bone mass at maturity and throughout adult life (Chung *et al.,* 2006). This phenotype is the result of a reduced number of active bone-forming cells and reduced bone formation rate. In addition to adult osteopenia, these animals exhibit a severely attenuated bone anabolic response to intermittent PTH administration, the consequence of a failure of Cx43 deficient osteoblasts to mount a full response to the hormone (Chung *et al.*, 2006). These results have important ramifications for translational research, as PTH analogs are currently used as anabolic therapy for osteoporosis (Neer *et al.*, 2001). It would be important to determine whether other bone anabolic stimuli, such as mechanical loading, are also attenuated in conditions of *Gja1* deficiency.

Recent and emerging data expand the role of Cx43 to the hematopoietic compartment of the bone marrow microenvironment. *Gja1* null marrow stromal cells fail to support hematopoietic cell differentiation (Cancelas *et al.*, 2000), and mice with conditional deletion of *Gja1* in bone marrow cells have severely impaired recovery of hematopoiesis after cytoablative treatment, (Presley *et al.*, 2005). These intriguing data, which require further development, suggest that Cx43 serves critical functions in both stromal and hematopoietic bone marrow stem cell commitment and differentiation.

Connexin43 Mutations and Oculodentodigital Dysplasia

The human autosomal dominant disorder oculodentodigital dysplasia (ODDD) has been linked to a number of mutations in the *GJA1* locus (Kjaer *et al.*, 2004; Paznekas *et al.*, 2003; Richardson *et al.*, 2004). Affected patients have neurologic abnormalities, heart conduction defects, but primarily skeletal malformations including widened mandibular alveolar ridge, microdontia, anodontia, and enamel hypoplasia, as well as cranial hyperostosis (Loddenkemper *et al.*, 2002; Schrander-Stumpel *et al.*, 1993). Some affected patients also have type III syndactyly of hands and feet, and/or hypoplasia or aplasia of the middle phalanges and broad tubular bones (Schrander-Stumpel *et al.*, 1993). Functional characterization of many ODDD *Gja1* mutants has demonstrated that most of the resultant proteins translocate to the plasma membrane but do not support intercellular communication, nor hemichannel activity (Lai *et al.*, 2006; Shibayama *et al.*, 2005). In fact, most of them function as dominant negative on wild type Cx43 (Roscoe *et al.*, 2005).

A mouse strain was identified from random N-ethyl N-nitrosourea mutagenesis with a phenotype closely resembling that of ODDD, including syndactyly, enamel hypoplasia, craniofacial abnormalities, and cardiac dysfunction (Flenniken *et al.*, 2005) (Fig. 5B–C). These mice ($Gja1^{Jrt/+}$) carry a G60S missense mutation of *Gja1*, never reported in patients with ODDD, generating a full-length Cx43 protein with dominant negative function. The $Gja1^{Jrt/+}$ mouse is only a partial phenocopy of human ODDD; the most notable difference is absence of thickened bones of the cranial vault, frequently seen in ODDD patients (Schrander-Stumpel *et al.*, 1993). On the other hand, abnormalities not typically described in ODDD are present in $Gja1^{Jrt/+}$ mice, such as severe osteopenia, myelofibrosis, and hematopoietic defects (Flenniken *et al.*, 2005). Such discrepancies might be related to species differences. Nonetheless, the fact that skeletal abnormalities represent the major phenotypic feature of ODDD, in humans and in a mouse model, provides genetic proof that skeletal development is one of the major sites of action of Cx43.

It is important to note that aside from skull hyperostosis, no actual data on whole body or regional bone mass have ever been reported in ODDD patients. Both $Gja1^{Jrt/+}$ and osteoblast *Gja1* ablated mice exhibit a similar degree of osteopenia throughout life (Chung *et al.*, 2006; Flenniken *et al.*, 2005); however, although *Gja1* ablation severely impairs osteoblast differentiation and mineralization potential, resulting in decreased new bone formation (Chung *et al.*, 2006), the cellular bases of the low bone mass in $Gja1^{Jrt/+}$ mice are unclear. Unlike *Gja1* deletion, expression of ODDD mutants in osteoblast-enriched calvaria cells does not lead to major functional abnormalities (McLachlan *et al.*, 2005). Thus, it is theoretically possible that the dominant negative effect of ODDD mutants may not be sufficient to alter differentiation of committed osteoblastic cells, although it may be able to interfere with earlier steps of osteogenesis. Preliminary results from a new mouse model in which *Gja1* is deleted embryonically in cells that give raise to chondro-osteoprogenitors support this hypothesis, showing much more severe osteogenic defects than osteoblast-specific *Gja1* ablated mice (Watkins *et al.*, 2006).

SHORT-RANGE INTERCELLULAR SIGNALING AMONG SKELETAL CELLS

Transient and oscillatory elevations of cytosolic free calcium concentrations ($[Ca^{2+}]_i$) initiate and modulate a number of cellular activities, including cell growth, motility, and secretion. Many studies have investigated intracellular calcium homeostasis and the mechanisms by which extracellular signals are transduced into intracellular calcium transients; less attention has been paid to the mechanisms by which groups of cells propagate calcium transients among themselves, and to the biologic significance of these short-range intercellular signals. Two mechanisms of intercellular calcium signaling have been identified: gap junctional communication and release of soluble mediators that act on nearby cells. The latter involves activation of P2 ("purinergic") receptors by extracellular adenosine triphosphate (ATP) and other nucleotides (Osipchuk and Cahalan, 1992; Schlosser *et al.*, 1996).

Purinergic Receptors in Bone

Specific receptors that recognize extracellular ATP and other phosphorylated nucleotides such as adenosine 5′-diphosphate (ADP), uridine 5′-triphosphate (UTP), and uridine 5'-diphosphate, but not adenosine, are termed P2 purinoceptors and are distinct from the P1 purinoceptors that bind adenosine. Two different families of P2 purinergic receptors exist, P2X and P2Y, which differ in structure and sensitivity to nucleotides (Brake *et al.*, 1996). Seven isotypes of P2X ($P2X_1$–$P2X_7$) and eight of P2Y receptors ($P2Y_{1,2,4,6,11,12,13,14}$) have been identified, all products of different genes (Ralevic and Burnstock, 1998). P2X receptors are ligand-gated ion channel receptors; ligand binding induces cell depolarization followed by a rapid increase in $[Ca^{2+}]_i$ via an influx of calcium ions through the channel. Conversely, P2Y receptors belong to the heptahelix G protein-coupled receptor superfamily (Dubyak and el-Moatassim, 1993) and are distributed more diffusely than P2X receptors. Binding of a ligand to a P2Y receptor activates the phospholipase C (PLC) system, with production of IP_3 and subsequent release of intracellular calcium from IP_3-sensitive stores.

The presence of P2 receptors in osteoblasts had been postulated by observations of transient $[Ca^{2+}]_i$ responses to extracellular ATP or ADP (Dixon *et al.*, 1997; Kumagai *et al.*, 1991; Schofl *et al.*, 1992). Indeed, several P2X and P2Y isotypes have been identified on the surface of human and rat osteoblastic cells, including $P2X_{1,4,5,6,7}$; $P2Y_{1,2,4,6,11}$ (Bowler *et al.*, 1995; Hoebertz *et al.*, 2000; Jørgensen *et al.*, 1997; Jørgensen *et al.*, 2002; Naemsch *et al.*, 2001). Osteoclasts also respond to extracellular nucleotides by increasing intracellular Ca^{2+} concentrations. Because both extracellular calcium influx and calcium release from intracellular stores are involved in the osteoclast response to ATP (Weidema *et al.*, 1997; Yu and Ferrier, 1993), both the P2X and P2Y families of receptors seem to be functional in these cells. Accordingly, $P2X_2$ (Hoebertz *et al.*, 2000), $P2X_4$ (Hoebertz *et al.*, 2000; Naemsch *et al.*, 1999), and $P2X_7$ (Hoebertz *et al.*, 2000) have been identified in rat osteoclasts. $P2Y_2$ is also expressed but it does not localize to the cell surface (Bowler *et al.*, 1995).

Function of Purinergic Receptors in Bone

In vitro studies demonstrate that extracellular nucleotides inhibit bone nodules formation and matrix mineralization in calvaria cultures (Hoebertz *et al.*, 2002; Jones *et al.*, 1997), and stimulate both osteoclast differentiation and bone resorption (Bowler *et al.*, 1995; Morrison *et al.*, 1998). The latter effect may be mediated by ATP upregulation of RANKL (Buckley *et al.*, 2002). Among all P2 receptors, $P2X_7$ has received particular attention as a potential regulator of osteoclast differentiation, based upon the observation that $P2X_7$ promotes giant cell formation from macrophages (Falzoni *et al.*, 1995). Initial *in vitro* studies supported this hypothesis, demonstrating that formation of multinucleated osteoclasts from human peripheral blood monocytes can be inhibited by a $P2X_7$ receptor blocking antibody (Gartland *et al.*, 2003a). Furthermore, osteoclast precursor cells lacking the $P2X_7$ receptor fail to form multinucleated osteoclast-like cells in response to RANKL (Hiken and Steinberg, 2004). However, apparently normal osteoclasts have been reported in two different mouse models of $P2X_7$ gene (*$P2X_7$*) ablation (Gartland *et al.*, 2003b; Ke *et al.*, 2003), thus suggesting that $P2X_7$ receptors are dispensable for osteoclast precursor fusion, or for the function of mature osteoclasts. In fact, osteoclast number is actually increased in one of the *$P2X_7$* null mouse models, an observation in keeping with decreased cortical and trabecular bone mass in these animals (Ke *et al.*, 2003). Intriguingly, cross-sectional area of long bones is smaller in these animals relative to wild type mice, arguing for an involvement of $P2X_7$ in periosteal bone formation (Ke *et al.*, 2003). On the other hand, only a modest increase in cortical thickness was described in the other *$P2X_7$* null model, without substantial changes in bone formation (Gartland *et al.*, 2003b). One possible explanation for such discrepancies might be related to the different strain backgrounds in which the mutations were introduced. In fact, the C57BL/6 strain carries a natural mutation of *$P2X_7$*, which reduces sensitivity to ATP (Adriouch *et al.*, 2002), and this genetic background is represented in different proportions in the two *$P2X_7$* null mouse strains. In any case, the relatively mild phenotype found in both models may also reflect compensatory mechanisms, as other P2X receptor isotypes may complement some of the functions of $P2X_7$.

Subsequent studies have shown that *$P2X_7$* null osteoclasts are less susceptible to apoptosis than are normal osteoclasts (Korcok *et al.*, 2004), findings consistent with the idea that lack of *$P2X_7$* may actually increase osteoclast activity *in vivo*. This notion is supported by results of a clinical study in postmenopausal Danish women demonstrating a threefold higher 10-year fracture rate in subjects homozygous for a single nucleotide polymorphism of *$P2X_7$* (Glu496Ala) relative to individuals with the "normal" allele (Ohlendorff *et al.*, 2007). Furthermore, the frequency of apoptosis in osteoclasts derived from peripheral blood monocytes of subjects homozygous for the Glu496Ala polymorphic variant is 50% lower compared to cells from individuals with the other genotypes (Ohlendorff *et al.*, 2007). Thus, it is possible that despite the relatively minor phenotype of *$P2X_7$* mice, this purinergic receptor may contribute to control bone homeostasis in humans.

It has been hypothesized that short-range autocrine signals may modulate the set point for activation of certain signal transduction pathways (Ostrom *et al.*, 2000). For example, nucleotides potentiate PTH-induced increases in $[Ca^{2+}]_i$, but not the effect of PTH on cAMP production (Buckley *et al.*, 2001; Kaplan *et al.*, 1995; Sistare *et al.*, 1995). Furthermore, activation of P2 receptors in primary human osteoblasts potentiates PTH-induced *c-fos* gene expression (Bowler *et al.*, 1999). In fact, PTH by itself might not be able to fully activate PLC in osteoblasts if P2 receptors are not activated

(Sistare *et al.,* 1995). Because ATP is released upon mechanical stimulation (Bowler *et al.,* 2001), such a potentiation of PTH action may represent a mechanism of local control of hormonal effects, by focusing the response in areas under active loading, while leaving unloaded areas with cells that remain less sensitive to PTH action.

Intercellular Signaling and Mechanotransduction in Bone

As already noted, bone and cartilage cells propagate $[Ca^{2+}]_i$ signals upon mechanical perturbation of the plasma membrane. One mechanism of $[Ca^{2+}]_i$ wave propagation is dependent on the passage of an unknown signaling molecule through gap junctions and regeneration of the calcium transient in neighboring cells by calcium-induced calcium release (Xia and Ferrier, 1992), or by depolarization of the plasma membrane of the neighboring cell and opening of voltage-operated calcium channels (Jørgensen *et al.,* 2003). Notably, these "slow" types of $[Ca^{2+}]_i$ waves occur in cells that express abundant Cx43. The other type of $[Ca^{2+}]_i$ waves propagate with faster dynamics, and are mediated by the autocrine action of an extracellular nucleotide, probably ATP, on P2 receptors on adjacent cells and are independent of Cx43 (Jørgensen *et al.,* 1997) (Fig. 6). The biological significance of two mechanisms of short-range signal propagation in bone cell networks remains to be fully clarified. Interestingly, primary cultures of human bone marrow stromal cells can propagate mechanically induced $[Ca^{2+}]_i$ waves via both the gap junction mediated and the P2 mediated mechanisms (Jørgensen *et al.,* 2000), but their activity seems to change with differentiation. In relatively immature stromal cells, wave propagation occurs primarily via the $P2Y_2$ mechanism, while the gap junction mediated wave propagation prevails after long-term culture (four months), even though the cells still respond to ATP stimulation with an increase in $[Ca^{2+}]_I$ (Henriksen *et al.,* 2006). Corroborating such conclusion, expression of P2 receptors and response to nucleotide stimulation vary during osteoblast differentiation, in a manner consistent with gradual loss of P2 dependent paracrine signaling upon differentiation (Orriss *et al.,* 2006).

ATP released from osteoblasts not only acts on other osteoblasts but also on osteoclasts. P2 receptors are present on both cell types, and calcium transients can be propagated bidirectionally between these two cell types. This signal requires the presence of functional $P2X_7$ receptors on the osteoclast (Jørgensen *et al.,* 2002). This raises the possibility that the activity of the two cell types can be regulated independently by modulation of either the $P2Y_2$ or the $P2X_7$ receptor subtype, resulting in modulation of either bone formation or resorption without affecting the other. Demonstrating the critical importance of these receptors in mechanotransduction, *in vivo* mechanical loading of the ulna produces a profoundly attenuated anabolic response in $P2X_7$ null mice relative to wild type mice (Li *et al.,* 2005).

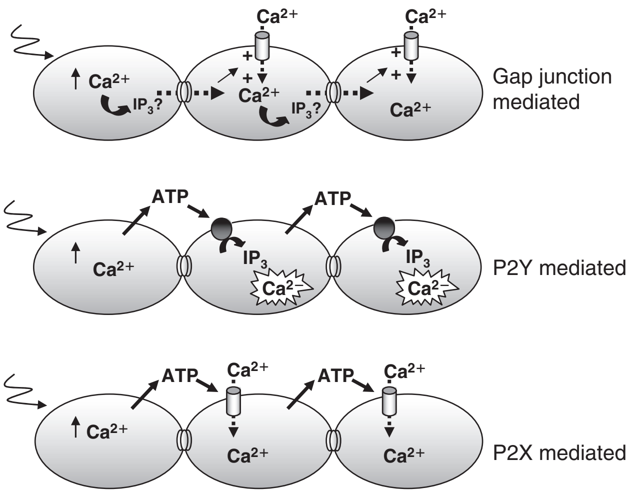

FIGURE 6 Mechanisms for the propagation of intercellular calcium signals. Gap junction-mediated calcium waves (top): the increase in intracellular-free calcium concentration caused by mechanical stimulation (jagged arrow) produces a signaling molecule, perhaps inositol-triphosphate (IP_3) that passes through the gap junction channel into adjacent cells where it releases calcium from intracellular stores, or induces depolarization of the plasma membrane and subsequent opening of voltage-operated calcium channels with an influx of calcium from the extracellular space. The intracellular calcium increase is then propagated to the next cell through the same mechanism, thus producing a calcium wave. Ligand-mediated calcium waves: mechanical stimulation increases intracellular-free calcium concentration in the stimulated cell. As a consequence, ATP or a related nucleotide is released to the extracellular space and binds to surface receptors on neighboring cells. If ATP binds to P2Y receptors (middle), IP_3 is generated, inducing the release of calcium from IP_3-sensitive intracellular calcium stores. If ATP binds to P2X receptors (bottom), conformational changes of the receptor/channel are induced, resulting in the opening of the channel, with a subsequent influx of extracellular calcium. In both cases, a calcium wave is generated by successive activation of P2 receptors in neighboring cells.

Articular chondrocytes can also propagate intercellular calcium waves in response to mechanical stimulation of single chondrocytes (D'Andrea *et al.,* 2000; Guilak *et al.,* 1999). Even in these cells, calcium wave propagation occurs via either gap junctional communication (D'Andrea *et al.,* 2000; D'Andrea and Vittur, 1996; Donahue *et al.,* 1995a), or via P2 receptor mediated mechanisms (Millward-Sadler *et al.,* 2004; Yellowley *et al.,* 1999). Mechanically induced calcium waves can also propagate from chondrocytes to synovial cells in culture via mechanisms involving both extracellular ATP release and gap junctions (D'Andrea *et al.,* 1998; Grandolfo *et al.,* 1993). The biologic significance of this intercellular signaling mechanism among chondrocytes remains to be determined.

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Chapter 22

Histomorphometric Analysis of Bone Remodeling

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INTRODUCTION

Iliac crest bone biopsy followed by histomorphometric analysis has provided invaluable information about the pathogenesis and treatment of metabolic bone diseases. The purpose of this chapter is threefold: first, to provide an overview of the methods used to procure, process, and analyze the biopsy; second, to summarize and illustrate the changes that occur in histomorphometric indices in common metabolic bone diseases; and third, to describe the effects of commonly used osteoporosis drugs, assessed by bone biopsy.

TETRACYCLINE LABELING AND THE SURGICAL PROCEDURE

Prelabeling the patient with tetracycline prior to biopsy allows the histomorphometrist to quantify precisely the rate of bone formation at the time of the biopsy (Frost, 1983). About 3 weeks prior to the biopsy, the patient is given a 3-day course of tetracycline. This is followed by a 12 drug-free days and then another 3-day course of tetracycline. This is termed a 3:12:3 sequence. The biopsy should not be performed until at least 5 days after the last tetracycline dose to prevent the last label from leaching out during the processing of the biopsy. This is often denoted as a 3:12:3:5 sequence. The tetracycline binds irreversibly to recently formed hydroxyapatite crystals at sites undergoing new deposition. When the histomorphometrist cuts and visualizes thin sections of the biopsy in a microscope equipped with ultraviolet illumination, the tetracycline fluoresces to label the sites of new bone formation (Fig. 1). The labels can be either double labels if bone formation at that site is continuous throughout the labeling sequence or single labels if formation started after the first, or stops before the second label is administered. Demeclocycline, tetracycline, and oxytetracycline can all be used as fluorochrome labels. In our laboratory, our preference is for demeclocycline, 600 mg/ day (4 × 150-mg tablets), taken on an empty stomach. Dairy products and antacids containing aluminum, calcium, or magnesium should be avoided because they impair absorption. Tetracyclines can cause nausea, vomiting, and diarrhea in some patients, and all patients should be cautioned to avoid excessive exposure to sunlight and UV light because tetracyclines can cause skin phototoxicity. Tetracyclines should not be given to children less than 8 years of age or to pregnant women because, just as it is incorporated into bones, it is incorporated into growing teeth and discolors them.

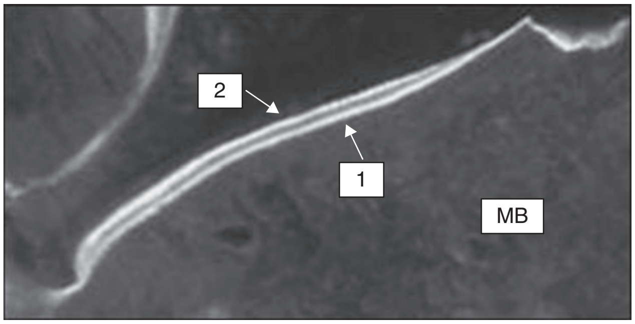

FIGURE 1 Double tetracycline label in an iliac crest bone biopsy. The patient was labeled with demeclocycline in a 3:12:3 sequence. 1, label 1; 2, label 2; MB, mineralized bone.

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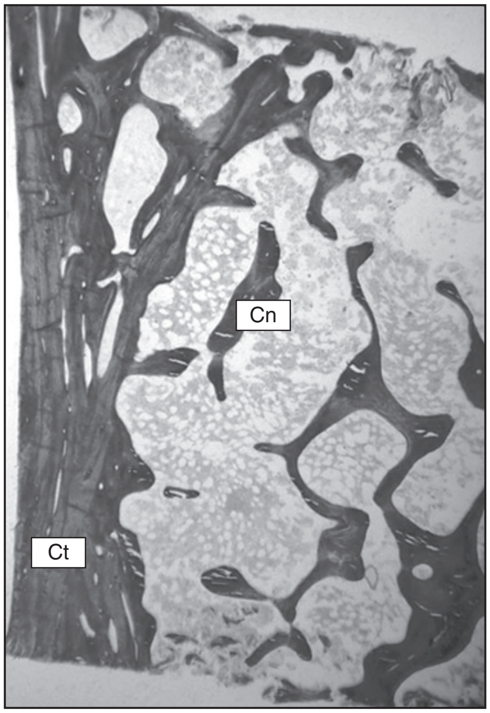

FIGURE 2 Low-power photomicrograph of an iliac crest bone biopsy section showing cancellous (Cn) and cortical (Ct) bone.

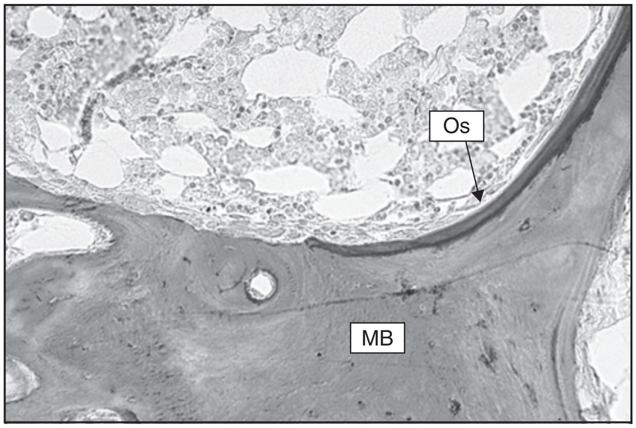

FIGURE 3 Photomicrograph of an iliac crest bone biopsy section showing an osteoid seam (Os) on the surface of mineralized bone.

Although the original site for bone biopsy was the rib, it is now performed exclusively at the anterior iliac crest, which is easily accessible, and the biopsy can be performed with minimal complications. This site also allows one to sample both cancellous and cortical bone in a single biopsy (Figs. 2 and 3). The structure and cellular activity at this site have been well characterized in a number of laboratories and have been shown to correlate with other clinically relevant skeletal sites, such as the spine and hip (Bordier *et al.*, 1964; Parfitt, 1983a; Rao, 1983; Dempster, 1988).

The biopsy generally is performed with a standard trephine with an internal diameter of at least 8 mm to obtain sufficient tissue and to minimize damage to the sample (Bordier *et al.*, 1964; Rao,1983). Immediately before the procedure, the patient should be sedated, usually with intravenous meperidine hydrochloride (Demerol) and diazepam (Valium). The skin, subcutaneous tissue, muscle, and, in particular, the periosteum covering both the lateral and the medial aspects of the ilium, should be thoroughly anesthetized with local anesthetic. Access to the iliac crest is achieved through a 2- to 3-cm skin incision made at a point 2 cm posterior and 2 cm inferior to the anterior superior iliac spine. It is important to locate this site carefully because there is considerable variation in bone structure around this location. In order to avoid damage to the biopsy, which could render it uninterpretable, the trephine should be rotated back and forth with gentle but firm pressure so that it cuts rather than pushes through the ilium. The patient should refrain from excessive activity for 24 hours after the procedure and a mild analgesic may be required. Significant complications from transiliac bone biopsy are rare. In an international multicenter study involving 9131 transiliac biopsies, complications were recorded in 64 patients (0.7%) (Rao, 1983). The most common complications were hematoma and pain at the biopsy site that persisted for more than 7 days; rarer complications included wound infection, fracture through the iliac crest, and osteomyelitis.

Sample Preparation and Analysis

The biopsy should be fixed in 70% ethanol because more aqueous fixatives may leach the tetracycline from the bone. After a fixation period of 4 to 7 days, the biopsy is dehydrated in ethanol, cleared in toluene, and embedded in methyl methacrylate without decalcification. The polymerized methyl methacrylate allows good-quality, thin (5 to 10 μm) sections to be cut on a heavy-duty microtome. The sections are then stained with a variety of dyes to allow good discrimination between mineralized and unmineralized bone matrix, which is termed “osteoid” (Figs. 4 and 5) and clear visualization of the cellular components of bone and marrow (see Fig. 7). Unstained sections are also mounted to allow observation of the tetracycline labels by fluorescence microscopy (see Fig. 1) (Baron *et al.*, 1983; Weinstein, 2002). The sections are subjected to morphometric analysis, according to standard stereological principles, using either simple “point-counting” techniques or computer-aided image analysis (Parfitt, 1983b; Malluche and Faugere, 1987; Compston, 1997).

Routine Histomorphometric Variables

A large number of histomorphometric variables can be measured or derived. Because the morphometric analysis is extremely time-consuming, the number of variables evaluated depends on whether the biopsy specimen is being

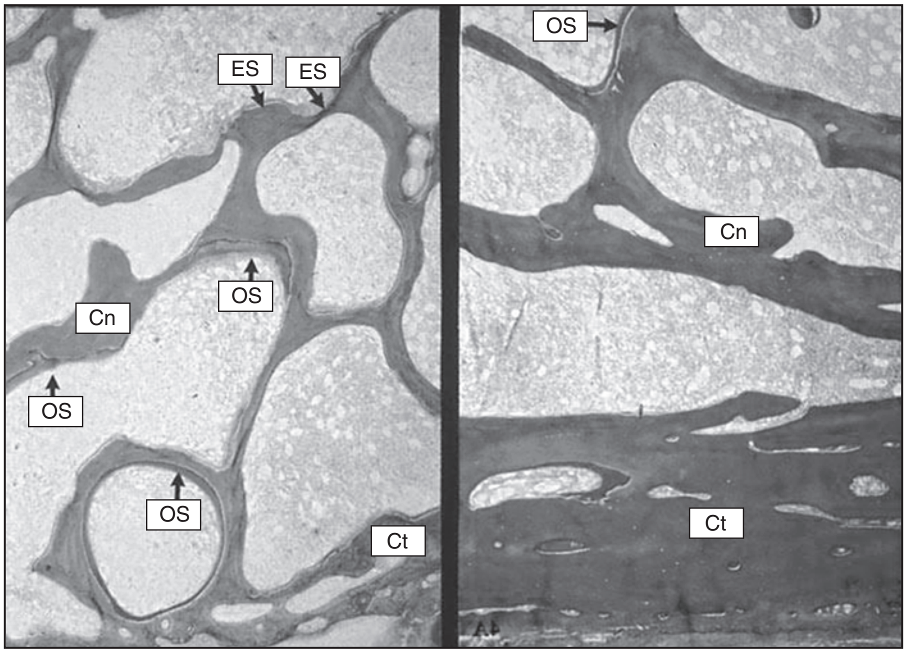

FIGURE 4 Photomicrographs of iliac bone biopsy sections from a subject with primary hyperparathyroidism (PHPT) (*left*), compared with a control subject (*right*). Note preservation of cancellous bone (Cn) and loss of cortical bone (Ct) in the subject with PHPT. Also note the marked extension of eroded surface (ES) and osteoid surface (OS) in PHPT.

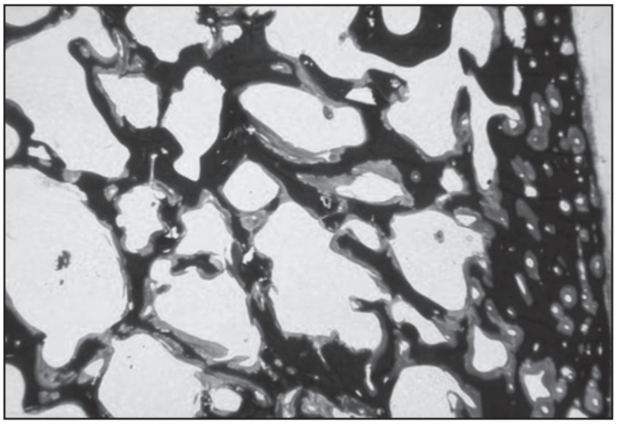

FIGURE 5 Photomicrograph of a bone biopsy section from a patient with severe osteomalacia. Note the dramatic extension of osteoid surface (stained light gray) and the increase in osteoid thickness.

analyzed for diagnostic or research purposes. Listed next are eight indices of trabecular bone that are of particular clinical relevance. For a detailed account of more theoretical aspects of bone biopsy analysis, see Parfitt (1983a) and Frost (1983).

It is conventional to divide histomorphometric parameters into two categories: Static variables yield information on the amount of bone present and the proportion of bone surface engaged the different phases of the remodeling cycle. Dynamic variables provide information on the rate of cell-mediated processes involved in remodeling. This category can only be evaluated in tetracycline-labeled biopsies. By measuring the extent of tetracycline-labeled surface and the distance between double-tetracycline labels, the bone formation rate can be computed directly in a single biopsy. Conversely, the resorption rate can only be calculated indirectly, using certain indices of bone formation, in a single biopsy specimen or from two sequential biopsy specimens (Frost, 1983; Eriksen, 1986).

Static Parameters

A list of five commonly used static variables is given here. The terms and abbreviations for all histomorphometric variables have been standardized by the Bone Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research, whose recommendations have been widely adopted (Parfitt *et al.*, 1987).

Cancellous bone volume (Cn-BV/TV, %). The fraction of a given volume of whole cancellous bone tissue (i.e., bone + marrow) that consists of mineralized and nonmineralized bone.

Osteoid volume (OV/BV, %). The fraction of a given volume of bone tissue (mineralized bone + osteoid bone) that is osteoid (i.e., unmineralized matrix).

Osteoid surface (OS/BS, %). The fraction of the entire trabecular surface that is covered by osteoid seams.

Osteoid thickness (O.Th, μm). The average thickness of osteoid seams.

Eroded surface (ES/BS, %). The fraction of the entire trabecular surface that is occupied by resorption bays (Howship lacunae), including both those with and without osteoclasts.

Dynamic Parameters

Following is a list of commonly use dynamic parameters:

Mineral apposition rate (MAR, μm/day). This is calculated by dividing the average distance between the first and second tetracycline labels by the time interval (e.g., 15 days) separating them. It is a measure of linear rate of production of calcified bone matrix by the osteoblasts.

Mineralizing surface (MS/BS, %). This is the fraction of trabecular surface bearing double-tetracycline-labeled plus one-half of the singly labeled surface. It is a measure of the proportion of bone surface on which new mineralized bone was being deposited at the time of tetracycline labeling.

Bone Formation Rate (BFR/BS, $\mu m^3/\mu m^2$ per day). This is the volume of mineralized bone made per unit surface of trabecular bone per year. It is calculated by multiplying the mineralizing surface by the mineral apposition rate.

In the following section, we will briefly review the remodeling process in normal bone and the changes that occur in a number of common disease states, as assessed by bone histomorphometry (Table I).

Normal Bone

Bone undergoes a continuous process of renewal with approximately 25% of trabecular bone and 3% of cortical bone being replaced annually. This remodeling process is referred to as a quantum phenomenon because it occurs in discrete units or “packets.” Osteoclasts resorb the old bone and osteoblasts replace it. The group of cells that work cooperatively to create one new packet of bone is called a bone-remodeling unit (BRU). In normal trabecular bone, approximately 900 bone remodeling units are initiated each day. In cortical bone, about 180 remodeling units are initiated per day (Frost, 1973; Parfitt, 1983a, 1988; Dempster, 2002).

Hyperparathyroidism

Increased circulating parathyroid hormone (PTH) levels increase the activation frequency of bone-remodeling units, resulting in increased osteoclast and osteoblast number. As a result, histomorphometric analysis of a biopsy from a patient with either primary or secondary hyperparathyroidism reveals increases in eroded surface, osteoid surface, and mineralizing surface (see Fig. 4) (Melsen *et al*., 1983; Malluche and Faugere, 1987; Parisien *et al*., 1990; Silverberg *et al*., 1990). Mineralizing surface is increased, but mineral apposition rate is slightly reduced. However, the increased mineralizing surface overcompensates for the decrease in mineral apposition rate, so that the bone formation rate, the product of these two variables, is increased. Bone turnover is higher in hyperparathyroid patients with vitamin D insufficiency (Silverberg *et al*., 1990). Cancellous bone volume and trabecular connectivity are preserved in primary hyperparathyroidism (Parisien *et al*., 1992). The elevated bone turnover is often accompanied by increased deposition of immature (woven) bone and marrow fibrosis, in particular, in cases of severe secondary hyperparathyroidism.

Because the biopsy reflects the long-term effects of excessive remodeling activity (e.g., increased eroded surface) it can be a sensitive indicator of parathyroid gland hyperactivity, especially when this is mild or intermittent. However, examination of the biopsy alone does not allow one to distinguish between primary and secondary hyperparathyroidism.

Osteomalacia

The hallmark of osteomalacia, regardless of the underlying pathogenetic mechanism, is inhibition of bone mineralization. Although mineralization is inhibited, the osteoblasts continue to synthesize and secrete organic matrix leading to an accumulation of osteoid (see Fig. 5). Although the cancellous bone volume is normal in osteomalacia, the amount of mineralized bone is actually reduced.

Careful analysis of the dynamic parameters is called for in suspected cases of osteomalacia. At some formation sites, mineral is still deposited, but at a reduced rate, resulting in low values for mineral apposition rate. At other sites, mineralization may be completely inhibited, resulting in reduced mineralizing surface. The decrease in both these variables markedly reduces bone formation rate. The accumulation of osteoid is reflected in increased osteoid thickness, osteoid surface, and osteoid volume. If PTH secretion is elevated, the activation frequency of bone-remodeling units is enhanced and the biopsy may show an increase in eroded surface. However, as osteoid surface increases, eroded surface often declines because osteoid is resistant to osteoclastic resorption. An elevated activation frequency, when accompanied by mineralization failure, will enhance the rate at which osteoid is deposited (Teitelbaum, 1980; Jaworski, 1983; Malluche and Faugere, 1987; Siris *et al*., 1987).

Renal Osteodystrophy

Chronic renal failure is usually accompanied by phosphate retention and hyperphosphatemia, which leads to a reciprocal decrease in serum-ionized calcium concentration and secondary hyperparathyroidism. Furthermore, as functional renal mass decreases, the plasma 1,25-dihydroxyvitamin D level falls, leading to impaired intestinal calcium absorption, which exacerbates hypocalcemia and ultimately may impair bone mineralization. As a result of these marked disturbances in metabolism, it is perhaps not surprising that the bone biopsy findings in renal osteodystrophy are heterogeneous (Malluche *et al*., 1976; Boyce

TABLE I Bone Biopsy Variables in a Variety of Disease States[a]

Disease state	Cancellous bone volume	Osteoid volume	Osteoid surface	Osteoid thickness	Eroded surface	Mineral apposition rate	Mineralizing surface	Bone formation rate
Hyperparathyroidism[b]	N or ↑	↑	↑	N	↑	↓	↑	↑
Osteomalacia[c]	N	↑	↑	↑	↑	↓	↓	↓
Renal osteodystrophy/dialysis[d]	↓ or ↑	↓ or ↑	↓ or ↑	↓ or ↑	↑	↓ or ↑	↓ or ↑	↓ or ↑
Postmenopausal or senile osteoporosis[e]	↓ or N	N or ↑	N or ↑	N or ↓	N or ↑	N or ↓	N, ↑ or ↓	N, ↑ or ↓
Cushing syndrome and corticosteroid-induced osteoporosis[f]	↓ or N	N	↑	↓	↑	↓	↓	↓
Paget's disease[g]	↑	↑	↑	↓	↑	↑	↑	↑
Thyrotoxicosis[h]	↓	↑	↑	↓	↑	↑	↑	↑
Hypothyroidism[h]	N	↓	N	↓	N	↓	↓	↓
Medullary thyroid carcinoma[h]	N	↑	↑	N	↑	↓	↑	N
Multiple myeloma[i]	N, ↑ or ↓	↑	↑	↓	↑	↓	↑	↑
Osteogenesis imperfect tarda[j]	↓	N	↑	↓	↑ or N	↓	N	↓

[a] *N, Normal; ↑, increased; ,↓, decreased.*

[b] *Melsen* et al., 1983; *Malluche and Faugere*, 1987; *Parisien* et al., 1990, 1992; *Silverberg* et al., 1990.

[c] *Teitelbaum*, 1980; *Jaworski*, 1983; *Malluche and Faugere*, 1987; *Siris* et al., 1987.

[d] *Malluche* et al., 1976; *Boyce* et al., 1982; *Hodsman* et al., 1982; *Charhon* et al., 1985; *Dunstan* et al., 1985; *Parisien* et al., 1988; *Salusky* et al., 1988; *Felsenfeld* et al., 1991; *Sherrard* et al., 1993; *Coburn and Salusky*, 2001; *Slatopolsky and Delmez*, 2002.

[e] *Meunier* et al., 1981; *Parfitt* et al., 1982; *Whyte* et al., 1982; *Civitelli* et al., 1988; *Meunier*, 1988; *Garcia Carasco* et al., 1989; *Arlot* et al., 1990; *Eriksen* et al., 1990; *Kimmel* et al., 1990; *Steiniche* et al., 1994; *Dempster*, 2000.

[f] *Bressot* et al., 1979; *Dempster*, 1989.

[g] *Meunier* et al., 1980.

[h] *Melsen* et al., 1983.

[i] *Valentin-Opran* et al., 1982.

[j] *Baron et al.*, 1983; *Ste-Marie et al.*, 1984.

et al., 1982; Hodsman *et al.*, 1982; Charhon *et al.*, 1985; Dunstan *et al.*, 1985; Parisien *et al.*, 1988; Salusky *et al.*, 1988; Moriniere *et al.*, 1989; Felsenfeld *et al.*, 1991; Hercz *et al.*, 1993; Sherrard *et al.*, 1993; Coburn and Salusky, 2001; Slatopolsky and Delmez, 2002). Indeed, in allowing a better understanding of the skeletal status in patients with chronic renal failure, the bone biopsy continues to play an important role in the management of this disease. Thus, renal osteodystrophy has been subdivided into two broad types, primarily on the basis of histomorphometric features. One type is characterized by normal or high bone turnover and a second is characterized by low bone turnover.

The most frequently observed biopsy changes in patients with end-stage renal disease are the result of chronic excess PTH secretion on the skeleton. These are classified as normal/high turnover, and include osteitis fibrosa, mild hyperparathyroidism, and mixed bone disease (Fig. 6). These features are characterized histomorphometrically by increased eroded surface, osteoid surface, and mineralizing surface. In osteitis fibrosa, however, woven osteoid is often present and there are variable amounts of peritrabecular marrow fibrosis in contrast to the minimal or absent fibrosis observed in mild hyperparathyroidism. On the other end of the spectrum, low bone turnover is frequently observed in patients undergoing dialysis, albeit less often than high turnover disease. The low turnover states are classified as osteomalacia and aplastic or adynamic bone disease. Patients with osteomalacia have evidence of reduced values for dynamic variables accompanied by the accumulation of excess osteoid, whereas patients with aplastic or adynamic disease have a reduced tetracycline-based bone formation rate, but normal or reduced osteoid volume. In the 1970s and 1980s, most symptomatic patients with osteomalacia or aplastic bone disease showed evidence of aluminum accumulation with more than 25% of surfaces displaying aluminum stain. They were considered to have aluminum-related bone disease (Boyce *et al.*, 1982; Hodsman *et al.*, 1982; Dunstan *et al.*, 1985; Parisien *et al.*, 1988). The aluminum was primarily derived from aluminum-containing phosphate binders used to control hyperphosphatemia and dialysis solutions that were contaminated with aluminum. Like tetracycline, aluminum accumulates at sites of new bone formation, where it may directly inhibit mineralization, which is manifested in an increase in osteoid thickness and osteoid surface. However, aluminum also is toxic to osteoblasts and may impair their ability both to synthesize and mineralize bone matrix, resulting in a decrease in mineral apposition rate and the mineralizing surface. However, if matrix production is also reduced, osteoid thickness will not be elevated. With appreciation of the sources of aluminum contamination and increased use of calcium-containing phosphate binders, aluminum-related bone disease has become much less common in recent years.

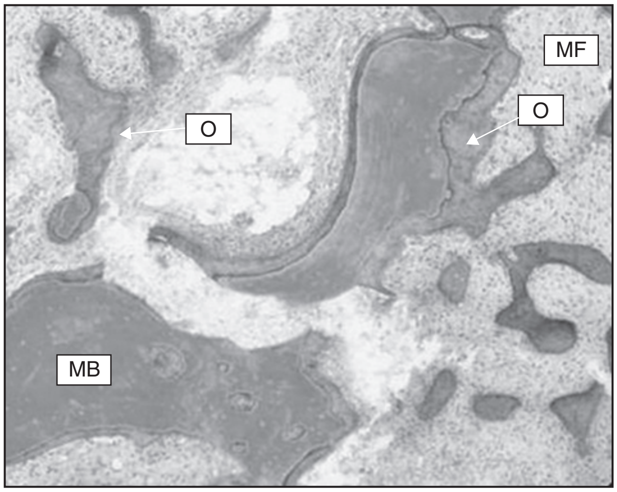

FIGURE 6 Photomicrograph of an iliac crest bone biopsy section from a patient with "mixed" renal osteodystrophy. Note deposition of woven osteoid (O) and marrow fibrosis (MF). MB, mineralized bone.

Another form of low turnover bone disease has been described that is not accompanied by significant aluminum accumulation. This is called idiopathic aplastic or adynamic bone disease. Its pathogenesis is unclear but may be related to various therapeutic maneuvers designed to prevent or reverse hyperparathyroidism in patients undergoing dialysis, including the use of dialysates with higher calcium concentrations (3.0 to 3.5 mEq/L), large doses of calcium-containing phosphate binders, and calcitriol therapy. As a rule, these patients have few symptoms of bone disease, and this "disease" ultimately may prove to be a histological rather than a clinically relevant form of bone disorder. However, it unknown whether patients with aplastic bone disease are at increased risk of the development of skeletal problems in the future.

Osteoporosis

The classic feature of bone biopsies in osteoporosis is the reduction in cancellous bone volume. Approximately 80% of patients with vertebral crush fractures have values that are lower than normal. In postmenopausal osteoporosis the reduction in cancellous bone volume is primarily caused by loss of entire trabeculae and, to a lesser degree, by the thinning of those that remain (Fig. 7) (Meunier *et al.*, 1981; Parfitt *et al.*, 1982; Whyte *et al.*, 1982; Meunier, 1988; Dempster, 2000).

With respect to the changes in the other static and dynamic variables in osteoporosis, there has been debate over whether patients can be stratified into high, normal, or low turnover groups. Even if they can, the pathogenetic and clinical significance of this so-called histological heterogeneity in patients with osteoporosis is unclear. In a study of 50 postmenopausal women with untreated osteoporosis, two subsets of patients were identified: one with normal turnover and one with high turnover, with the high turnover

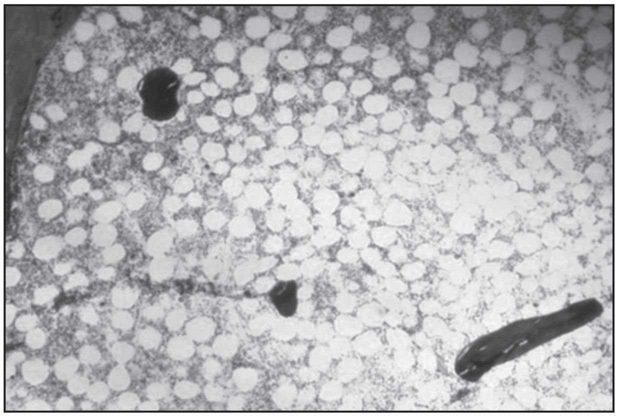

FIGURE 7 Photomicrograph of an iliac crest bone biopsy section from a patient with severe osteoporosis. Note marked reduction in cancellous bone volume in isolated trabecular profiles (arrowheads), which are cross-sections of thin, rod-like structures in three dimensions.

representing 30% of the cases (Arlot *et al*., 1990). However, this conclusion was based on the finding of a bimodal distribution in the static parameter, osteoid surface. The tetracycline-based bone formation rate, a dynamic measure of turnover rate, displayed a normal distribution. Based on the interval between the 10th percentile and the 90th percentile for calculated bone resorption rate in a group of normal postmenopausal women, another study classified 30% of women with untreated postmenopausal osteoporosis as having high turnover, whereas 64% and 6% had normal and low turnover, respectively (Eriksen *et al*., 1990). When bone formation rate was used as the discriminant variable, 19% were classified as having high turnover, 72% as having normal turnover, and 9% as having low turnover. On the other hand, in two studies of postmenopausal women with osteoporosis and their normal counterparts, the same wide variation in turnover indices was found in both groups, leading the investigators to conclude that there were no important subsets of patients with osteoporosis (Garcia Carasco *et al*., 1989; Kimmel *et al*., 1990). These studies, however, confirmed earlier observations that some patients with osteoporosis show profoundly depressed formation with little or no tetracycline uptake (Whyte *et al*., 1982).

From a clinical viewpoint, the desire to classify patients with osteoporosis according to their turnover status stems from the notion that the turnover rate may influence the response to particular therapeutic agents. For example, patients with high turnover rates may respond better to anticatabolic treatments. There was early evidence that this was the case for calcitonin (Civitelli *et al*., 1988). However, in clinical practice, the biopsy is an impractical way to determine turnover status. It was once believed that biochemical markers of bone resorption and formation would be useful in this regard, but this has yet to materialize.

Note that, in most cases, bone biopsy is performed when the disease is severe, with multiple fractures having already occurred. It is probable that, in many cases, the disturbances in bone metabolism that led to the reduction in bone mass and strength took place several years before the time of the biopsy and are no longer evident (Steiniche *et al*., 1994). Another confounding factor is that most patients who undergo biopsy for osteoporosis have already received treatment with one or more pharmaceutical agents.

Clinical Indications for Bone Biopsy

In general, a bone biopsy is only helpful in metabolic bone diseases. Only rarely is a biopsy indicated in patients with localized skeletal disease such as Paget's disease of bone, primary bone tumors, or bone metastases involving the iliac crest. The biopsy usually does not provide significantly greater insight into the disease process in postmenopausal women with osteoporosis. However, bone biopsy can be useful in patients who are less frequently affected by osteoporosis, such as young men and premenopausal women. Patients with osteopenia or women with postmenopausal osteoporosis should not have biopsies simply to measure cancellous bone volume to confirm the diagnosis of osteoporosis. The intraindividual and interindividual variability in cancellous bone volume is too great, and there is too much overlap between cancellous bone volume in patients with clinical osteoporosis and normal subjects to make this useful. However, bone biopsy is useful to exclude subclinical osteomalacia. In one study, 5% of patients with vertebral fractures displayed definitive evidence of osteomalacia on biopsy despite normal biochemical and radiological findings (Meunier, 1981). Moreover, the biopsy can be useful in identifying more precisely the probable cause of bone loss in individual patients with osteoporosis. For example, if the biopsy reveals or confirms a high bone turnover rate, it would be important to rule out endocrine disorders, such as hyperthyroidism and hyperparathyroidism. Finally, the biopsy is the best available way to evaluate the effect of various therapeutic maneuvers on bone cell function (e.g., Holland *et al*., 1994a,b; Marcus *et al*., 2000). This is discussed in detail in the context of therapies for osteoporosis in the following section.

As noted earlier, the bone biopsy can be extremely useful in patients with renal osteodystrophy, although the large number of patients with renal disease precludes its use in every case. In general, if a symptomatic patient has biochemical evidence of secondary hyperparathyroidism (hyperphosphatemia, hypocalcemia, and markedly elevated intact PTH levels), biopsy is not necessary because one can predict with reasonable certainty that it would reveal osteitis fibrosa. However, patients with renal disease who have bone pain and fractures without the biochemical profile of secondary hyperparathyroidism should undergo biopsy to determine whether they have osteomalacia or idiopathic, aplastic bone disease. Although aluminum accumulation is much less common nowadays, the biopsy will also permit the physician to determine whether it is a significant contributory factor. Although the biopsy can be useful in the clinical management of certain patients with bone disease, its principal use today is as a research tool.

HISTOMORPHOMETRIC STUDIES OF THE EFFECTS OF OSTEOPOROSIS DRUGS

In this section we will review what histomorphometry has revealed about the effects of drugs used to treat osteoporosis. The drugs will be covered under the headings of their two principal mechanisms of action: anticatabolic, also known as antiresorptive, and anabolic (Riggs and Parfitt, 2005).

Anticatabolic Agents

Calcitonin

Intranasal calcitonin is approved to reduce the risk of vertebral fractures, but its efficacy in nonvertebral fractures has not been established (Silverman, 2003). There have been several histomorphometric studies of the effects of calcitonin in subjects with osteoporosis or with rheumatoid arthritis (Gruber *et al.*, 1984; Marie and Caulin, 1986; Alexandre *et al.*, 1988; Palmieri *et al.*, 1989; Kroger *et al.*, 1992; Gruber *et al.*, 2000; Pepene *et al.*, 2004; Chesnut *et al.*, 2005). In cancellous bone, calcitonin treatment reduced eroded surface (Kroger *et al.*, 1982; Gruber *et al.*, 1984) and active resorption surface (Alexandre *et al.*, 1988) and mean resorption rate (Chesnut *et al.*, 2005) with no observed decrease in osteoclasts (Marie and Caulin, 1986; Palmieri *et al.*, 1989; Gruber *et al.*, 2000). Most studies failed to reveal any differences in bone formation parameters, e.g., osteoblast number and perimeter, osteoid perimeter and thickness, mineralized perimeter, mineral apposition rate (Gruber *et al.*, 1984; Marie and Caulin, 1986; Alexandre *et al.*, 1988; Chesnut *et al.*, 2005). However, one report (Gruber *et al.*, 2000;) suggested that bone formation was not reduced to the same extent as resorption. Cancellous bone volume was shown to be unchanged (Alexandre *et al.*, 1988; Gruber *et al.*, 2000; Chesnut *et al.*, 2005) or increased (Gruber *et al.*, 1984; Alexandre *et al.*, 1988; Palmieri *et al.*, 1989; Marie and Caulin, 1986; Kroger *et al.*, 1992).

Hormone Therapy (HT)

Bone histomorphometry has been used by several investigators to assess the effects of HT on both cancellous and, in some studies, cortical bone of the ilium (Steiniche *et al.*, 1989; Lufkin *et al.*, 1992; Holland *et al.*, 1994; Eriksen *et al.*, 1999; Vedi and Compston, 1996, 2003). One of the most interesting studies was by Eriksen *et al.* (1999) who showed that two years of HT decreased resorption parameters, reducing bone formation at the BMU level. Cancellous wall thickness was similar in treated and placebo groups, but there was a significant reduction in resorption rate in the HT group. This was in contrast to the placebo group which showed a significant increase in erosion depth and a modest increase in resorption rate. The reduction in the size of the resorption cavity with HT was confirmed in a later study (Vedi *et al.*, 1996), although that study also demonstrated a compensatory decrease in the wall width of trabecular bone packets. These findings were not replicated in a study (Steiniche *et al.*, 1989) in which HT was only given for one year.

Estrogen treatment has been shown to stimulate bone formation in animal models (Chow *et al.*, 1992a, 1992b; Edwards *et al.*, 1992) but this remains controversial in humans (Steiniche *et al.*, 1989; Lufkin *et al.*, 1992; Holland *et al.*, 1994; Vedi and Compston, 1996; Wahab *et al.*, 1997; Eriksen *et al.*, 1999; Patel *et al.*, 1999; Vedi *et al.*, 1999, 2003). Standard doses of HT reduce osteoid and mineralizing surfaces and bone formation rate, with no change or a decrease in wall width (Steiniche *et al.*, 1989; Lufkin *et al.*, 1992; Holland *et al.*, 1994; Vedi and Compston, 1996; Eriksen *et al.*, 1999; Patel *et al.*, 1999; Vedi *et al.*, 2003). On the other hand, long-term, high-dose HT was reported to increase cancellous wall width and to decrease eroded cavity area. Similarly, six years of subcutaneous HT increased cancellous bone volume with an increment in trabecular thickness and number as well as wall width (Khastgir *et al.*, 2001). Such anabolic actions of HT have also been reported in Turner's syndrome treated with HT (Khastgir *et al.*, 2003). The improvements in bone structure demonstrated by two-dimensional histomorphometric analysis are supported by micro-CT findings of a higher ratio of plate- to rod-like structures (Jiang *et al.*, 2005). In addition to these changes in histomorphometric variables, HT has also been shown to increase the degree of collagen cross-linking and bone mineralization, consistent with its primary action to lower bone turnover (Walters and Eyre, 1980; Holland *et al.*, 1994a,b; Rey *et al.*, 1995; Yamauchi, 1996; Khastgir *et al.*, 2001; Boivin and Meunier, 2002; Burr *et al.*, 2003; Paschalis *et al.*, 2004; Boivin *et al.*, 2005).

Selective Estrogen Receptor Modulators (SERMs)

Selective estrogen receptor modulators (SERMs) bind to the estrogen receptor and exhibit agonist actions in some tissues, such as bone, and antagonist actions in others, such as breast (Lindsay *et al.*, 1997). Bone histomorphometry studies are primarily limited to raloxifene. Two years of raloxifene treatment in the MORE trial (Ettinger *et al.*, 1999) decreased the bone formation rate, without changes in eroded surface and osteoclast number at the 60-mg dose, whereas the dose of 120 mg also decreased the bone formation rate and showed a trend toward a decrease in eroded surface and osteoclast number (Ott *et al.*, 2000). Cancellous bone volume, trabecular thickness, and cortical width were unchanged compared with baseline and the placebo group (Ott *et al.*, 2000). A significant decrease in activation frequency was observed with a higher dose (150 mg) of raloxifene (Weinstein *et al.*, 2003). In that study, raloxifene was shown to have effects similar to those of HT. However, a 6-month treatment with 60 mg of raloxifene did not suppress activation frequency and bone formation rate to the same extent as HT (Prestwood *et al.*, 2000). Reductions in activation frequency, bone formation rate, and resorption cavity area have also been demonstrated for another SERM, tamoxifen (Wright *et al.*, 1994). In contrast to HT, raloxifene had little effect on mineralization density as assessed by quantitative microradiography of the biopsy sections (Boivin *et al.*, 2003).

Bisphosphonates

The bisphosphonates have been the mainstay of osteoporosis therapy and will continue to be so for some time to come (Fleisch, 1998). The effects of alendronate, the first bisphosphonate to be approved in the United States, have been investigated in patients with postmenopausal osteoporosis (Bone *et al.*, 1997; Chavassieux *et al.*, 1997; Arlot *et al.*, 2005), as well as in patients with glucocorticoid-induced osteoporosis (Chavassieux *et al.*, 2000). Alendronate reduced osteoid surface and thickness, mineralizing surface, bone formation rate, and activation frequency. The mineral apposition rate was unchanged (Bone *et al.*, 1997; Chavassieux *et al.*, 1997; Arlot *et al.*, 2005). Although the primary target of bisphosphonates is the osteoclast, alendronate, like other anticatabolic agents, had little, if any effect on histomorphometric variables of bone resorption, including eroded surface and volume, osteoclast number, and erosion depth. This is inconsistent with the marked reductions seen in biochemical markers of bone resorption. The discrepancy is most likely explained by the fact that histomorphometric indices of bone resorption are static parameters, in contrast to bone formation indices, which are dynamic. In one study (Chavassieux *et al.*, 1997), wall thickness of trabecular bone packets was increased after two years of treatment, but this effect was not observed after three years. Histomorphometric studies failed to show an improvement in cancellous bone microarchitecture compared with placebo-treated subjects, but such an effect has been reported for three-dimensional structural parameters obtained by microcomputed tomography (Recker *et al.*, 2005), with the assumption that alendronate prevented the loss of structural integrity experienced by the placebo-treated patients. Consistent with its primary action to reduce the activation frequency, alendronate increased the degree of mineralization of the matrix (Meunier and Boivin, 1997; Boivin *et al.*, 2000; Hernandez *et al.*, 2001; Roschger *et al.*, 1997, 2001).

There have also been extensive studies of the effects of risedronate on the bone biopsy. Here, a paired biopsy design was employed with biopsies being obtained before and after treatment in the same subjects (Eriksen *et al.*, 2002; Dufresne *et al.*, 2003; Borah *et al.*, 2004, 2005, 2006; Seeman and Delmas, 2006; Zoehrer *et al.*, 2006). Like alendronate, three years of risedronate treatment decreased mineralizing surface, bone formation rate, and activation frequency (Eriksen *et al.*, 2002). Again, no significant change was noted in eroded surface and depth, but there was a significant decrease in resorption rate after risedronate treatment, with a significant increase in erosion depth in placebo-treated subjects. Also similar to alendronate's effects, risedronate preserved cancellous microarchitecture, as assessed by microcomputed tomography (Dufresne *et al.*, 2003; Borah *et al.*, 2004, 2005). No significant changes were seen in three-dimensional structural variables compared to baseline in risedronate-treated women, whereas trabecular microstructure deteriorated significantly in a subset of placebo-treated women who exhibited higher bone turnover at baseline (Borah *et al.*, 2004). Furthermore, the degree of structural deterioration was positively correlated with the bone turnover, confirming that high turnover has a deleterious effect on bone structure. Similar results were reported for early postmenopausal women who were treated for just one year with risedronate (Dufresne *et al.*, 2003). The reduction in bone turnover was associated with an increase in bone mineralization density, but there was no evidence of an abnormally high degree of mineralization, even when treatment was extended to five years (Borah *et al.*, 2006; Durchschlag *et al.*, 2006; Seeman and Delmas, 2006; Zoehrer *et al.*, 2006).

There are a number of other studies on the effects of different bisphosphonates, such as zoledronate and ibandronate, on iliac bone (Recker *et al.*, 2004, 2008). In general, the data obtained in patients with osteoporosis treated with these bisphosphonates are similar to those obtained with alendronate and risedronate (Recker *et al.*, 2004, 2008). It should also be noted that there is evidence of dramatic improvements in bone structure and turnover in children with osteogenesis imperfecta treated with bisphosphonates (Munns *et al.*, 2005).

Anabolic Therapies

Bone histomorphometry has confirmed that the mechanism of action of anabolic agents is fundamentally different from that of anticatabolic drugs (Frost, 1983). Rather than reducing the activation frequency of bone remodeling, anabolic agents elevate it with a positive bone balance. In each bone-remodeling unit, more bone is formed than was resorbed. Bone formation is increased prior to the increase in bone resorption. Consequently, anabolic agents are able to improve, rather than simply preserve cancellous and cortical bone microarchitecture.

PTH(1–34) and PTH(1–84)

The first bone biopsy studies of the effects of PTH(1–34) were conducted in postmenopausal women with osteoporosis who were treated concurrently with PTH(1–34) and HT for six or twelve months (Reeve *et al.*, 1980, 1991; Bradbeer *et al.*, 1992). Hodsman and colleagues (1993, 2000) also used bone biopsy to study the effects of a cyclical regimen of 28 days of PTH(1–34) every three months, with or without sequential calcitonin, for two years. Dempster *et al.* (2001) and Misof *et al.* (2003) performed paired biopsies in men with osteoporosis treated with PTH(1–34) for eighteen months, as well as in postmenopausal women treated with a combination of PTH(1–34) and HT for three years. Biopsy studies of the effects of monotherapy with PTH(1–34) were completed as part of a multinational fracture trial (Neer *et al.*, 2001; Jiang *et al.*, 2003; Dobnig *et al.*, 2005; Paschalis *et al.*, 2005; Ma *et al.*, 2006). Arlot and colleagues (2005) compared the effects of PTH(1–34) with those of alendronate in postmenopausal women with osteoporosis. The effects of the two agents on activation frequency and bone formation rate were diametrically opposed. Compared with appropriate reference ranges (Arlot *et al.*, 1990; Chavassieux *et al.*, 1997), the bone formation rate was 10% of normal in the alendronate-treated group and 150% higher than normal in the PTH(1–34)-treated group. The higher activation frequency in the PTH(1–34) group led to an increase in cortical porosity, which may explain observations of transient reductions in bone mineral density following treatment with PTH(1–34) (Neer *et al.*, 2001; Finkelstein *et al.*, 2003; Ettinger *et al.*, 2004). A recent study (132) used a novel, quadruple tetracycline-labeling regimen to perform a longitudinal study of the early effects of PTH(1–34) treatment on bone formation. Within 4 weeks of treatment, PTH(1–34) increased mineralized perimeter, mineral apposition rate, and bone formation rate (Hodsman *et al.*, 1993, 2000; Lindsay *et al.*, 2006). Lindsay *et al.*'s study (2006) suggested that PTH(1–34) stimulates osteoblastic activity in preexisting remodeling units. This could be accomplished by a variety of mechanisms, including an increase in the work rate of preexisting osteoblasts, enhanced recruitment of new osteoblasts, or a prolongation of osteoblast life span (Jilka *et al.*, 1999). Regardless of the mechanism, one noteworthy feature of PTH(1–34) is its ability to extend formation to quiescent surfaces surrounding the original remodeling unit (Fig. 8) (Lindsay *et al.*, 2006). Bone-remodeling indices were increased after 1 month (Holland *et al.*, 1994b), 2 months (Chow *et al.*, 1992b), and 6 months (Reeve *et al.*, 1980; Arlot *et al.*, 2005) of treatment, and they returned toward baseline between 12 (Reeve *et al.*, 1991) and 36 months of continuous treatment (Hodsman *et al.*, 2000; Dempster *et al.*, 2001). This temporal sequence of remodeling activation and deactivation, derived from biopsy studies, is confirmed by parallel changes in bone markers (Lindsay *et al.*, 1997; Kurland *et al.*, 2000; Cosman *et al.*, 2001; Arlot *et al.*, 2005; McClung *et al.*, 2005).

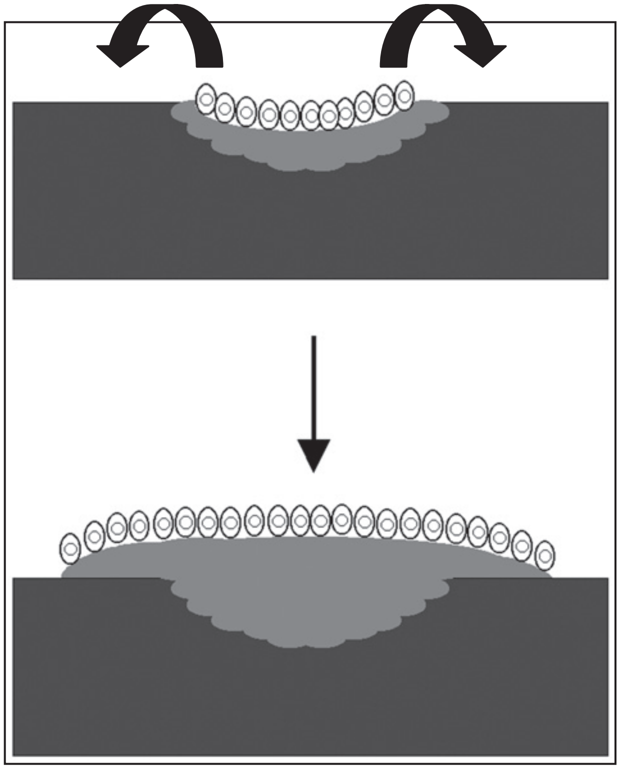

FIGURE 8 Proposed mechanism whereby PTH(1–34) could extend bone formation beyond the limits of the remodeling unit to annex the surrounding bone surface. Reproduced with permission from Lindsay *et al.* (2006).

The striking stimulation of bone formation by PTH (1–34) provides a mechanism for the reported increases in wall thickness of bone packets on cancellous and endocortical surfaces (Bradbeer *et al.*, 1992; Hodsman *et al.*, 2000; Dempster *et al.*, 2001; Ma *et al.*, 2006), which in turn leads to improvements in cancellous bone mass, trabecular connectivity, and cortical thickness (Dempster *et al.*, 2001; Jiang *et al.*, 2003) (Figs. 9 and 10). These improvements in cancellous bone structure were correlated with the early increases in bone formation markers (Dobnig *et al.*, 2005). The deposition of new bone brought about an increase in the proportion of bone matrix with lower

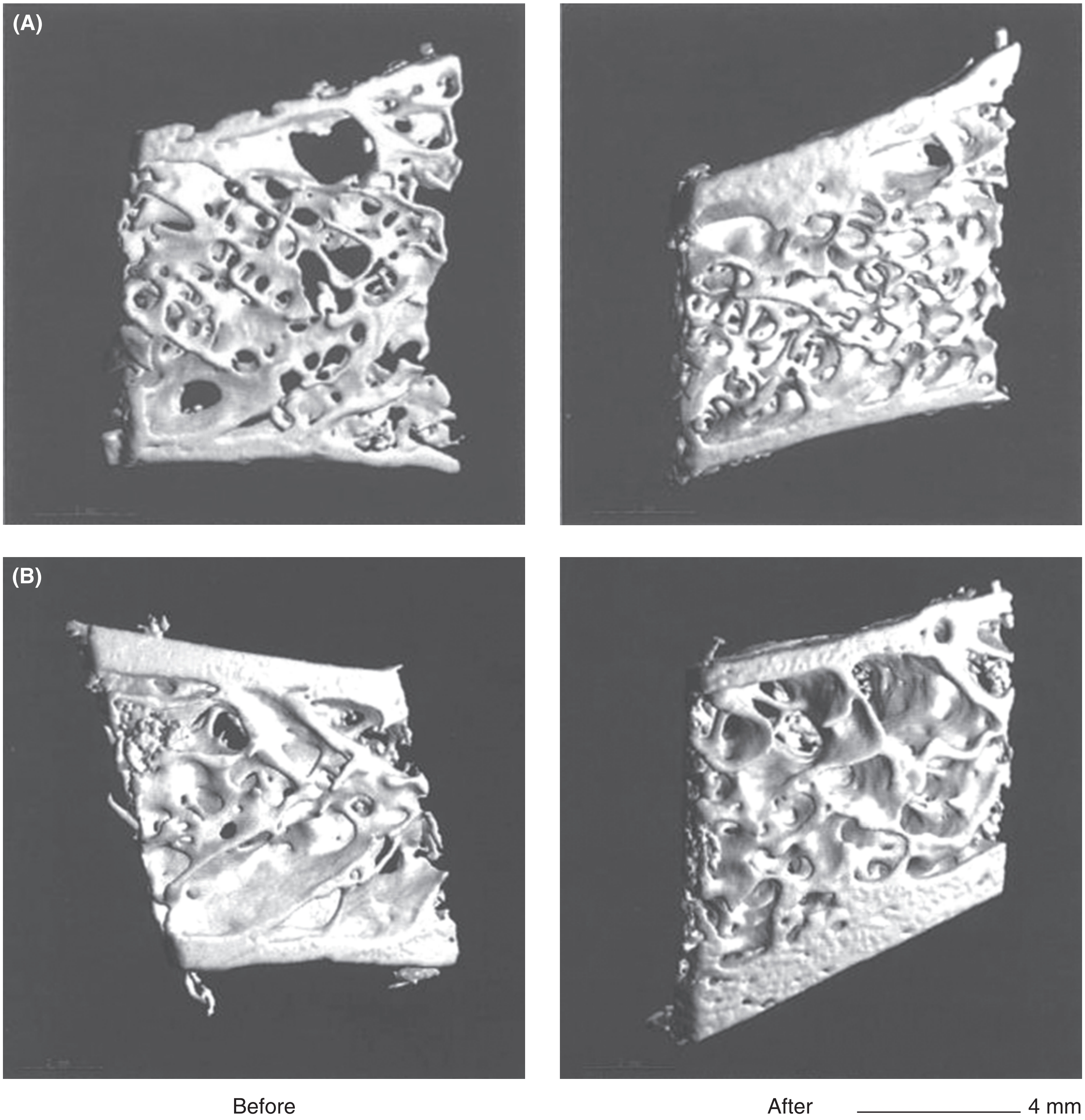

FIGURE 9 Microcomputed tomographic images of paired biopsies before (*left*) and after (*right*) treatment with PTH(1–34) in a 64-year-old woman (**A**) and a 47-year-old man (**B**). Note improvement in cancellous and cortical bone structure after treatment. Reproduced with permission from Dempster *et al*. (2001).

mineralization, mineral crystallinity, and collagen cross-link ratio (Misof *et al*., 2003; Paschalis *et al*., 2005).

The first study of the effects of PTH(1–34) raised the specter that the improvement in cancellous bone mass and structure may have been gained at the expense of cortical bone (Reeve *et al*., 1980). This was not confirmed in animal models where cortical thickness and diameter were improved by PTH(1–34) treatment (Hirano *et al*., 1999, 2000; Jerome *et al*., 1999; Burr *et al*., 2001; Mashiba *et al*., 2001). Histomorphometric and microcomputed tomographic studies in humans revealed an increase in cortical thickness at the iliac crest, which was accompanied by stimulation of bone formation on the endosteal surface (see Figs. 9 and 10) (Dempster *et al*., 2001; Jiang *et al*., 2003; Lindsay *et al*.,

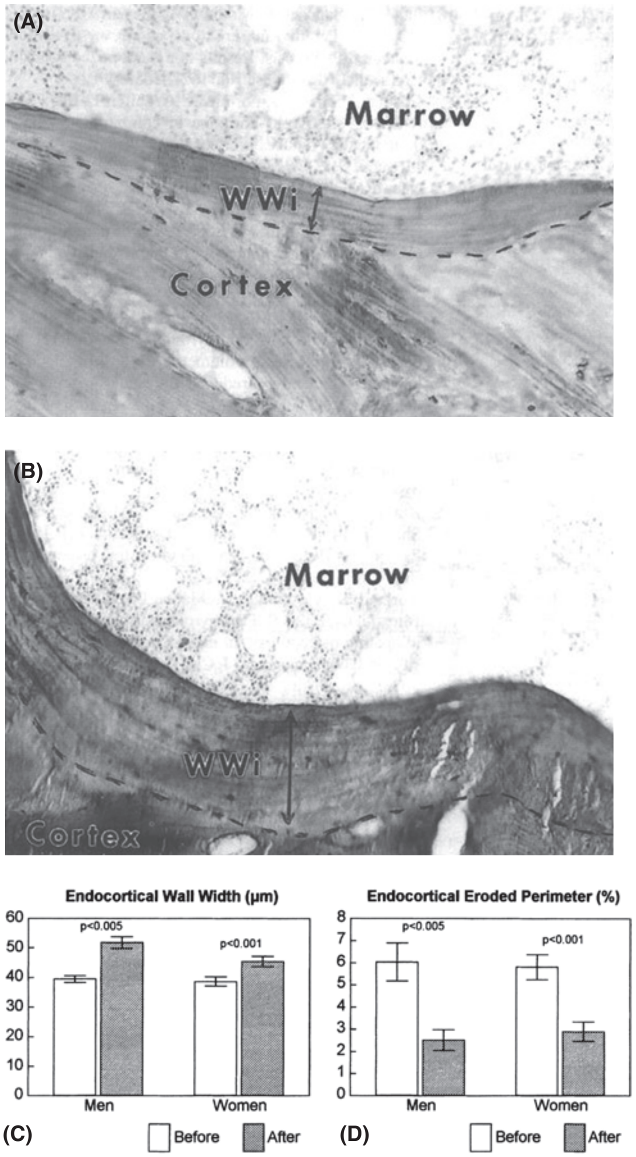

FIGURE 10 Bone packets on the endocortical surface of a 52-year-old man before (**A**) and after (**B**) 18 months of treatment with PTH(1–34). Note that after treatment the packet is almost twice as wide as the one before treatment. (**C** and **D**) Endocortical wall width and eroded perimeter before and after treatment of men and women with teriparatide. Note the increase in wall width and reduction in eroded perimeter after treatment. Reproduced with permission from Dempster *et al*. (2001).

2006). However, whether PTH(1–34) is able to stimulate periosteal bone formation in humans as it does in animals is not yet clear. Noninvasive techniques have yielded conflicting data on the effects of PTH(1–34) on bone diameter in humans (Zanchetta *et al*., 2003; Uusi-Rasi *et al*., 2005). However, recent biopsy studies suggest that PTH(1–34) can enhance periosteal bone formation (Ma *et al*., 2006; Lindsay *et al*., 2007). Although there are currently few data, the effects of PTH(1–84) on the human ilium appear to be broadly similar to those of PTH(1–34) (Fox *et al*., 2005).

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Chapter 23

Phosphorus Homeostasis and Related Disorders

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Phosphorus plays an important role in cellular physiology and skeletal mineralization, serving as a constituent of nucleic acids and hydroxyapatite, a source of the high-energy phosphate in adenosine triphosphate, an essential element of the phospholipids in cell membranes, and a factor influencing a variety of enzymatic reactions (e.g., glycolysis) and protein functions (e.g., the oxygen-carrying capacity of hemoglobin by regulation of 2,3-diphosphoglycerate synthesis). Indeed, phosphorus is one of the most abundant components of all tissues, and disturbances in phosphate homeostasis can affect almost any organ system. Most phosphorus within the body is in bone (600–700 g); the remainder is largely distributed in soft tissue (100–200 g). As a consequence, less than 1% of the total is in extracellular fluids. The plasma contains about 12 mg/dL of phosphorus, of which approximately 8 mg is organic and contained in phospholipids, a trace is an anion of pyrophosphoric acid, and the remainder is inorganic phosphate (P_i) (Yanagawa *et al.*, 1994). Inorganic phosphate is present in the circulation as divalent monohydrogen phosphate and monovalent dihydrogen phosphate. At normal pH, the relative concentrations of monohydrogen and dihydrogen phosphate are 4:1.

The critical role that phosphorus plays in cell physiology has resulted in development of elaborate mechanisms designed to maintain phosphate balance. These adaptive changes are manifest by a constellation of measurable responses, the magnitude of which is modified by the difference between metabolic P_i need and exogenous P_i supply. Such regulation maintains plasma and extracellular fluid phosphorus within a relatively narrow range and depends primarily on gastrointestinal absorption and renal excretion as mechanisms to affect homeostasis. Although investigators have recognized a variety of hormones that influence these various processes, in concert with associated changes in other metabolic pathways, the sensory system, the messenger, and the mechanisms underlying discriminant regulation of P_i balance remain incompletely understood.

Whereas long-term changes in P_i balance depend on these variables, short-term changes in phosphate concentrations can occur as a result of redistribution of phosphate between the extracellular fluid and either bone or cell constituents. Such redistribution results secondary to various mechanisms, including elevated levels of insulin and/or glucose; increased concentrations of circulating catecholamines; respiratory alkalosis; enhanced cell production or anabolism; and rapid bone remineralization. In many of these circumstances, hypophosphatemia manifests in the absence of phosphorus depletion or deprivation.

REGULATION OF PHOSPHATE HOMEOSTASIS

Phosphate is sufficiently abundant in natural foods that phosphate deficiency is unlikely to develop except under conditions of extreme starvation, as a consequence of administration of phosphate binders, or secondary to renal phosphate wasting. Indeed, the major proportion of ingested phosphate is absorbed in the small intestine, and hormonal regulation of this process plays only a minor role in normal phosphate homeostasis. In contrast, absorbed phosphate, in response to complex regulatory mechanisms, is eliminated by the kidney, incorporated into organic forms in proliferating cells, or deposited as calcium-phosphate complexes in soft tissue and/or as a component of bone mineral (hydroxyapatite). The vast majority of the absorbed phosphate, however, is excreted in the urine. Thus, under usual conditions, phosphate homeostasis depends for the most part on the renal mechanisms that regulate tubular phosphate transport. Alternatively, during times of severe phosphate deprivation, the phosphate contained in bone mineral provides a source of phosphate

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TABLE I Summary of Known Parameters of the Type II and Type III Na/P_i Transporters

	Type II			
	Type IIa	Type IIc	Type IIb	Type III
Chromosomal location (human)	5	9	4	2 (P_iT-1) 8 (P_iT-2)
Amino acids	~640	601	~690	679 656
Function	Na–P_i cotransport; electrogenic, pH dependent	Na–P_i cotransport; neutral	Na–P_i cotransport; electrogenic	Na–P_i cotransport; electrogenic
Substrate	P_i	P_i	P_i	P_i
Affinity for P_i	0.1–0.2 mM	0.1–0.2 mM	0.05 mM	0.025 mM
Affinity for Na	50–70 mM	50 mM	33 mM	40–50 mM
Na^+–P_i coupling	3	2	3	3
Tissue expression (mRNA, protein)	Kidney, parathyroid	Kidney	Small intestine, lung, other tissues	Ubiquitous
Major regulator(s)	PTH, dietary P_i, calcitriol, FGF-23	PTH, dietary P_i, FGF-23	Dietary P_i, calcitriol, estrogen, epidermal growth factor, glucocorticoids	Dietary P_i

FGF, *fibroblast growth factor*; PTH, *parathyroid hormone*.

for the metabolic needs of the organism. The specific role that the intestine and kidney play in this complex process is discussed later.

Mechanism of Phosphate Transport

Cells obtain phosphorus in the form of negatively charged P_i from the extracellular environment by means of secondary-active transport. In vertebrates, P_i transporters use the inwardly directed electrochemical gradient of Na^+ ions, established by the Na^+,K^+-ATPase, to drive P_i influx. Two unrelated families of Na^+-dependent P_i transporters manage the active transport (Virkki *et al.*, 2007). The SCL34 or type II Na^+/P_i family prefer divalent HPO_4^{2-} and comprises both electrogenic and electroneutral members that are expressed in various epithelia and other polarized cells. Through regulated activity in apical membranes of the gut and kidney, they maintain body P_i homeostasis, and in salivary and mammary glands, liver, and testes they play a role in modulating the P_i content of luminal fluids. The SLC20 or type III Na^+/P_i family transport monovalent $H_2PO_4^-$ and consist of P_iT-1 and P_iT-2 receptors, which are electrogenic and ubiquitously expressed, likely serving a housekeeping role for cell P_i homeostasis. However, more specific roles are emerging for these transporters in bone P_i metabolism and vascular calcification.

The two families of Na^+–P_i cotransporters share no significant homology in their primary amino acid sequence and exhibit substantial variability in substrate affinity, pH dependence, and tissue expression (Table I). The tissue expression, relative renal abundance, and overall transport characteristics of type II and III Na–P_i cotransporters suggest that the type II transporters play a key role in brush border membrane P_i flux (Miyamoto *et al.*, 2007).

The most detailed studies of the cellular events involved in P movement from the luminal fluid to the peritubular capillary blood have been performed in proximal tubules and cultured cells derived from them. These investigations indicate that P reabsorption occurs principally by a unidirectional process that proceeds transcellularly with minimal intercellular backflux from the plasma to the lumen. Across the luminal membrane, P_i entry into the tubular cell proceeds by a saturable active transport system that is sodium dependent (Fig. 1).

The active transport of phosphate across the apical membrane in renal proximal tubules is mediated by two members of the SLC34 family of solute carriers, NaP_i-IIa (*SLC34A1*) and NaP_i-IIc (*SLC34A3*; see Table I). These receptors mediate the reabsorption of phosphate from the urine by using the free energy provided by the electrochemical gradient for Na^+. NaP_i-IIa is electrogenic and transports divalent P_i preferentially. It functions with a Na^+: P_i stoichiometry of 3:1, which results in the net movement inward of one positive charge per cotransport cycle. In contrast, NaP_i-IIc is electroneutral and exhibits a 2:1 stoichiometry. In mice, NaP_i-IIa is the protein primarily responsible for P_i reabsorption in the adult kidney, whereas

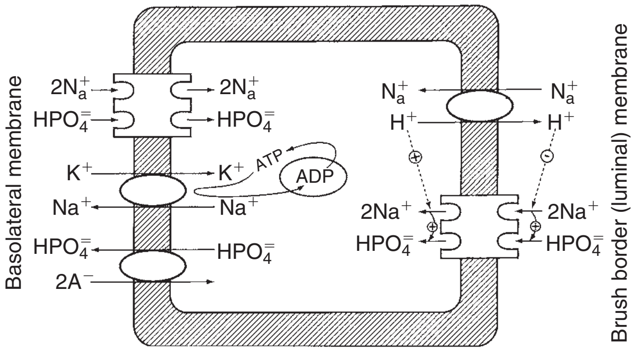


FIGURE 1 Model of inorganic phosphate ($HPO_4^=$) transcellular transport in the proximal convoluted tubule of the mammalian kidney. On the brush border or luminal membrane, a Na^+/H^+ exchanger and $Na/HPO_4^=$ cotransporters operate. ($HPO_4^=$) that enters the cell across the luminal surface mixes with the intracellular metabolic pool of phosphate and is eventually transported out of the cell across the basolateral membrane via an anion (A-) exchange mechanism. On the basolateral membrane there is also a $2Na/HPO_4^=$ cotransporter and a Na^+/K^+-ATPase system. The ATPase transports the Na^+ out of the cell, maintaining the Na^+ gradient-driving force for luminal phosphate entry.

NaP_i-IIc is seemingly more important in weanling animals. The phenotype of NaP_i-IIa knockout mice suggests that that this cotransporter is responsible for the bulk of renal P_i reabsorption with a very small percentage potentially due to the NaP_i-IIc transporter. However, recent data indicate that in humans, NaP_i-IIc may have a previously unpredicted importance (see the later subsection on Hereditary Hypophosphatemic Rickets with Hypercalciuria: Pathophysiology and Genetics). The expression of the sodium-dependent phosphate cotransporters is regulated to adapt the renal reabsorption of P_i to the needs of the organism. Thus, the phosphaturic effects associated with parathyroid hormone or the phosphatonins, such as fibroblast growth factor (FGF)-23, are due to the membrane retrieval of both cotransporters, whereas in conditions of P_i deprivation their expression is increased.

The phosphate that enters the tubule cell plays a major role in governing various aspects of cell metabolism and function and is in rapid exchange with intracellular phosphate. Under these conditions, the relatively stable free P_i concentration in the cytosol implies that P_i entry into the cell across the brush border membrane must be tightly coupled with its exit across the basolateral membrane (see Fig. 1). The transport of phosphate across the basolateral membrane is mediated by a transporter that remains unidentified. Regardless, basolateral P_i transport serves at least two functions: (1) complete transcellular P_i reabsorption when luminal P_i entry exceeds the cellular P_i requirements and (2) guaranteed basolateral P_i influx if apical P_i entry is insufficient to satisfy cellular requirements (Schwab *et al.*, 1984).

Gastrointestinal Absorption of Phosphorus

The average dietary phosphate intake in humans, derived largely from dairy products, meat, and cereals, is 800 to 1600 mg/day, one and one-half to threefold greater than the estimated minimum requirement. This phosphate is in both organic and inorganic forms, but the organic forms, except for phytates, are degraded in the intestinal lumen to inorganic phosphate, which is the form absorbed. Absorption occurs throughout the small intestine with transport greatest in the jejunum and ileum and less in the duodenum. Essentially no absorption occurs in the colon (Walling, 1977).

The absorption of phosphorus in the intestine is dependent on the amount and availability of phosphorus present in the diet. In normal subjects, net P absorption is a linear function of dietary P intake. Indeed, for a dietary P range of 4 to 30 mg/kg/day, the net P absorption averages 60 to 65% of the intake (Lee *et al.*, 1986). Intestinal P absorption occurs via two routes: transcellular and paracellular pathways. Transcellular phosphorus flux is regulated by a variety of hormones and metabolic factors, whereas the paracellular pathway appears dependent on the magnitude of the electrochemical gradients across the intestinal epithelium. The latter transport pathway is still controversial and may predominate at high intraluminal concentrations of phosphorus, such as during the intake of a meal.

Transcellular intestinal absorption of phosphorus is initiated by the sodium-dependent transport of P_i across the apical (brush border) membrane. Phosphate incorporated into intestinal cells by this mechanism is ferried from the apical pole to the basolateral pole likely through restricted channels such as microtubules. At the basolateral membrane, phosphate is released from intestinal cells by a passive mechanism, which is carrier mediated and occurs in accord with the electrochemical gradient. The apparent K_m for P_i is between 0.1 and 0.2 mM, and lowering the external pH value results in a modest increase of the transport rate.

The transepithelial transport of P_i occurs predominantly in the small intestine and is determined largely by the abundance of the Na/P_i-IIb transporter in the brush border membrane vesicles (see Table I). Thus, regulation of transepithelial intestinal absorption of P_i is mainly explained by alterations in the apical abundance of Na/P_i-IIb. Indeed, modulation of P_i transport through the cotransporter is regulated by various physiological effectors. Epidermal growth factor, and glucocorticoids inhibit intestinal sodium-dependent absorption and Na/P_i-IIb gene expression, whereas $1,25(OH)_2D$, estrogen and dietary P_i deprivation stimulate sodium-dependent absorption and Na/P_i-IIb gene expression.

To date numerous studies have shown that the intestinal absorption of P_i is regulated by $1,25(OH)_2D$. The effects of $1,25(OH)_2D$ on this process are modulated by the calcitriol-induced transcription of messenger RNA. Because low dietary intake of P_i stimulates the synthesis of calcitriol, the enhanced P_i absorption that occurs concomitantly has been attributed to the effects of this vitamin D metabolite on the abundance of the apical Na/P_i-IIb protein. Because in the adult mouse, the calcitriol dependent increase in the Na/P_i-IIb protein is paralleled by an increase in Na/P_i-IIb mRNA, the upregulation of the receptors in response to this stimulus has been considered secondary to a genomic mechanism possibly involving the VDR. However, recent studies (Capuano *et al.,* 2005) indicate that upregulation of the Na/P_i-IIb receptors by a low P_i diet occurs normally in VDR$^{-/-}$ and 25(OH)D-1α-hydroxylase-deficient mice, indicating that the increase in receptors likely involves a genomic mechanism that does not require VDR and is not dependent on an increased 1,25(OH)$_2$D level. The mechanism for this effect, however, remains unknown.

Although the active transport systems are responsive to various hormones (Lee *et al.*, 1986; Rizzoli *et al.*, 1977; Xu *et al.,* 2001), such hormonal provocation plays a relatively minor role in normal phosphate homeostasis (Takeda *et al.,* 2004). For example, during vitamin D deficiency, the percentage of P absorbed from the diet is reduced by only 15%. Moreover, a substantial portion of this decline is secondary to the failure to absorb calcium, which results from vitamin D deficiency and the resultant formation of calcium phosphate that reduces the free phosphate concentration.

The minimal effects of hormone stimulation on P_i absorption are expected, because the vast majority of phosphate absorption occurs via the process of diffusional absorption. This results as a consequence of the relatively low K_m of the active transport process (2 mM); the luminal P content during feeding, which generally exceeds 5 mM throughout the intestine; and the occurrence of net diffusional absorption of P whenever luminal P concentration exceeds 1.8 mM (a concentration generally exceeded even when fasting; Karr and Abbott, 1935; Walton and Gray, 1979; Wilkinson, 1976). Given these conditions, the active component of transport becomes important only under unusual circumstances, such as when dietary P or vitamin D is extremely low. Regardless, the bulk of intestinal P absorption is mediated by a diffusional process, presumably through the paracellular space, and therefore is primarily a function of P intake. Because most diets contain an abundance of P, the quantity of phosphate absorbed almost always exceeds the need both under normal circumstances and disease states such as uremia. Thus, active, transcellular P absorption becomes predominant only under conditions of low luminal P availability, such as dietary P deprivation and/or excessive luminal P binding (Lee *et al.,* 1979; Kurnik and Hruska, 1984). Factors that may influence the diffusional process adversely are the formation of nonabsorbable calcium, aluminum, or magnesium phosphate salts in the intestine and age, which reduces P absorption by as much as 50%.

Renal Excretion of Phosphorus

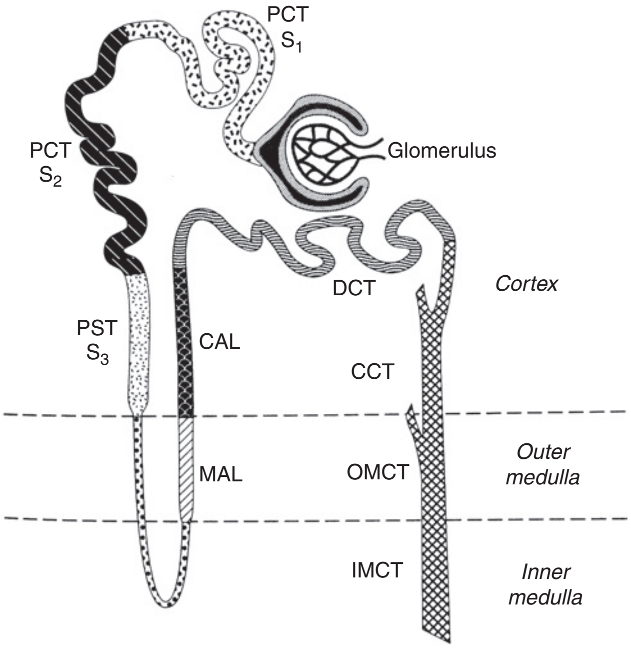
The kidney is immediately responsive to changes in serum levels or dietary intake of phosphate. Renal adaptation is determined by the balance between the rates of glomerular filtration and tubular reabsorption (Mizgala and Quamme, 1985).

The concentration of phosphate in the glomerular ultrafiltrate is approximately 90% of that in plasma because not all of the phosphate in plasma is ultrafilterable (Harris *et al.,* 1977). Nondiffusable phosphorus includes plasma P that is protein bound and a small fraction of plasma P that complexes with calcium and magnesium. With increasing serum calcium levels, the calcium–phosphate–protein colloid complex increases, reducing the ultrafilterable plasma P to as little as 75% (Rasmussen and Tenenhouse, 1995). Because the product of the serum phosphorus concentration and the glomerular filtration rate (GFR) approximates the filtered load of phosphate, a change in the GFR may influence phosphate homeostasis if uncompensated by commensurate changes in tubular reabsorption.

Normally, 80% to 90% of the filtered phosphate load is reabsorbed, primarily in proximal tubules, with higher rates at early segments (S_1/S_2 versus S_3) and in deep nephrons (Agus, 1983; Cheng and Jacktor, 1981; Dousa and Kempson, 1982; Suki and Rouse, 1996). The transcellular transport of phosphate is a carrier-mediated, saturable process limited by a transfer maximum or T_{max}. The T_{max} varies considerably as dietary phosphorus changes, and the best method to approximate this variable is to measure maximum phosphate reabsorption per unit volume of glomerular filtrate (T_mP/GFR) during acute phosphate infusions. Alternatively, the nomogram developed by Bijvoet allows estimation of the T_mP/GFR with measurement of phosphate and creatinine excretion and plasma phosphate concentration (Walton and Bijvoet, 1975).

The major site of phosphate reabsorption is the proximal convoluted tubule, at which 60% to 70% of reabsorption occurs (Fig. 2). Along the proximal convoluted tubule the transport is heterogeneous. In the most proximal portions, the S_1 segment, phosphate reabsorption exceeds that of sodium and water, whereas more distally, phosphate reabsorption parallels that of fluid and sodium. Additional reabsorption in the proximal straight tubule accounts for 15% to 20% of phosphate reclamation. In contrast, there is little evidence to suggest net P transport in the thin and thick ascending loops of Henle. However, increasing, but not conclusive, data support the existence of a P reabsorptive mechanism in the distal tubule. Currently, however, definitive proof for tubular secretion of phosphate in humans is lacking (Knox and Haramati, 1981).

At all three sites of phosphate reabsorption—the proximal convoluted tubule, proximal straight tubule, and distal tubule—several investigators have mapped parathyroid hormone (PTH)-sensitive adenylate cyclase (see Fig. 2;



	Phosphate reabsorption	PTH sensitive adenylate cyclase	PTH inhibited phosphate reabsorption	Calcitonin sensitive adenylate cyclase	Calcitonin inhibited phosphate reabsorption
PCT	60–70%	++	++	–	++
PST	15–20%	++	++	–	++
DCT	5–10%	++	++	++	–
CAL	0%	++	–	++	–
MAL	0%	–	–	++	–

FIGURE 2 Model of the renal tubule and distribution of phosphate reabsorption and hormone-dependent adenylate cyclase activity throughout the structure. The renal tubule consists of a proximal convoluted tubule (PCT), composed of an S_1 and S_2 segment, a proximal straight tubule (PST), also known as the S_3 segment, the loop of Henle, the medullary ascending limb (MAL), the cortical ascending limb (CAL), the distal convoluted tubule (DCT), and three segments of the collecting tubule: the cortical collecting tubule (CCT), the outer medullary collecting tubule (OMCT), and the inner medullary collecting tubule (IMCT). Phosphate reabsorption occurs primarily in the PCT but is maintained in the PST and DCT as well. In general, parathyroid hormone (PTH) influences phosphate reabsorption at sites where PTH-dependent adenylate cyclase is localized. FGF-23 modifies phosphate reabsorption in the PCT, where receptors for the phosphatonin are located. However, colocalization of the klotho protein, which is necessary for receptor activation, is controversial, because it is found primarily in the DCT. Calcitonin alters phosphate transport at sites distinct from those where calcitonin-dependent adenylate cyclase is present, suggesting that response to this hormone occurs by a distinctly different mechanism.

Knox and Haramati, 1981; Morel, 1981). Not surprisingly, there is clear evidence that PTH decreases phosphate reabsorption at these loci by a cAMP-dependent process, as well as a cAMP-independent signaling mechanism. Likewise, several investigators have identified FGF-23 receptors in the proximal tubule, the primary site at which phosphatonins decrease phosphate reabsorption. However, the colocalization of klotho proteins at these sites is controversial despite the need for these proteins to activate the FGF-23 receptors. In fact, the primary localization of the klotho proteins is the DCT. Calcitonin-sensitive adenylate cyclase maps to the medullary and cortical thick ascending limbs and the distal tubule (see Fig. 2; Berndt and Knox, 1984). Nevertheless, calcitonin inhibits phosphate reabsorption in the proximal convoluted and proximal straight tubule, certainly by a cAMP-independent mechanism that may be mediated by a rise in intracellular calcium (Murer *et al.*, 2000). An action of calcitonin on the distal tubule is uncertain, despite the abundant calcitonin-sensitive adenylate cyclase.

Hormonal/Metabolic Regulation of Phosphate Transport

Many hormonal and nonhormonal factors regulate renal reabsorption of P_i. The effects of PTH and dietary P_i, and more recently phosphatonins (most notably FGF-23), on this process have been the subject of detailed investigation. These studies suggest that NaP_i-IIa receptor regulation depends on its shuttling to/from the brush border membrane of the renal tubule. Thus, reduced P_i reabsorption (on PTH or phosphatonin release) is achieved by downregulation of the receptors at the brush border membranes. Such downregulation is dependent on endocytic removal of the receptors from the membranes and subsequent degradation in lysosomes. As a consequence, subsequent restoration of P_i transport upon removal of the hormonal stimulus depends on *de novo* synthesis. The detailed mechanism for recycling of the receptors is provided in a review by Forster *et al.* (2006). In concert with these findings, studies indicate that expression of the Na/P_i-IIa protein at renal tubular sites is increased in parathyroidectomized rats and decreased after PTH treatment. In addition, Northern blot analysis of total RNA shows that the abundance of Na/P_i-IIa-specific mRNA is not changed by parathyroidectomy, but is decreased minimally in response to the administration of parathyroid hormone. These data are consistent with the concept that parathyroid hormone regulation of renal Na^+–P_i cotransport is determined predominantly by changes in expression of the Na/P_i-IIa protein in the renal brush border membrane (Kempson *et al.*, 1995).

In contrast, a requirement for increased P_i reabsorption (in response to a sustained low P_i diet) is met by increasing expression of NaP_i-IIa (and NaP_i-IIc) at the brush border membrane. Such upregulation is independent of changes in transcription or translation. Therefore, increased expression of the receptors is due to both the stabilization of the transporter at the membrane and an increased rate of insertion in the membrane. Thus, dietary-induced upregulation is modulated by the presence of scaffolding proteins, which stabilize action, and the microtubule network, which facilitates an increased rate of insertion.

Despite these advances in understanding the mechanisms regulating the abundance of Na/P_i receptors, there remains a void in our knowledge regarding the manner in which the organism maintains overall P_i balance. Although historically PTH is accepted as the most important physiologic influence on renal P excretion and the major determinant of plasma P concentrations through its effect on P_i transport, repeated observations have confirmed that the balance between urinary excretion and dietary input of P is maintained not only in normal humans but in patients with hyper- and hypoparathyroidism. In fact, the renal tubule has a seemingly intrinsic ability to adjust the reabsorption rate of P according to dietary P_i intake and the need and availability of P_i to the body (Levi *et al.*, 1994). Thus P_i reabsorption is increased under conditions of greater P need, such as rapid growth, pregnancy, lactation, and dietary restriction. Conversely, in times of surfeit, such as slow growth, chronic renal failure, or dietary excess, renal P reabsorption is curtailed. This adaptive response is localized in the proximal convoluted tubule and involves an alteration in the apparent V_{max} of the high-affinity Na^+–phosphate cotransport systems. Such changes in response to chronic changes in P_i availability are characterized by parallel changes in Na^+–phosphate cotransport activity, the Na/P_i transporter mRNA level, and protein abundance. In contrast, the acute adaptation to altered dietary P_i is marked by parallel changes in Na^+–phosphate cotransporter activity and Na/P_i-IIa protein abundance in the absence of a change in mRNA. Thus, in response to chronic conditions, protein synthesis is requisite in the adaptive response, whereas under acute conditions, the number of Na/P_i-IIa cotransporters is changed rapidly by mechanisms independent of *de novo* protein synthesis, such as insertion of existing transporters into the apical membrane or internalization of existing transporters. Although the signal for the adaptive alteration in phosphate transport is not yet known, several lines of evidence suggest that renal adaptations to changes in dietary phosphate occur independently of the known regulators of renal phosphate transport. In this regard, adaptations to changes in dietary phosphate mediated by the vitamin D endocrine system, PTH, and the phosphatonins generally occur over a period of hours to days and cannot account for the modulation of renal phosphate excretion that rapidly occurs after a phosphate-containing meal. Recently, Berndt *et al.* (2007) presented data that support the existence of an intestinal-renal axis specific for phosphate that is mediated by an as yet unknown factor, an intestinal "phosphatonin." These observations suggest that the rapid renal response to increased dietary phosphate concentrations is due to the regulated production in the duodenum of a hormone, which increases the renal excretion of phosphate. This response dampens large increases in serum phosphate concentrations that could have a deleterious effect by enhancing the precipitation of calcium phosphate salts in soft tissues.

CLINICAL DISORDERS OF PHOSPHATE HOMEOSTASIS

The variety of diseases, therapeutic agents, and physiological states that affect phosphate homeostasis are numerous and reflect a diverse pathophysiology. Indeed, rational choice of an appropriate treatment for many of these disorders depends on determining the precise cause for the abnormality. The remainder of this chapter reviews several clinical states that represent primary disorders of phosphate homeostasis. These include X-linked hypophosphatemic rickets/osteomalacia (XLH); autosomal-dominant hypophosphatemic rickets (ADHR); autosomal recessive hypophosphatemic rickets (ARHR); tumor-induced osteomalacia (TIO); hereditary hypophosphatemic rickets with hypercalciuria (HHRH); Dent's disease; Fanconi's syndrome (FS), types I and II; and tumoral calcinosis (TC). Table II documents the full spectrum of diseases in which disordered phosphate homeostasis occurs. Many of these are discussed in other chapters.

Impaired Renal Tubular Phosphate Reabsorption

X-Linked Hypophosphatemic Rickets

X-linked hypophosphatemic rickets/osteomalacia is the archetypal phosphate-wasting disorder, characterized in general by progressively severe skeletal abnormalities and growth retardation. The syndrome occurs as an X-linked dominant disorder with complete penetrance of a renal tubular abnormality resulting in phosphate wasting and consequent hypophosphatemia (Table III). The clinical expression of the disease is widely variable even in members of the same family, ranging from a mild abnormality, the apparent isolated occurrence of hypophosphatemia, to severe bone disease (Lobaugh *et al.*, 1984). On average, disease severity is similar in males and females, indicating minimal, if any, gene dosage effect (Whyte *et al.*, 1996). The most common clinically evident manifestation is short stature. This height deficiency is a consequence of abnormal lower extremity growth, averaging 15% below normal. In contrast, upper segment growth is not affected. The majority of children with the disease exhibit enlargement of the wrists and/or knees secondary to rickets, as well as bowing of the lower extremities. Additional signs of the disease may include late dentition, tooth abscesses secondary to poor mineralization of the interglobular dentin, enthesopathy (calcification of tendons, ligaments, and joint capsules), and premature cranial synostosis. However, many of these features may not become apparent until age 6 to 12 months or older (Harrison *et al.*, 1966). Despite marked variability in the clinical presentation, bone biopsies in affected children and adults invariably reveal osteomalacia, the severity of which has no relationship to sex, the extent of the biochemical abnormalities, or the severity

TABLE II Diseases of Disordered Phosphate Homeostasis

- Increased phosphate
  - Reduced renal phosphate excretion
    - Renal failure
    - Hypoparathyroidism
    - Tumoral calcinosis[a]
    - Hyperthyroidism
    - Acromegaly
    - Diphosphonate therapy
  - Increased phosphate load
    - Vitamin D intoxication
    - Rhabdomyolysis
    - Cytotoxic therapy
    - Malignant hyperthermia
- Decreased phosphate
  - Decreased gastrointestinal absorption
    - Phosphate deprivation
    - Gastrointestinal malabsorption
  - Increased renal phosphate excretion
    - Hyperparathyroidism
    - X-linked hypophosphatemic rickets/osteomalacia[a]
    - Fanconi's syndrome, type I[a]
      - Familial idiopathic
      - Cystinosis (Lignac–Fanconi disease)
      - Hereditary fructose intolerance
      - Tyrosinemia
      - Galactosemia
      - Glycogen storage disease
      - Wilson's disease
      - Lowe's syndrome
    - Fanconi's syndrome, type II[a]
    - Vitamin D–dependent rickets
    - Autosomal-dominant hypophosphatemic rickets[a]
    - Autosomal-recessive hypophosphatemic rickets[a]
    - Dent's disease (X-linked recessive hypophosphatemic rickets)[a]
    - Tumor-induced osteomalacia[a]
    - Hereditary hypophosphatemic rickets with hypercalciuria[a]
  - Transcellular shift
    - Alkalosis
    - Glucose administration
  - Combined mechanisms
    - Alcoholism
    - Burns
    - Nutritional recovery syndrome
    - Diabetic ketoacidosis

[a] *Primary disturbance of phosphate homeostasis.*

of the clinical disability. In untreated youths and adults, serum 25(OH)D levels are normal and the concentration of $1,25(OH)_2D$ is in the low-normal range (Haddad *et al.*, 1973; Lyles *et al.*, 1982). The paradoxical occurrence of hypophosphatemia and normal serum calcitriol levels is due to the aberrant regulation of renal 25(OH)D-1α-hydroxylase activity as a direct result of abnormal phosphate transport or of elevated circulating levels of phosphatonins (e.g., FGF-23), independent of the effects on phosphate transport. Studies in *hyp*-mice, the murine homologue of the human disease, have established that defective regulation is confined to enzyme localized in the proximal convoluted tubule, the site of the abnormal phosphate transport (Lobaugh and Drezner, 1983; Nesbitt *et al.*, 1986, 1987; Nesbitt and Drezner, 1990).

Pathophysiology

Investigators generally agree that the primary inborn error in XLH results in an expressed abnormality of the renal proximal tubule that impairs P_i reabsorption. This defect has been indirectly identified in affected patients and directly demonstrated in the brush border membranes of the proximal nephron in *hyp*-mice. Until recently, whether this renal abnormality is primary or secondary to the elaboration of a humoral factor has been controversial. In this regard, demonstration that renal tubule cells from *hyp*-mice maintained in primary culture exhibit a persistent defect in renal P_i transport (Bell *et al.*, 1988; Dobre *et al.*, 1990), likely due to decreased expression of the Na^+–phosphate cotransporter mRNA and immunoreactive protein (Tenenhouse *et al.*, 1994, 1995; Collins and Ghishan, 1994), supported the presence of a primary renal abnormality. In contrast, transfer of the defect in renal P_i transport to normal and/or parathyroidectomized normal mice parabiosed to *hyp*-mice implicated a humoral factor in the pathogenesis of the disease (Meyer *et al.*, 1989a, 1989b). Subsequent studies, however, have provided compelling evidence that the defect in renal P_i transport in XLH is secondary to the effects of a circulating hormone or metabolic factor. Thus, immortalized cell cultures from the renal tubules of *hyp*-mice exhibit normal Na^+–phosphate transport, suggesting that the paradoxical effects observed in primary cultures may represent the effects of impressed memory and not an intrinsic abnormality (Nesbitt *et al.*, 1995, 1996). Moreover, the report that cross-transplantation of kidneys in normal and *hyp*-mice results in neither transfer of the mutant phenotype nor its correction unequivocally established the humoral basis for XLH (Nesbitt *et al.*, 1992). Subsequent efforts, which resulted in localization of the gene encoding the primary renal Na^+–phosphate cotransporter to chromosome 5, further substantiated the conclusion that the renal defect in brush border membrane phosphate transport is not intrinsic to the kidney (Kos *et al.*, 1994). Although these data establish the presence of a humoral abnormality in XLH, the identity of the putative

TABLE III Biochemical and Genetic Characteristics of Primary Disturbances of Phosphate Homeostasis[a]

	XLH	HHRH	ADHR	ARHR	Dent's Disease	TIO	FS I	FS II	TC
Calcium metabolism									
Serum Ca	N/LN	N/HN	N/LN	N/LN	N	N/LN	N/LN	N/HN	N/HN
Urine Ca	↓	↑	↓	↓	↑	↓	↓	↑	↑
Serum PTH	N	N/LN	N	N	N/LN	N	N	N/LN	N
Phosphate metabolism									
Serum P	↓	↓	↓	↓	N/↓	↓	↓	↓	↑
T_mP/GFR	↓	↓	↓	↓	N/↓	↓	↓	↓	↑
GI function									
P absorption	↓	↑	↓	↓	?	↓	↓	↑	↑
Ca absorption	↓	↑	↓	↓	↑	↓	↓	↑	↑
Serum biochemistries									
Alkaline phosphatase	N/↑	N/↑	N/↑	N/↑	N/↑	N/↑	N/↑	N/↑	N
Vitamin D metabolism									
Serum 25(OH)D	N	N	N	N	N	N	N	N	N
Serum 1,25(OH)$_2$D	(↓)	↑	(↓)	(↓)	↑	↓	(↓)	↑	↑
Gene(s) mutation	PHEX	Na/Pi-IIC	FGF-23	DMP1	CLCN5	–	–	–	FGF-23, GLANT3 KLOTHO

[a] *Modified from* Econs et al. (1992). XLH, *X-linked hypophosphatemic rickets*; HHRH, *hereditary hypophosphatemic rickets with hypercalciuria*; ADHR, *autosomal-dominant hypophosphatemic rickets*; ARHR, *autosomal recessive hypophosphatemic rickets*; TIO, *tumor-induced osteomalacia*; FS I, *Fanconi's syndrome type* I; FS II, *Fanconi's syndrome type* II; TC, *tumoral calcinosis*. N, *normal*; LN, *low normal*; HN, *high normal*; ↑, *increased*; ↓, *decreased*; (↓), *decreased relative to the serum phosphorus concentration*.

factor, the spectrum of its activity, and the cells producing it have not been definitively elucidated until recently.

Genetic Defect

Efforts to better understand XLH have more recently included attempts to identify with certainty the genetic defect underlying this disease. In 1986, Read and coworkers and Machler and colleagues reported linkage of the DNA probes DXS41 and DXS43, which had been previously mapped to Xp22.31–p21.3, to the *HYP* gene locus. In subsequent studies, Thakker *et al.* (1987) and Albersten *et al.* (1987) reported linkage to the *HYP* locus of additional polymorphic DNA, DXS197, and DXS207 and, using multipoint mapping techniques, determined the most likely order of the markers as Xpter-DXS85-(DXS43/-DXS197)-*HYP*-DXS41-Xcen and Xpter-DXS43-*HYP*-(DXS207/DXS41)-Xcen, respectively. The relatively small number of informative pedigrees available for these studies prevented definitive determination of the genetic map along the Xp22–p21 region of the X chromosome and only allowed identification of flanking markers for the *HYP* locus 20 cM apart. However, the HYP consortium (HYP Consortium, 1995), in a study of some 20 multigenerational pedigrees, used a positional cloning approach to refine mapping of the Xp22.1–p21 region of the X chromosome, identify tightly linked flanking markers for the *HYP* locus, construct a YAC contig spanning the *HYP* gene region, and eventually clone and identify the disease gene as *PHEX*, a phosphate-regulating gene with homologies to endopeptidases located on the X chromosome. The *PHEX* locus was mapped to Xp22.1 and more than 140 loss-of-function *PHEX* mutations (see http://www.envbiocourse.rutgers.edu/eu-us/pages/extras/software/DatabasesListNAR2002/summary/145.html) have been reported to date in patients with XLH (Holm *et al.*, 1997). However, no genotype/phenotype correlations have been recognized, albeit changes in conserved amino acids more often result in clinical disease than changes in nonconserved amino acids (Filisetti *et al.*, 1999). Further studies documented that the murine homologue of the human disease, the *hyp*-mouse, has a phenotype identical to that evident in patients with XLH and is due to a large deletion of the 3' region of the *Phex* gene (Beck *et al.,* 1997). Collectively, these findings indicate that a mutation in the *PHEX/Phex* gene is responsible for the phenotypic changes in patients with XLH and the *hyp*-mouse.

The *PHEX* gene has 22 exons and encodes for a 749-amino-acid glycoprotein, which has homology with members of the M13 membrane-bound zinc metalloendopeptidase family. Investigation of murine tissues and cell cultures revealed that *Phex* is predominantly expressed in bones and teeth (Du *et al.,* 1996; Ruchon *et al.,* 1998), although detectable Phex mRNA and/or protein have been found in lung, brain, muscle gonads, skin and parathyroid glands. The *PHEX/Phex* expression in bone is limited to cells of the osteoblast lineage (Ruchon *et al.,* 1998; Thompson *et al.,* 2002), osteoblasts and osteocytes.

The physiological function of the *PHEX/Phex* gene product and the mechanisms that lead to the biochemical and skeletal abnormalities evident in patients with XLH and in the *hyp*-mouse remain ill-defined. Because *PHEX/Phex* codes for a membrane-bound enzyme, several groups have postulated that *PHEX/Phex* activates or degrades a putative phosphate- and bone mineralization-regulating factor(s), "phosphatonin" or "minhibin," which are involved in the regulation of phosphate and mineralization processes (Rasmussen and Tenenhouse 1995; Nesbitt *et al.,* 1992; Ecarot *et al.,* 1992; Econs and Drezner 1994; Bowe *et al.,* 2001). Studies in patients with XLH and the *hyp*-mouse, as well as in a wide variety of other P_i wasting disorders, have identified several candidate proteins as the phosphatonin(s) or minhibin(s), including FGF-23 (Fukomoto and Yamashita 2002; Liu *et al.,* 2006), sFRP4 (Kumar, 2002; Berndt *et al.,* 2003), MEPE (Argiro *et al.,* 2001; Rowe *et al.,* 2004) and FGF-7 (Carpenter *et al.,* 2005). Because abnormal production or circulating levels of several of these factors have been identified in patients with XLH and in *hyp*-mice, until recently it remained unknown whether a single phosphatonin is responsible for the physiological abnormalities of the disease or a family of proteins works cooperatively to create the *HYP* phenotype.

Progress in this regard has been limited by unsuccessful efforts to rescue the *HYP* phenotype and determine the physiologically relevant site for the *PHEX* mutation. In attempts to determine whether abnormal *PHEX/Phex* expression in bone cells alone is the determining abnormality underlying the pathogenesis of XLH, several investigators have used osteoblast/osteocyte targeted overexpression of *Phex* in order to normalize osteoblast mineralization *in vitro* and rescue the *HYP* phenotype *in vivo* (Liu *et al.,* 2002; Bai *et al.,* 2002). Surprisingly, however, these studies documented that restoration of *Phex* expression and enzymatic activity to immortalized *hyp*-mouse osteoblasts, by retroviral mediated transduction, does not restore their capacity to mineralize extracellular matrix *in vitro*, under conditions supporting normal mineralization. Moreover, in complementary studies Liu *et al.* (2002) and Bai *et al.* (2002) found that transgenic *hyp*-mice maintained characteristic hypophosphatemia and abnormal vitamin D metabolism, as well as histological evidence of osteomalacia, despite expressing abundant Phex mRNA and enzyme activity in mature osteoblasts and osteocytes, under the control of the bone-specific promoters osteocalcin and ColA1 (2.3 kb). These findings suggest that expression of *Phex* at sites other than bone is responsible for the *Hyp* phenotype. However, similar studies performed with transgenic mice in which *Phex* overexpression was under the regulation of the ubiquitous human β-actin promoter likewise failed to normalize the phosphate homeostasis (Erben *et al.,* 2005).

In recent studies, however, Yuan *et al.* (2007) attempted to explore this apparent paradox by evaluating whether conditional inactivation of *Phex* in osteoblasts and osteocytes generates a biochemical phenotype similar to that in mice with a global *Phex* knockout and comparable to that in *hyp*-mice. To accomplish this goal they generated mouse lines with global and targeted deletion of exon 17 from the *Phex* gene, which codes a portion of the protein crucial for bioactivity. Their studies established unequivocally that the mutation of the *Phex* gene in osteoblasts and osteocytes alone is sufficient to generate the classical *HYP* phenotype. Indeed, the targeted knockout mice exhibit a biochemical phenotype (phosphorus homeostatic abnormalities and aberrant vitamin D metabolism) no different than that of the global knockout mice and indistinguishable from that in the *hyp*-mouse. Moreover, investigation of the bone pathology in the targeted knockout mice further established that mutation of the *Phex* gene in the osteoblasts and osteocytes is a sufficient abnormality to produce the *HYP* phenotype. Hence, bone biopsies from the targeted knockout mice displayed histomorphological evidence of osteomalacia and cannicular disorganization indistinguishable from those observed in bone biopsies from the global knockout mice, as well as those from *hyp*-mice.

Although these observations provide compelling evidence that aberrant *PHEX/Phex* function in osteoblasts and osteocytes alone underlies the characteristic biochemical and pathological phenotype in *hyp*-mice and likely XLH, a surprising discordance between the biochemical phenotype in the targeted knockout mice from that in both the global knockout and *hyp*-mice became apparent with further study. Previous investigations have implicated the phosphatonins as critical factors in the pathogenesis of XLH and have identified that the production rate and/or the circulating level of FGF-23, MEPE, and sFRP-4, are increased in affected humans and/or the *hyp*-mouse. However, Yuan *et al.* (2007) found that the targeted knockout mice had only an increased osseous production rate and serum level of FGF-23, whereas the global knockout and *hyp*-mice exhibited increased production and serum levels of FGF-23, MEPE, and sFRP-4. These observations provide the first successful attempt to discern whether integrated effects of these hormones or the activity of a single phosphatonin is essential for expression of the disease phenotype in *hyp*-mice and affected patients with XLH. Indeed, the data suggest that increased bone production and serum levels of MEPE and sFRP-4 are not critical for development of the classical *HYP* phenotype, whereas increased osseous production and serum FGF-23 concentration appear requisite for this biological function. Therefore, FGF-23 is likely the phosphatonin pivotal to the pathogenesis of XLH, and the role of MEPE and sFRP-4, if any, remains uncertain.

Pathogenesis

Despite the remarkable advances that have been made in understanding the genetic abnormality and pathophysiology of XLH, the detailed pathogenetic mechanism underlying this disease remains unknown. Nevertheless, several observations suggest the likely cascade of events that result in the primary abnormalities characteristic of the syndrome. In this regard, the X-linked dominant expression of the disorder with little, if any, gene dosage effect likely results from PHEX mutations that result in a haploinsufficiency defect, in which one half the normal gene product (or null amounts) causes the phenotype. The alternative possibility that the PHEX gene results in a dominant-negative effect is unlikely because, inconsistent with this prospect, several mutations reported in affected humans (Francis *et al.*, 1997) and the murine *Gy* mutation almost certainly result in the lack of message production (Meyer *et al.*, 1998). In any case, it is tempting to speculate that the PHEX gene product acts directly or indirectly on a phosphaturic factor (e.g., FGF-23) that regulates renal phosphate handling. Given available data, the PHEX gene product, a putative cell membrane-bound enzyme, may function normally to inactivate “phosphatonin,” a phosphaturic hormone. However, data from parabiotic studies of normal and *hyp*-mice argue strongly that extracellular degradation of the phosphaturic factor does not occur. Indeed, such activity would preclude transfer of the *hyp*-mouse phenotype to parabiosed normals. Alternatively, the PHEX gene product may function intracellularly to regulate phosphatonin production. In this regard, Jalal *et al.* (1991) reported the internalization of neutral endopeptidase and a potential role for this enzyme in intracellular metabolism. In addition, Thompson *et al.* (2002) reported that the PHEX protein in osteoblasts is found predominantly in the Golgi apparatus and the endoplasmic reticulum.

In any of these cases, a defect in the PHEX gene will result in overproduction and circulation of phosphatonin and consequent decreased expression of the renal Na^+–phosphate cotransporter, the likely scenario in the pathogenesis of XLH. The possible mechanism by which PHEX regulates the production of FGF-23, the requisite phosphatonin for the expression of the *HYP* phenotype, was recently discovered by Yuan and Drezner (unpublished observations). Their studies indicate that the loss of function *Phex* mutation in *hyp*-mice results in decreased production of 7B2, an intracellular chaperone protein, which proteolytically activates subtilin-like prohormone convertase 2 (SPC2), enabling cleavage of hormones to inactive peptides and prohormones to active hormones. The decreased activity of the 7B2–SPC2 enzyme complex decreases FGF-23 degradation in the osteoblast, increasing the concentration of the intact active FGF-23. In addition, the decreased 7B2–SPC2 activity limits conversion of DMP1 (104 kDa) to active DMP1 (57 kDa), the relative absence of which increases FGF-23 mRNA and consequent protein production. These concordant effects on FGF-23 degradation and production result in the increased circulating FGF-23 and production of the *HYP* phenotype.

Treatment

In past years, physicians employed pharmacologic doses of vitamin D as the cornerstone for treatment of XLH. However, long-term observations indicate that this therapy fails to cure the disease and poses the serious problem of recurrent vitamin D intoxication and renal damage. Current treatment strategies for children directly address the combined calcitriol and phosphorus deficiency characteristic of the disease. Generally, the regimen includes a period of titration to achieve a maximum dose of calcitriol, 40 to 60 ng/kg/day in two divided doses, and phosphorus, 1 to 2 g/day in four to five divided doses (Friedman and Drezner 1991, 1993). Such combined therapy often improves growth velocity, normalizes lower extremity deformities, and induces healing of the attendant bone disease. Of course treatment involves a significant risk of toxicity that is generally expressed as abnormalities of calcium homeostasis and/or detrimental effects on renal function secondary to abnormalities such as nephrocalcinosis. In addition, refractoriness to the growth-promoting effects of treatment is often encountered, particularly in youths presenting at below the 5th percentile in height (Friedman *et al.*, 1993). Several studies, however, indicate that the addition of growth hormone to conventional therapy increases growth velocity significantly. Unfortunately, such a benefit is realized more frequently in younger patients, and disproportionate growth of the trunk often continues to manifest. Moreover, the definitive impact of growth hormone treatment on adult height remains unknown (Wilson, 2000).

More recently, several investigators have suggested that the effects of combination therapy with calcitriol and phosphate may be enhanced with the concurrent use of cinacalcet, a calcimimetic agent, which reduces parathyroid hormone. Use of cinacalcet decreases the elevated parathyroid hormone observed occasionally in untreated patients and frequently in treated patients, limiting the phosphaturic effects of PTH and permitting more optimal restoration to normal of the renal TmP/GFR and serum phosphorus concentration in treated patients.

Therapy in adults is reserved for episodes of intractable bone pain and refractory nonunion bone fractures.

Hereditary Hypophosphatemic Rickets with Hypercalciuria

This rare genetic disease is characterized by hypophosphatemic rickets with hypercalciuria (Tieder *et al.*, 1985). The cardinal biochemical features of the disorder include hypophosphatemia due to increased renal phosphate clearance and normocalcemia. In contrast to other diseases in which renal phosphate transport is limited, patients with HHRH exhibit increased 1,25$(OH)_2$D production (see Table III). The resultant elevated serum calcitriol levels enhance gastrointestinal calcium absorption, which in turn increases the filtered renal calcium load and inhibits parathyroid secretion (Tieder *et al.*, 1985). These events cause the hypercalciuria observed in affected patients.

The clinical expression of the disease is heterogeneous, although initial symptoms, evident at 6 months to 7 years of age, generally consist of bone pain and/or deformities of the lower extremities. The bone deformities vary from genu varum or genu valgum to anterior external bowing of the femur and coxa vara. Additional features of the disease include short stature, muscle weakness, and radiographic signs of rickets or osteopenia. These various symptoms and signs may exist separately or in combination and may be present in a mild or severe form. Relatives of patients with evident HHRH may exhibit an additional mode of disease expression. These subjects manifest hypercalciuria and hypophosphatemia, but the abnormalities are less marked and occur in the absence of discernible bone disease (Tieder *et al.*, 1987). Bone biopsies in children with characteristic HHRH exhibit classical osteomalacia, but the mineralization defect appears to vary in severity with the magnitude of the hypophosphatemia. Histological measurements are within the normal range in family members with idiopathic hypercalciuria.

Pathophysiology and Genetics

Liberman and co-workers (Tieder *et al.*, 1985, 1987 Liberman, 1988) have presented data that indicate the primary inborn error underlying this disorder is an expressed abnormality in the renal proximal tubule, which impairs phosphate reabsorption. They propose that this pivotal defect results in enhanced renal 25(OH)D-1α-hydroxylase, thus promoting the production of 1,25$(OH)_2$D and increasing its serum levels. Consequently, intestinal calcium absorption is augmented, resulting in the suppression of parathyroid function and an increase of the renal filtered calcium load. The concomitant prolonged hypophosphatemia diminishes osteoid mineralization and accounts for the ensuing rickets and/or osteomalacia.

The suggestion that abnormal phosphate transport results in increased calcitriol production remains untested. Indeed, the elevation of 1,25$(OH)_2$D in patients with HHRH is a unique phenotypic manifestation of the disease that distinguishes it from other disorders in which abnormal phosphate transport is likewise manifest. Such heterogeneity in the phenotype of these disorders suggests that disease at variable anatomical sites along the proximal convoluted tubule uniformly impairs phosphate transport but not 25(OH)D-1α-hydroxylase activity. Alternatively, the aberrant regulation of vitamin D metabolism in other hypophosphatemic disorders may occur independently (e.g., in XLH secondary to the PHEX gene abnormality or FGF-23) and override the effects of the renal phosphate transport.

Extensive studies in a large consanguineous Bedouin kindred have established that HHRH is transmitted in an autosomal recessive inheritance pattern. Under such circumstances, the variability in this disorder may be explained by assuming that individuals with HHRH or idiopathic hypercalciuria are homozygous and heterozygous, respectively, for the same mutant allele. Early studies focused on Na/P_i-IIa as a candidate gene underlying HHRH. Indeed, Beck *et al.* (1998) reported that homozygous ablation of this murine gene (*SLC34a1*) leads to hypophosphatemia, renal phosphate wasting, increased serum 1,25$(OH)_2$D levels, and hypercalciuria. These findings were evident at weaning, but the magnitude of the changes decreased with increasing age. Furthermore, Na/P_i-IIa-ablated mice lack the typical features of rickets (Beck *et al.*, 1998; Gupta *et al.*, 2001; Tenenhouse, 2005). Therefore, it was not surprising that *SLC34A1* mutations were excluded in genomic DNA from affected members of several kindred with HHRH (Jones *et al.*, 2001; van den Heuvel *et al.*, 2001). More recently, Bergwitz *et al.* (2006) performed a genome-wide linkage scan, combined with a homozygosity mapping approach in an extended Bedouin kindred to map the genetic defect responsible for HHRH to a 1.6-Mbp interval in chromosome region 9q34. Na/P_i-IIc (*SLC34A3*), a plausible candidate gene in this region, was sequenced and all individuals with HHRH were homozygous for a 1-nt deletion, c 228delC. This mutation produces a nonfunctional Na/P_i-IIc protein that is truncated within the first membrane-spanning domain. Consistent with these observations, several compound heterozygous missense mutations or deletions were found in affected individuals from three additional unrelated kindreds, which were not present in healthy controls. Moreover, several investigators (Ichikawa *et al.*, 2006; Lorenz-Depiereux *et al.*, 2006) have confirmed mutations in *SLC34A3* in multiple additional families.

Although the function of the NaPi-IIc receptor in the regulation of renal phosphate transport was considered minimal, the identification of a mutation in the *SLC34A3* gene as the cause of HHRH has brought new perspective to the role of this receptor. Indeed, a recent series of studies (Tenenouse *et al.*, 2003; Miyamoto *et al.*, 2004) provide undeniable evidence that NaP_i-IIC appears to be critically involved in regulating phosphate homeostasis.

Treatment

In accord with the hypothesis that a singular defect in renal phosphate transport underlies HHRH, affected patients have been treated successfully with high-dose phosphorus (1–2.5 g/day in five divided doses) alone. In response to therapy, bone pain disappears and muscular strength improves substantially. Moreover, the majority of treated subjects exhibit accelerated linear growth and radiologic signs of rickets disappear completely within 4 to 9 months. Concordantly, serum phosphorus values increase toward normal, the 1,25(OH)$_2$D concentration decreases, and alkaline phosphatase activity declines. Despite this favorable response, limited studies indicate that such treatment does not heal the associated osteomalacia. Therefore, further investigation is necessary to determine whether phosphorus alone is truly sufficient for this disorder.

Autosomal-Dominant Hypophosphatemic Rickets

Several studies have documented an autosomal-dominant inheritance, with incomplete penetrance, of a hypophosphatemic disorder similar to XLH (Harrison and Harrison, 1979). The phenotypic manifestations of this disorder include lower extremity deformities and rickets/osteomalacia. Indeed, affected patients display biochemical and radiographic abnormalities indistinguishable from those of individuals with XLH. These include hypophosphatemia secondary to renal phosphate wasting and normal levels of parathyroid hormone and 25(OH)D, as well as inappropriately normal (relative to the serum phosphorus concentration) 1,25(OH)$_2$D (see Table III). However, unlike patients with XLH, some with ADHR display variable incomplete penetrance and delayed onset of penetrance (Econs and McEnery, 1997). Thus, long-term studies indicate that a few of the affected female patients exhibit delayed penetrance of clinically apparent disease and an increased tendency for bone fracture, uncommon occurrences in XLH. Moreover, these individuals present in the second through the fourth decade with weakness and bone pain but do not have lower extremity deformities. Further, other patients with the disorder present during childhood with phosphate wasting, rickets, and lower extremity deformity but manifest postpubertal loss of the phosphate-wasting defect. Finally, a few apparently unaffected individuals have been identified, who seemingly are carriers for the ADHR mutation.

An apparent forme fruste of this disease, (autosomal-dominant) hypophosphatemic bone disease, has many of the characteristics of XLH and ADHR, but reports indicate that affected children display no evidence of rachitic disease (Scriver *et al.*, 1977, 1981). Because this syndrome is described in only a few small kindreds and radiographically evident rickets is not universal in children with familial hypophosphatemia, these families may have ADHR. Further observations are necessary to discriminate this possibility.

Pathophysiology and Genetics

The primary inborn error in ADHR results in an expressed abnormality of the renal proximal tubule that impairs P$_i$ reabsorption. Until recently, whether this renal abnormality is primary or secondary to the elaboration of a humoral factor has been controversial. However, identification of the genetic defect underlying this disease has established that hormonal dysregulation is the pivotal abnormality in this disorder. In this regard, studies localized the ADHR gene to a 6.5-cM interval on chromosome 12p13 (Econs *et al.*, 1997). Moreover, extending these studies, the ADHR Consortium (2000) used a positional cloning approach to identify 37 genes within 4 Mb of the genomic sequence in the 6.5-cM interval and identified missense mutations in a gene encoding FGF-23. Three different mutations were found in four kindreds, each resulting in amino acid substitutions in the FGF-23 at R176Q, R179Q, and R179W. These mutations occur at a protease cleavage site (RXXR) and the resulting mutant FGF-23 molecules are resistant to cleavage (White *et al.*, 2001a, 2001b; Shimada *et al.*, 2002; Bai *et al.*, 2003). The impact of these mutations is the inability to degrade the FGF-23, a relevant abnormality because only the full-length molecule has biological effects *in vivo* (Shimada *et al.*, 2002). Hence the resulting abundance of full-length FGF-23 results in the hypophosphatemia and attendant rickets and osteomalacia characteristic of the disorder. The relationship of the elevated FGF-23 concentration to active disease has recently been investigated by Imel *et al.* (2007). They discovered that FGF-23 concentrations are not universally elevated in ADHR. Rather, the ADHR symptoms and disease severity likely fluctuate with the FGF-23 concentration and the ability to alter the FGF-23 concentration changes the clinical phenotype, resulting in the observed delayed penetrance, and also in at least temporary resolution of the phenotype in some individuals. Such variability likely involves an intrinsic but unknown physiological alteration in the metabolism of FGF-23 leading to resolution of ADHR features.

Treatment

Although studies of treatment in patients with ADHR are not available, the pathogenetic role of FGF-23 in the development of the disease suggests that therapy effective in patients with XLH will likewise benefit those with ADHR. Hence, treatment with calcitriol and phosphorus supplementation has been employed in a limited number of the affected patients. Evaluation of treatment outcome will likely reveal only partial success, similar to that in patients with XLH.

Autosomal Recessive Hypophosphatemic Rickets

Recent studies have identified ARHR in two unrelated, consanguineous kindreds as a genetically transmitted form of hypophosphatemic rickets. Affected individuals present with renal phosphate wasting, rachitic changes, and lower limb deformity. In family 1 there were three affected sisters and in family 2 a single affected female. The parents and siblings of these individuals did not exhibit any clinical or biochemical evidence of the disease.

At presentation members of family 1 in mid- to late infancy had radiographic evidence of rickets or clinically evident rickets with progressive lower limb deformity. In family 2 the affected individual presented at 8 years of age with a mild genu valgum. Biochemical abnormalities at baseline included hypophosphatemia, renal phosphate wasting, normal parathyroid function, normocalcemia, normal urinary calcium, and elevated alkaline phosphatase. Serum 1,25$(OH)_2$D levels were inappropriately normal in the presence of hypophosphatemia (see Table III). Bone biopsy in an affected individual confirmed severe osteomalacia, marked by excess osteoid and extended mineralization lag time, as well as a disrupted osteocyte lacunocanalicular system. Serum FGF-23 levels were overtly elevated or overlapped the upper normal range. Biochemical studies obtained during adulthood showed persistent renal phosphate wasting, and consequent hypophosphatemia and linear growth was heterogeneous. Testing for *FGF-23* and *PHEX* mutations was negative.

Studies of the *Dmp1*-null mouse provided insight to the genetic defect underlying ARHR. Dmp1 is a member of the SIBLING family (Fisher and Fedarko 2003) that is primarily expressed in mineralized tissues, most notably osteocytes (Toyosawa *et al.,* 2001). Absence of this protein in null mice results in mild hypocalcemia and severe hypophosphatemia secondary to increased renal phosphate clearance. The enhanced renal phosphate clearance is due to an elevated serum FGF-23 concentration, which is associated with increased FGF-23 mRNA expression in bone. Moreover, newborn mice lacking *Dmp1* develop radiological evidence of rickets and an osteomalacia phenotype with age (Ling *et al.,* 2005). These observations highlight that genetic removal of *Dmp1* from the skeletal matrix in mice leads to altered skeletal mineralization and disturbed phosphate homeostasis associated with increased FGF-23 production, abnormalities similar to those observed in individuals with ARHR.

Thus, Feng *et al.* (2006) undertook a candidate gene approach using direct sequence analyses to test ARHR families for a mutation in *DMP1*. In the affected individuals in family 1, they detected a homozygous deletion of nucleotides 1484–1490 (1484–1490del) in DMP1 exon 6, resulting in a frameshift that replaced the conserved C-terminal 18 residues with 33 unrelated residues. The affected individual in family 2 had a biallelic nucleotide substitution in the DMP1 start codon (ATG to GTG, or A1→G) and a consequent substitution of the initial methionine with valine (M1V), resulting in predicted loss of the highly conserved 16-residue DMP1 signal sequence. These mutations segregated with the disorder in both kindreds, and neither DMP1 mutation was found in 206 control alleles. Mutational analyses of an additional 19 hypophosphatemic individuals negative for PHEX and FGF-23 mutations, with no known history of consanguinity, did not uncover any disease-causing changes in DMP1.

Treatment

Studies in the *Dmp1*-null mice revealed that restoring serum phosphate to normal level by feeding a high-phosphate diet rescues the radiological appearance of rickets owing to correction of the mineralization defect at the growth plate. However, although the osteomalacia improved with a high-phosphate diet, the bone phenotype was not completely rescued, consistent with similar observations in *hyp*-mice. Thus, the treatment strategy for ARHR will likely include use of high-dose phosphate supplements and calcitriol, albeit complete bone healing is not anticipated.

Tumor-Induced Osteomalacia

Since 1947 there have been reports of well more than 200 patients in whom rickets and/or osteomalacia has been induced by various types of tumors. In at least half of the reported cases a tumor has been clearly documented as causing the rickets/osteomalacia, because the metabolic disturbances improved or disappeared completely on removal of the tumor. In the remainder of cases, patients had inoperable lesions, and investigators could not determine the effects of tumor removal on the syndrome or surgery did not result in complete resolution of the evident abnormalities during the period of observation.

Affected patients generally present with bone and muscle pain, muscle weakness, rickets/osteomalacia, and occasionally recurrent fractures of long bones. Additional symptoms common to younger patients are fatigue, gait disturbances, slow growth, and bowing of the lower extremities. Biochemistries include hypophosphatemia secondary to renal phosphate wasting and normal serum levels of calcium and 25(OH)D. Serum 1,25$(OH)_2$D is overtly low or low relative to the prevailing hypophosphatemia (see Table III). Aminoaciduria, most frequently glycinuria, and glucosuria are occasionally present. Radiographic abnormalities include generalized osteopenia, pseudofractures, and coarsened trabeculae, as well as widened epiphyseal plates in children. The histologic appearance of trabecular bone in affected subjects most often reflects the presence of low-turnover osteomalacia. In contrast, bone biopsies from the few patients who have tumors that secrete a nonparathyroid hormone factor(s), which activates adenylate cyclase, exhibit changes consistent with enhanced bone turnover, including an increase in osteoclast and osteoblast number.

The large majority of patients with this syndrome harbor tumors of mesenchymal origin and include primitive-appearing, mixed connective tissue lesions, osteoblastomas, nonossifying fibromas, and ossifying fibromas. However, the frequent occurrence of Looser zones in the radiographs of moribund patients with carcinomas of epidermal and endodermal derivation indicates that the disease may be secondary to a variety of tumor types. Indeed, the observation of tumor-induced osteomalacia concurrent with breast carcinoma (Dent and Gertner, 1976), prostate carcinoma (Lyles *et al.*, 1980; Murphy *et al.*, 1985; Hosking *et al.*, 1975), oat-cell carcinoma (Leehey *et al.*, 1985), small-cell carcinoma (Shaker *et al.*, 1995), multiple myeloma, and chronic lymphocytic leukemia (McClure and Smith, 1987) supports this conclusion. In addition, the occurrence of osteomalacia in patients with widespread fibrous dysplasia of bone (Dent and Gertner, 1976; Saville *et al.*, 1995), neurofibromatosis (Weidner and Cruz, 1987; Konishi *et al.*, 1991), and linear nevus sebaceous syndrome (Carey *et al.*, 1986) could also be tumor induced. Although proof of a causal relationship in these disorders has been precluded in general by an inability to excise the multiplicity of lesions surgically, in one case of fibrous dysplasia, removal of virtually all of the abnormal dysplastic lesions did result in appropriate biochemical and radiographic improvement.

Regardless of the tumor cell type, the lesions at fault for the syndrome are often small and difficult to locate and present in obscure areas, which include the nasopharynx, a sinus, the popliteal region, and the suprapatellar area. In any case, a careful and thorough examination is necessary to document or exclude the presence of such a tumor. Indeed, attempts at PET/CT and/or MRI localization of the tumor are often required. In addition, several groups have used octreotide scanning and other forms of nucleotide scintigraphy to identify suspected, but nonlocalized, tumors.

Pathophysiology

The relatively infrequent occurrence of this disorder has confounded attempts to determine the pathophysiological basis for TIO. Nevertheless, most investigators agree that tumor production of a humoral factor(s) that may affect multiple functions of the proximal renal tubule, particularly phosphate reabsorption, is the probable pathogenesis of the syndrome. This possibility is supported by (1) the presence of phosphaturic activity in tumor extracts from three of four patients with TIO (Aschinberg *et al.*, 1977; Yoshikawa *et al.*, 1977; Lau *et al.*, 1979); (2) the absence of parathyroid hormone and calcitonin from these extracts and the apparent cyclic AMP-independent action of the extracts; (3) the occurrence of hypophosphatemia and increased urinary phosphate excretion in heterotransplanted tumor-bearing athymic nude mice (Miyauchi *et al.*, 1988); (4) the demonstration that extracts of the hetero-transplanted tumor inhibit renal 25-hydroxyvitamin D-1 α-hydroxylase activity in cultured kidney cells (Miyauchi *et al.*, 1988); and (5) the coincidence of aminoaciduria and glycosuria with renal phosphate wasting in some affected subjects, indicative of complex alterations in proximal renal tubular function (Drezner and Feinglos, 1977). Indeed, partial purification of "phosphatonin" from a cell culture derived from a hemangioscleroma causing tumor-induced osteomalacia has reaffirmed this possibility (Cai *et al.*, 1994). These studies revealed that the putative phosphatonin may be a peptide with a molecular mass of 8 to 12 kDa that does not alter glucose or alanine transport, but inhibits sodium-dependent phosphate transport in a cyclic AMP-independent fashion. However, studies, which document the presence in various disease states of additional phosphate transport inhibitors (and stimulants), indicate that the tumor-induced osteomalacia syndrome may be heterogeneous. In this regard, excessive tumor production and secretion of FGF-23 (White *et al.*, 2001b) and matrix extracellular phosphoglycoprotein (MEPE) have been identified in large numbers of patients with tumor-induced osteomalacia. Moreover, secreted frizzled related protein 4 (sFRP4) has recently been identified as a putative phosphatonin in patients with TIO. Indeed, the gene for this protein was reported as a highly expressed transcript in hemangiopericytomas that caused TIO and a differential expression cloning approach also identified sFRP4 as highly expressed in TIO tumors. Thus, it seems certain that ectopic hormone production of a phosphatonin by a tumor underlies the syndrome. As a result, suspicion of the syndrome is often confirmed by measurement of serum FGF-23. Indeed, Imel *et al.* (2006) studied 22 patients with suspected TIO, 13 of whom had confirmed tumors. They found elevated FGF-23 concentrations in 16 of the 22 patients, using the Immunotopics C-terminal assay (sensitivity 73%), in 5 of the 22 patients using the Immunotopics Intact assay (sensitivity 23%), and in 19 of the 22 patients using the Kainos Intact assay (sensitivity 86%). In the 13 patients with confirmed tumors, the sensitivity was higher with all assays. Of course, the presence of normal FGF-23 levels in some patients with putative TIO suggests that FGF-23 is not responsible for the hypophosphatemia in all affected patients.

In contrast to these observations, patients with TIO secondary to hematogenous malignancy manifest abnormalities of the syndrome due to a distinctly different mechanism. In these subjects the nephropathy induced with light-chain proteinuria or other immunoglobulin derivatives results in the decreased renal tubular reabsorption of phosphate characteristic of the disease. Thus, light-chain nephropathy must be considered a possible mechanism for the TIO syndrome.

Treatment

The first and foremost treatment of TIO is complete resection of the tumor. However, recurrence of mesenchymal tumors, such as giant-cell tumors of bone, or inability to resect certain malignancies completely, such as prostate carcinoma, has resulted in the development of alternative therapeutic intervention for the syndrome. In this regard, administration of calcitriol alone or in combination with phosphorus supplementation has served as effective therapy for TIO. Doses of calcitriol required range from 1.5 to 3.0 μg/day, whereas those of phosphorus are 2 to 4 g/day. Although little information is available regarding the long-term consequences of such treatment, the high doses of medicine required raise the possibility that nephrolithiasis, nephrocalcinosis, and hypercalcemia may frequently complicate the therapeutic course. Indeed, hypercalcemia secondary to parathyroid hyperfunction has been documented in at least five treated subjects. All of these patients received phosphorus as part of a combination regimen, which may have stimulated parathyroid hormone secretion and led to parathyroid autonomy. Thus, a careful assessment of parathyroid function, serum and urinary calcium, and renal function is essential to ensure safe and efficacious therapy.

In an effort to diminish the complications of high-dose calcitriol and phosphorus therapy, addition of cinacalcet to the therapeutic regimen has been tested (Geller *et al.*, 2007). The resultant decrease of serum PTH resulted in an increase of renal phosphate reabsorption and serum phosphorus and allowed for a decrease in phosphate supplementation to a dose that was more tolerable. Moreover, one such treated patient exhibited significant bone healing, indicated by resolution of activity on a bone scan and lack of osteomalacia as assessed by histomorphometry.

Dent's Disease (X-Linked Recessive Hypophosphatemic Rickets)

In the past several decades, multiple syndromes have been described that are characterized by various combinations of renal proximal tubular dysfunction (including renal phosphate wasting), proteinuria, hypercalciuria, nephrocalcinosis, nephrolithiasis, renal failure, and rickets; these disorders, referred to as Dent's disease, include X-linked recessive hypophosphatemic rickets (see Table III), X-linked recessive nephrolithiasis with renal failure, and low-molecular-weight proteinuria with nephrocalcinosis. The spectrum of phenotypic features in these diseases is remarkably similar, except for differences in the severity of bone deformities and renal impairment. The observation that all of these syndromes are caused by mutations affecting a chloride channel clarifies the interrelationship between them and establishes that they are variants of a single disease (Scheinman, 1998).

Urinary loss of low-molecular-weight proteins is the most consistent abnormality in the disease, present in all affected males and in almost all female carriers of the disorder (Wrong *et al.*, 1994; Reinhart *et al.*, 1995). Other signs of impaired solute reabsorption in the proximal tubule, such as renal glycosuria, aminoaciduria, and phosphate wasting, are variable and often intermittent. Hypercalciuria is an early and common feature, whereas hypokalemia occurs in some patients. Urinary acidification is normal in over 80% of affected subjects, and when it is abnormal, the defect has been attributed to hypercalciuria or nephrocalcinosis (Wrong *et al.*, 1994; Reinhart *et al.*, 1995; Buckalew *et al.*, 1974). The disease apparently does not recur after kidney transplantation (Scheinman, 1998).

These phenotypic features indicate the presence of proximal tubular dysfunction but do not suggest its pathophysiologic basis. The gene causing Dent's disease, *CLCN5*, encodes the chloride/proton antiporter, CLC-5, which is expressed predominantly in the kidney and in particular the proximal tubule, the thick ascending limb of Henle, and the alpha intercalated cells of the collecting duct (Scheinman, 1998; Lloyd *et al.*, 1996; Hoopes *et al.*, 1998; Igarashi *et al.*, 1998). Mapping studies have established linkage of this gene to the short arm of the X chromosome (Xp11.22; Scheinman *et al.*, 1997). The gene is a member of a family of genes encoding voltage-gated chloride channels and is critical for acidification in the endosomes that participate in solute reabsorption and membrane recycling in the proximal tubule. CLC-5 also alters membrane trafficking via the receptor-mediated-endocytic pathway that involves megalin and cubulin. CLC-5 mutations associated with Dent's disease impair chloride flow and likely lead to impaired acidification of the endosomal lumen, and thereby disruption of endosome trafficking to the apical surface, resulting in abnormal solute reabsorption by the renal tubule.

It is not clear how this process leads to the increased intestinal calcium absorption and high serum 1,25$(OH)_2$D levels in this disorder (Reinhart *et al.*, 1995), because the 25(OH)D-1α-hydroxylase that catalyzes calcitriol formation is located in the mitochondria of proximal tubular cells, whereas C1C-5 is expressed in the thick ascending limb of Henle's loop (Devuyst *et al.*, 1999), a major site of renal calcium reabsorption. The role of this channel in the reabsorption of calcium in the thick ascending limb remains unknown.

Fanconi Syndrome

Rickets and osteomalacia are frequently associated with Fanconi syndrome, a disorder characterized by phosphaturia and consequent hypophosphatemia, aminoaciduria, renal glycosuria, albuminuria, and proximal renal tubular acidosis (De Toni, 1933; McCune *et al.*, 1943; Brewer, 1985; Chan and Alon, 1985; Chesney, 1990). Damage to the renal proximal tubule, secondary to genetic disease (see Table II) or environmental toxins, represents the common underlying mechanism of this disease. Resultant dysfunction results in renal wasting of those substances primarily reabsorbed at the proximal tubule. The associated bone disease in this disorder is likely secondary to hypophosphatemia and/or acidosis, abnormalities that occur in association with aberrantly (Fanconi syndrome, type I) or normally regulated (Fanconi syndrome, type II) vitamin D metabolism.

Type I

The type I disease resembles in many respects the more common genetic disease, X-linked hypophosphatemic rickets (see Table III). In this regard, the occurrence of abnormal bone mineralization appears dependent on the prevailing renal phosphate wasting and resultant hypophosphatemia. Indeed, subtypes of the disease in which isolated wasting of amino acids, glucose, or potassium occur are not associated with rickets and/or osteomalacia. Further, in the majority of patients studied, affected subjects exhibit abnormal vitamin D metabolism, characterized by serum 1,25$(OH)_2$D levels that are overtly decreased or abnormally low relative to the prevailing serum phosphorus concentration (Chesney *et al.*, 1984). Although the aberrantly regulated calcitriol biosynthesis may be due to the abnormal renal phosphate transport, proximal tubule damage and acidosis may play important roles.

A notable difference between this syndrome and XLH is a common prevailing acidosis, which may contribute to the bone disease. In this regard, several studies indicate that acidosis may exert multiple deleterious effects on bone. Such negative sequelae may be related to the loss of bone calcium that occurs due to calcium release for use in buffering. Alternatively, several investigators have reported that acidosis may impair bone mineralization secondary to the direct inhibition of renal 25(OH)D-1α-hydroxylase activity. Others dispute these findings and claim that acidosis does not cause rickets or osteomalacia in the absence of hypophosphatemia. Most likely, hypophosphatemia and abnormally regulated vitamin D metabolism are the primary factors underlying rickets and osteomalacia in this form of the disease.

Type II

Tieder *et al.* (1988) have described two siblings (from a consanguineous mating) who presented with classic characteristics of Fanconi syndrome, including renal phosphate wasting, glycosuria, generalized aminoaciduria, and increased urinary uric acid excretion. However, these patients had appropriately elevated (relative to the decreased serum phosphorus concentration) serum 1,25$(OH)_2$D levels and consequent hypercalciuria (see Table III). Moreover, treatment with phosphate reduced the serum calcitriol in these patients into the normal range and normalized the urinary calcium excretion. In many regards, this syndrome resembles HHRH and represents a variant of Fanconi syndrome, referred to as type II disease. The bone disease in affected subjects is likely due to the effects of hypophosphatemia. In any case, the existence of this variant form of disease is probably the result of renal damage to a unique segment of the proximal tubule. Further studies will be necessary to confirm this possibility.

Treatment

Ideal treatment of the bone disease in this disorder is correction of the pathophysiological defect influencing proximal renal tubular function. In many cases, however, the primary abnormality remains unknown. Moreover, efforts to decrease tissue levels of causal toxic metabolites by dietary (such as in fructose intolerance) or pharmacological means (such as in cystinosis and Wilson's syndrome) have met with variable success. Indeed, no evidence exists that indicates whether the proximal tubule damage is reversible on relief of an acute toxicity. Thus, for the most part, therapy of this disorder must be directed at raising the serum phosphorus concentration, replacing calcitriol (in type I disease) and reversing an associated acidosis. However, use of phosphorus and calcitriol in this disease has been limited. In general, such replacement therapy leads to substantial improvement or resolution of the bone disease (Schneider and Schulman, 1983). Unfortunately, growth and developmental abnormalities, more likely associated with the underlying genetic disease, remain substantially impaired. More efficacious therapy, therefore, is dependent on future research into the causes of the multiple disorders that cause this syndrome.

Tumoral Calcinosis

Tumoral calcinosis, also referred to as hyperphosphatemic familial tumoral calcinosis, is a rare genetic disease characterized by periarticular cystic and solid tumorous calcifications. Biochemical markers of the disorder include hyperphosphatemia and a normal or an elevated serum 1,25$(OH)_2$D concentration (see Table III). Using these criteria, evidence has been presented for autosomal recessive inheritance of this syndrome. However, an abnormality of dentition, marked by short bulbous roots, pulp stones, and radicular dentin deposited in swirls, is a phenotypic marker of the disease that is variably expressed (Lyles *et al.*, 1985). Thus, this disorder may have multiple formes frustes that could complicate genetic analysis. Indeed, using the dental lesion, as well as the more classic biochemical and clinical hallmarks of the disease, an autosomal dominant pattern of transmission has been documented.

The hyperphosphatemia characteristic of the disease results from an increase in capacity of renal tubular phosphate reabsorption. Hypocalcemia is not a consequence of this abnormality, however, and the serum parathyroid hormone concentration is normal. Moreover, the phosphaturic and urinary cAMP responses to parathyroid hormone are not disturbed. Thus, the defect does not represent renal insensitivity to parathyroid hormone, or hypoparathyroidism. Rather, the genetic basis for the disease has recently been revealed with inactivating mutations encoding the *FGF-23*, UDP-*N*-galactosamine polypeptide *N*-acetylgalactosaminyl transferase 3 (*GALNT3*), or Klotho genes.

- The mutations of *FGF-23* lead to destabilized protein and resultant increased intracellular proteolysis of FGF-23, most likely due to furin-like proteases (Araya *et al.*, 2005). This process increases circulating levels of the bioinactive C-terminal fragment of FGF-23 and markedly decreases concentration of the bioactive intact molecule. Such absence of FGF-23 bioactivity results in the enhanced renal phosphate reabsorption and hyperphosphatemia observed in affected patients. Indeed, several investigators have discovered that FGF-23 gene knockout mice develop hyperphosphatemia and severe soft-tissue calcification as expected.
- GALNT3 is a Golgi-associated enzyme that initiates *O*-glycosylation of mature polypeptides This enzyme

selectively *O*-glycosylates a furin-like convertase recognition sequence in FGF-23, thereby impairing the normal function of this protein (Ichikawa *et al.,* 2005; Specktor *et al.,* 2006). This process decreases circulating intact, bioactive FGF-23 as described earlier for the destabilizing mutations of FGF-23.

- Recent studies revealed that FGF-23 requires an additional cofactor, klotho (KL), to bind and signal through its cognate FGF receptors. Not surprisingly, therefore, diminished *KL* expression in mice results in a phenotype characterized by severe hyperphosphatemia, increased serum 1,25$(OH)_2$D levels, and ectopic vascular and soft-tissue calcifications. Indeed the *KL*-deficient phenotype broadly overlaps with the phenotype of *Fgf-23*-null mice, reaffirming the functional crosstalk between KL and FGF-23 and underscoring the observed interactions between KL, FGF-23 and its cognate FGF receptors. In accord, Ichikawa *et al.* (2007)have recently reported a point mutation in the *KL* gene, which attenuates the ability of KL to support FGF-23 signaling and thereby causes the deranged phosphate, vitamin D and calcium homeostasis, characteristic of tumoral calcinosis.

Clearly, therefore, the basis of the disease is diminished FGF-23 function, which results in enhanced renal phosphate reabsorption and increased calcitriol production. Undoubtedly, calcific tumors result from the elevated calcium–phosphorus product. The observation that long-term phosphorus depletion alone or in association with administration of acetazolamide, a phosphaturic agent, leads to resolution of the tumor masses supports the assumption that an increased calcium–phosphorus product underlies the calcific tumor formation.

An acquired form of this disease is rarely seen in patients with end-stage renal failure. Affected patients manifest hyperphosphatemia in association with either (1) an inappropriately elevated calcitriol level for the degree of renal failure, hyperparathyroidism, or hyperphosphatemia or (2) long-term treatment with calcium carbonate, calcitriol, or high-calcium-content dialysates. Calcific tumors again likely result from an elevated calcium–phosphorus product. Indeed, complete remission of the tumors occurs on treatment with vinpocetine, a mineral scavenger drug, dialysis with low-calcium-content dialysate, and renal transplantation

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Chapter 24

Magnesium Homeostasis

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INTRODUCTION

Magnesium (Mg) is the fourth most abundant cation and the second most abundant intracellular cation in vertebrates. Mg is involved in numerous biological processes and is essential for life (Rude and Shils, 2006). This mineral has evolved to become a required cofactor in literally hundreds of enzyme systems (Laires *et al.*, 2004; Maguire and Cowan, 2002; Romani, 2006; Rude and Shils, 2006). Examples of the physiological role of Mg are shown in (Table I). Mg may be required for enzyme substrate formation. For example, enzymes that utilize ATP do so as the metal chelate, MgATP. Free Mg^{2+} also acts as an allosteric activator of numerous enzyme systems, as well as playing a role in ion channels and for membrane stabilization. Mg is therefore critical for a great number of cellular functions, including oxidative phosphorylation, glycolysis, DNA transcription, and protein synthesis.

TABLE I Examples of the Physiological Role of Magnesium

I. Enzyme substrate (ATP Mg, GTP Mg)
A. ATPase or GTPase (Na^+, K^+-ATPase, Ca^{2+}-ATPase)
B. Cyclases (adenylate cyclase, guanylate cyclase)
C. Kinase (hexokinase, creatine kinase, protein kinase)
II. Direct enzyme activation
A. Adenylate cyclase
B. Phospholipase C
C. Na^+, K^+-ATPase
D. Ca^{2+}-ATPase
E. K^+, H^+-ATPase
F. G proteins
G. 5′-Nucleotidase
H. Creatine kinase
I. Phosphofructokinase
J. 5-Phosphoribosyl-pyrophosphate synthetase
K. Lipoprotein lipase
III. Influence membrane properties
A. K^+ channels
B. Ca^{2+} channels
C. Nerve conduction

MAGNESIUM METABOLISM

The normal adult total body Mg content is approximately 25 grams (2000 mEq or 1 mol) of which 50% to 60% resides in bone (Elin, 1987; Wallach, 1988). Mg constitutes 0.5% to 1% of bone ash (200 mmol/kg ash weight). One-third of skeletal Mg is surface limited and exchangeable, and this fraction may serve as a reservoir for maintaining a normal extracellular Mg concentration (Wallach, 1988; Rude and Shils, 2006; Laires *et al.*, 2004). The remainder of Mg in bone is an integral component of the hydroxyapatite lattice, which may be released during bone resorption. The rest of body Mg is mainly intracellular. The Mg content of soft tissues varies between 6 and 25 mEq/kg wet weight (Elin, 1987; Rude and Shils, 2006). In general, the higher the metabolic activity of the cell, the higher the Mg content. The concentration of Mg within cells is in the order of 5–20 mmol/liter, of which 1% to 5% is ionized or free (Elin, 1987; Romani, 2006; Rude and Shils, 2006). The distribution of Mg in the body is shown in (Table II) and (Fig. 1).

Extracellular Mg accounts for about 1% of total body Mg. Mg concentration or content may be reported as mEq/liter, mg/dl, or mmol/liter. Values reported as mEq/liter can be converted to mg/dl by multiplying by 1.2 and to mmol/liter by dividing by 0.5. The normal serum Mg concentration is 1.5–1.9 mEq/liter (0.7–1.0 mmol/liter) (Elin, 1987; Rude and Shils, 2006). About 70% to 75% of plasma Mg is ultrafilterable, of which the major portion (55% of total serum Mg) is ionized or free, and the remainder is complexed to citrate, phosphate, and other anions as represented schematically in (Fig. 2). The remainder is protein bound; 25% of total serum Mg is bound to albumin and 8% to globulins.

Intestinal Mg Absorption

Mg homeostasis is dependent on the amount of Mg ingested, efficiency of Mg intestinal absorption, and renal reabsorption and excretion. Intestinal Mg absorption is proportional

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to the amount ingested (Fine *et al.,* 1991; Schweigel and Martens, 2000). The mechanism(s) for intestinal Mg absorption includes passive diffusion and active transport (Fine *et al.,* 1991; Kayne and Lee, 1993; Bijvelds *et al.,* 1998; Schweigel and Martens, 2000). Shown in (Fig. 3) is a schematic model (Schlingmann *et al.*, 2004) as suggested by rat (Ross, 1962) and human studies (Fine *et al.,* 1991; Kayne and Lee, 1993), which may account for the higher fractional intestinal Mg absorption at low dietary Mg intakes. Others have concluded that intestinal Mg absorption in humans increases linearly with Mg intake (for review, see Schweigel and Martens, 2000). The report of a patient with primary hypomagnesemia who was shown to malabsorb Mg during low Mg concentration in the intestine suggested an active transport process (Milla *et al.,* 1979). A familial defect in intestinal Mg absorption has been mapped to chromosome 9q22 (Walder *et al.,* 1997; Schlingmann *et al.*, 2002; Schlingmann *et al.*, 2004). This gene encodes for TMP6 in the intestinal epithelium for an Mg permeable channel (Schlingmann and Gudermann, 2005), which is evidence for at least one active Mg transport process. TMPR7 has also been demonstrated to play a role in Mg transport (Schlingmann and Gudermann, 2005; Chubanov *et al.,* 2004). Under normal dietary conditions in healthy individuals, approximately 30% to 50% of ingested Mg is absorbed (Brannan *et al.,* 1976; Hardwick *et al.,* 1990; Fine *et al.,* 1991; Kayne and Lee, 1993; Schweigel and Martens, 2000). Mg is absorbed along the entire intestinal tract, including the large and small bowel, but the sites of maximal Mg absorption appear to be the ilium and distal jejunum (Hardwick *et al.,* 1990; Fine *et al.,* 1991; Kayne and Lee, 1993; Schweigel and Martens, 2000).

The recommended daily allowance for Mg is 420 mg per day for adult males and 320 mg per day for adult females

TABLE II Distribution of Magnesium in Adult Humans[a]

Tissue	Body mass, kg (wet wt)	Mg concentration, mmol/kg (wet wt)	Mg content (mmol)	% of total body Mg
Serum	3.0	0.85	2.6	0.3
Erythrocyte	2.0	2.5	5.0	0.5
Soft tissue	22.7	8.5	193.0	19.3
Muscle	30.0	9.0	270.0	27.0
Bone	12.3	43.2	530.1	52.9
Total	70.0		1000.7	100.0

[a] *Adapted from* Elin (1987).

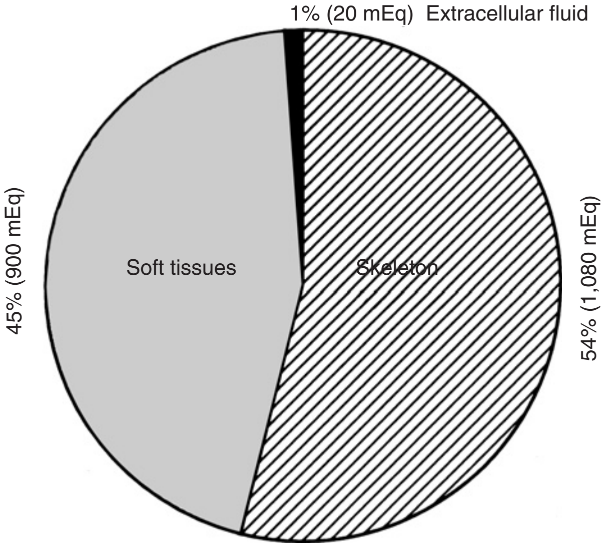

FIGURE 1 Distribution of magnesium in the body.

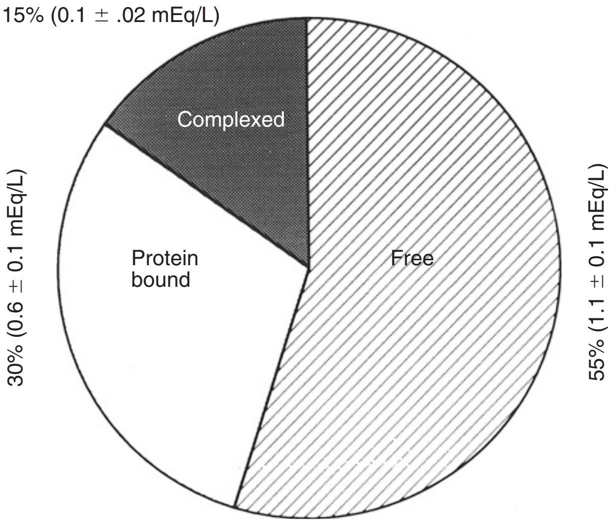

FIGURE 2 Physicochemical states of magnesium in normal plasma.

(Institute of Medicine, 1997). The dietary Mg intake in Western culture, however, appears to fall below that in a large section of the population across all ages, ranging from approximately 150 to 350 mg per day, suggesting that occult Mg depletion may be relatively prevalent (Morgan *et al.,* 1985; Marier, 1986). A recent study, however, has suggested that the dietary Mg requirement may be lower (Hunt and Johnson, 2006). The major sources of Mg are nuts, cereals, green leafy vegetables, and meats.

A principal factor that regulates intestinal Mg transport has not been described. Vitamin D, as well as its metabolites 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] have been observed in some studies to enhance intestinal Mg absorption but to a much lesser extent than they do calcium absorption (Brannan *et al.,* 1976; Hodgkinson *et al.,* 1979; Krejs *et al.,* 1983). Although net intestinal calcium absorption in humans correlates with plasma $1,25(OH)_2D$ concentrations, Mg does not (Wilz *et al.,* 1979). A low Mg diet has been shown to increase intestinal calbindin-D_{9k}, suggesting that this vitamin D-dependent, calcium-binding protein may play a role in intestinal Mg absorption (Hemmingsen *et al.,* 1994).

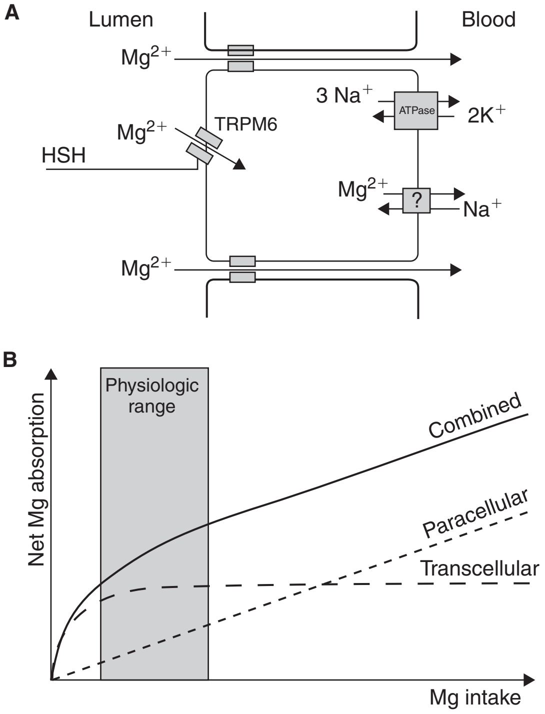

FIGURE 3 **A**. Schematic model of intestinal magnesium absorption via two independent pathways: passive absorption via the paracellular pathway and active transcellular transport consisting of an apical entry through a putative magnesium channel and a basolateral exit mediated by a putative sodium-coupled exchange. **B**. Kinetics of human intestinal magnesium absorption. Paracellular transport linearly rising with intraluminal concentration (dotted line) and saturable active trancellular transport (dashed line) together yield a curvilinear function for net magnesium absorption (solid line). (Schlingmann *et al.,* 2004, with permission).

Bioavailability of Mg may also be a factor in Mg intestinal absorption as other nutrients may affect Mg absorption. Although dietary calcium has been reported to both decrease and increase Mg absorption, human studies have shown no effect (Brannan *et al.,* 1976; Fine *et al.,* 1991). The presence of excessive amounts of substances such as free fatty acids, phytate, oxalate, polyphosphates, and fiber may bind Mg and impair absorption (Seelig, 1981; Franz, 1989).

Renal Mg Handling

The kidney is the principal organ involved in Mg homeostasis (Cole and Quamme, 2000; Satoh and Romero, 2002). During Mg deprivation in normal subjects, the kidney conserves Mg avidly and less than 1–2 mEq is excreted in the urine per day (Barnes *et al.,* 1958). Conversely, when excess Mg is taken, it is excreted into the urine rapidly (Heaton and Parson, 1961). The renal handling of Mg in humans is a filtration–reabsorption process; there appears to be no tubular secretion of Mg. Micropuncture studies of the nephron in several mammalian species have indicated that Mg is absorbed in the proximal tubule, thick ascending limb of Henle, and distal convoluted tubule (Quamme and De Rouffignac, 2000; Cole and Quamme, 2000), as illustrated in (Fig. 4).

About 2,400 mg of Mg is filtered daily through the normal adult. Of this, approximately 15% to 20% of filtered Mg is reabsorbed in the proximal convoluted tubule. Current data suggest that Mg transport in this segment is reabsorbed passively through the paracellular pathway (Cole and Quamme, 2000; Satoh and Romero, 2002). The majority, approximately 65% to 75%, of filtered Mg is reclaimed in the loop of Henle with the major site at the cortical thick ascending limb. Magnesium transport in this segment appears to be dependent on the transepithelial potential generated by NaCl absorption (Cole and Quamme, 2000; Satoh and Romero, 2002). Mutation of the CLDN16 gene, which encodes for a defect in paracellin-1 in the thick asending limb of Henle, results in renal Mg and calcium wasting (Schlingmann *et al.,* 2002; Konrad *et al.,* 2004). Micropuncture studies have also demonstrated that hypermagnesemia or hypercalcemia will decrease Mg reabsorption in this segment independent of NaCl transport (Cole and Quamme, 2000). Studies suggest that the concentration of calcium and/or Mg in the extracellular fluid may regulate absorption of Mg in the thick ascending limb of Henle by activation of the Ca^{2+}-sensing receptor in this segment of the nephron (Brown and Hebert, 1996; Cole and Quamme, 2000). Inactivating and activating mutations of the Ca^{2+}-sensing receptor will result in hypermagnesemia and hypercalcemia with decreased urine excretion of both cations or hypomagnesemia and hypocalcemia with increased excretion of both cations, respectively (Konrad *et al.,* 2004). Approximately 5% to 10% of Mg is reclaimed in the distal tubule where reabsorption is trancellular and active in

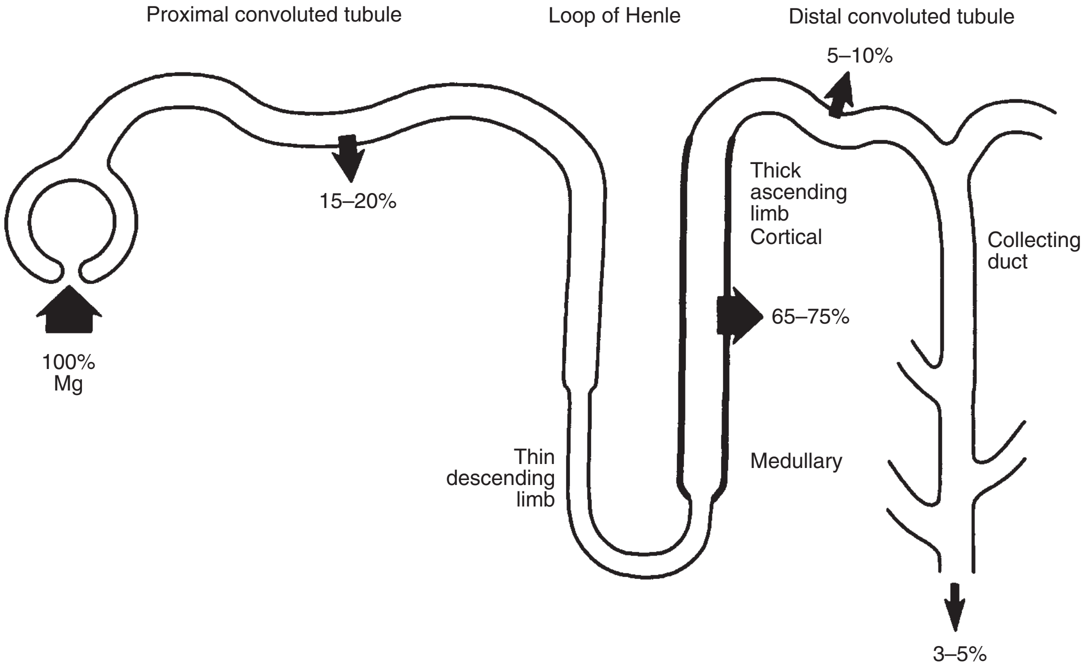

FIGURE 4 Summary of the tubular handling of magnesium. Schematic illustration of the cellular transport of magnesium within the thick ascending limb of the loop of Henle (Cole and Quamme, 2000).

nature (Cole and Quamme, 2000). A genetic defect on chromosome 11q23, gene FXYD2 results in a defect in Na-K-ATPase resulting in renal Mg wasting (Konrad *et al.*, 2004; Meij *et al.*, 2002). TRPM6, TRMP7, and TRMP9, members of the TRMP family of cation channels, may also affect Mg transport as genetic defects of these proteins have resulted in renal Mg wasting (Schmitz *et al.*, 2003; Schlingmann *et al.*, 2002; Schlingmann *et al.*, 2004). It is speculated that transport at this site may be regulated hormonally and serve to finely regulate Mg homeostasis.

Micropuncture studies performed during a time in which the concentration of Mg was increased gradually, in either the tubular lumen or in the extracellular fluid, have failed to demonstrate a tubular maximum for Mg (TmMg) in the proximal tubule (Quamme and De Rouffignac, 2000). The rate of Mg reabsorption is dependent on the concentration of Mg in the tubule lumen. Similarly, a TmMg was not reached in the loop of Henle during a graduated increase in the luminal-filtered Mg load. Hypermagnesemia, as discussed earlier, however, results in a marked depression of Mg resorption in this segment. *In vivo* studies in animals and humans, however, have demonstrated a TmMg that probably reflects a composite of tubular reabsorption processes, as shown in Fig. 5 (Rude *et al.*, 1980; Rude and Ryzen, 1986).

During Mg deprivation, Mg virtually disappears from the urine (Barnes *et al.*, 1958). Despite the close regulation of Mg by the kidney, there has been no hormone or factor described that is responsible for renal Mg homeostasis. Micropuncture studies have shown that PTH changes the potential difference in the cortical thick ascending limb and increases Mg reabsorption (Quamme and De Rouffignac, 2000; Cole and Quamme, 2000). When given in large doses in humans or other species, PTH decreases urinary Mg excretion (Bethune *et al.*, 1968; Massry *et al.*, 1969). However, patients with either primary hyperparathyroidism or hypoparathyroidism usually have a normal serum Mg concentration and a normal TmMg, suggesting that PTH is not an important physiological regulator of Mg homeostasis (Rude *et al.*, 1980). Glucagon, calcitonin, and ADH also affect Mg transport in the loop of Henle in a manner similar to PTH (Quamme and De Rouffignac, 2000; Cole and Quamme, 2000); the physiological relevance of these actions is unknown. Little is known about the effect of vitamin D on renal Mg handling. An overall view of Mg metabolism is shown in (Fig. 6).

Intracellular Mg

Within the cell, Mg is compartmentalized and most of it is bound to proteins and negatively charged molecules such as ATP, ADP, RNA, and DNA; in the cytoplasm, about 80% of Mg is complexed with ATP (Gupta and Moore, 1980; Frausto da Silva and Williams, 1991; Romani, 2006). Significant amounts of Mg are found in the nucleus, mitochondria, and endoplasmic and sarcoplasmic reticulum as well as in the cytoplasm (Gunther, 1986; Romani, 2006).

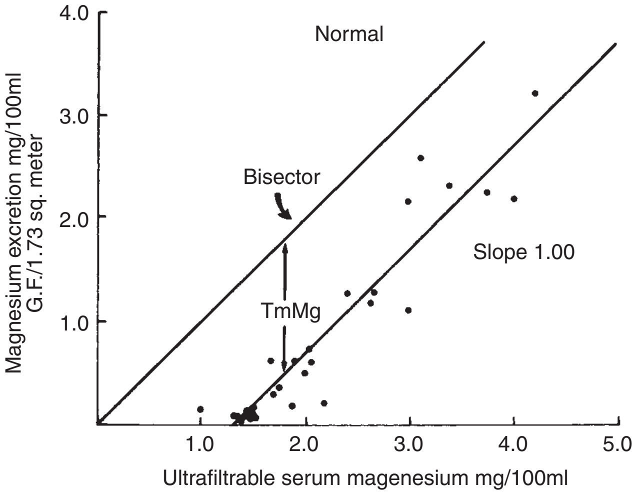

FIGURE 5 Urinary magnesium excretion is plotted against ultrafiltrable serum Mg in normal subjects before and during magnesium infusion. Data are related to a bisector that corresponds to the theoretical value of magnesium excretion if no magnesium were reabsorbed (Rude *et al.*, 1980).

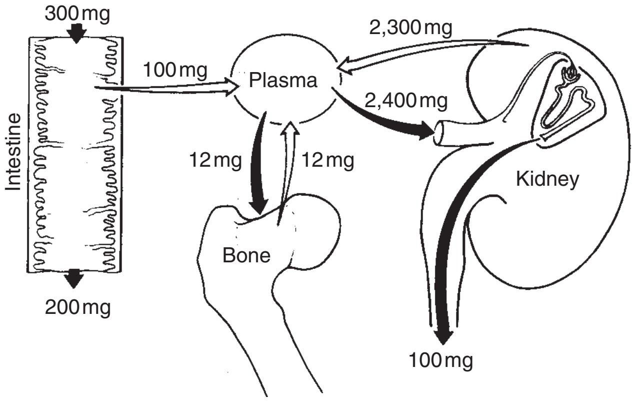

FIGURE 6 Schematic representation of magnesium metabolism.

Total cell Mg concentration has been reported to range between 5 and 20 mM (Gunther, 1986; Romani, 2006). The concentration of free ionized Mg^{2+}, which has been measured in the cytoplasm of mammalian cells, has ranged from 0.2 to 1.0 mM, depending on cell type and means of measurement (Raju *et al.*, 1989; London, 1991; Romani and Scarpa, 1992; Romani, 2006). It constitutes 1% to 5% of the total cellular Mg. The Mg^{2+} concentration in the cell cytoplasm is maintained relatively constant even when the Mg^{2+} concentration in the extracellular fluid is experimentally varied to either high or low nonphysiological levels (Dai and Quamme, 1991; Quamme *et al.*, 1993; Romani *et al.*, 1993). The relative constancy of the Mg^{2+} in the intracellular milieu is attributed to the limited permeability of the plasma membrane to Mg and to the operation of specific Mg transport systems, which regulate the rates at which Mg is taken up by cells or extruded from cells (Flatman, 1984; Romani, 2006; Murphy, 2000). Although the concentration differential between the cytoplasm and the extracellular fluid for Mg^{2+} is minimal, Mg^{2+} enters cells down an electrochemical gradient because of the relative electronegativity of the cell interior. Maintenance of the normal intracellular concentrations of Mg^{2+} requires that Mg be actively transported out of the cell (Murphy, 2000; Romani, 2006).

Studies in mammalian tissues and isolated cells suggest the presence of specific Mg transport systems. Early *in vivo* studies, using the radioactive isotope ^{28}Mg, suggested that tissues vary with respect to the rates at which Mg exchange occurs and the percentage of total Mg that is readily exchangeable (Rogers and Mahan, 1959). The rate of Mg exchange in heart, liver, and kidney exceeded that in skeletal muscle, red blood cells, brain, and testis (Romani, 2006). These studies do show that, albeit slow in some tissues, there is a continuous equilibration of Mg between cells and the extracellular fluid. Buffering of intracellular Mg by ATP and other negatively charged molecules may also play a role in maintaining a constant intracellular Mg concentration (Romani, 2006). An increased cellular Mg content has been reported for rapidly proliferating cells, indicating a possible relationship between the metabolic state of a cell and the relative rates of Mg transport into and out of cells (Cameron *et al.*, 1980).

Mg transport out of cells appears to require the presence of carrier-mediated transport systems, possibly regulated by the concentration of Mg^{2+} within the cell (Romani, 2006; Gunther, 1993; Gunther, 2006). The efflux of Mg from the cell is coupled to Na transport and requires energy (Romani, 2006; Gunther, 1993; Murphy, 2000; Gunther, 2006). Muscle tissue, which is incubated in isotonic sucrose, a low sodium buffer, or in the presence of ouabain as a metabolic inhibitor, has been shown to accumulate large amounts of Mg. These studies also suggest that the efflux of Mg from the cell is coupled with the movement of sodium down its electrochemical gradient into the cell. Maintenance of this process would require the subsequent extrusion of sodium by the Na^+, K^+-ATPase. There is also evidence for a Na-independent efflux of Mg, however (Gunther, 1993, 2006). Mg influx appears to be linked to Na and HCO_3 transport, but by a different mechanism than efflux (Gunther, 1993; Gunther and Hollriegl, 1993; Romani, 2006). The molecular characteristics of the Mg transport proteins have not been described. Studies in prokaryotes, however, have identified three separate transport proteins for Mg (Smith and Maguire, 1993). TRPM6, TRPM7, and TRMP9, members of the TRPM family of cation channels, have also been demonstrated to influence Mg transport across the plasma membrane (Schmitz *et al.*, 2003; Schlingmann *et al.*, 2002, Schlingmann *et al.*, 2004).

Mg transport in mammalian cells is influenced by hormonal and pharmacological factors. Mg^{2+} efflux from isolated perfused rat heart and liver (Romani and Scarpa, 1990; Gunther *et al.*, 1991; Gunther, 1993) or thymocytes

(Gunther and Vormann, 1990) is stimulated after short-term acute exposure to β-agonists and permeant cAMP. Because intracellular Mg^{2+} does not change, a redistribution from the mitochondria was suggested, as cAMP can induce Mg^{2+} release from this compartment (Romani and Scarpa, 1992) or by altered buffering of Mg within the cell (Murphy, 2000). In contrast, Mg^{2+} influx was stimulated by β-agonists after a more prolonged exposure in hepatocytes, as well as in adipocytes and vascular smooth muscle, presumably mediated by protein kinase A (Zama and Towns, 1986; Ziegler *et al.*, 1992; Gunther, 1993; Romani *et al.*, 1993). However, the rate of Mg uptake by the mouse lymphoma S49 cell line is inhibited by β-adrenergic agents (Maguire, 1984). Activation of protein kinase C by diacyl-glycerol or by phorbol esters also stimulates Mg^{2+} influx and does not alter efflux (Grubbs and Maguire, 1986; Romani, 2006). Mg^{2+} extrusion is also elicited by ∝1-adrenergic receptors in a cAMP-independent manner (Fagen and Romani, 2001; Romani, 2006).

Growth factors may also influence Mg^{2+} uptake by cells. Epidermal growth factor has been shown to increase Mg transport into a vascular smooth muscle cell line (Grubbs, 1991). Insulin and dextrose were found to increase ^{28}Mg uptake by a number of tissues, including skeletal and cardiac muscle, in which total cellular Mg content increased as well (Lostroh and Krahl, 1973). Increased amounts of total intracellular Mg following treatment with insulin *in vitro* have been reported in uterine smooth muscle and chicken embryo fibroblasts (Aikawa, 1960; Sanui and Rubin, 1978). An insulin-induced transport of Mg into cells could be one factor responsible for the fall in the serum Mg concentration observed during insulin therapy of diabetic ketoacidosis (Kumar *et al.*, 1978). The effect of insulin on total cellular Mg may differ from its effects on intracellular-free Mg^{2+}. Measurements of intracellular-free Mg^{2+} in frog skeletal muscle failed to show an effect of insulin (Gupta and Moore, 1980); however, other studies demonstrated that insulin increases Mg^{2+} in human red blood cells, platelets, lymphocytes, and heart (Hwang *et al.*, 1993; Barbagallo *et al.*, 1993; Hua *et al.*, 1995; Romani *et al.*, 2000).

It is hypothesized that this hormonally regulated Mg uptake system controls intracellular Mg^{2+} concentration in cellular subcytoplasmic compartments. The Mg^{2+} concentration in these compartments would then serve to regulate the activity of Mg-sensitive enzymes.

Risk Factors and Causes of Magnesium Deficiency

Prevalence

The many risk factors for magnesium depletion shown in (Table III) suggest that this condition is not a rare occurrence in acutely or chronically ill patients. A survey of 2,300 patients in a Veterans Administration hospital found 6.9% were hypomagnesemic; 11% of patients having routine magnesium determinations were hypomagnesemic (Whang *et al.*, 1984. Similar high rates of depletion have been reported in studies of ICU patients (Whang *et al.*, 1994; Ryzen *et al.*, 1985; Chernow *et al.*, 1989; Fiaccadori *et al.*, 1988). The true prevalence of magnesium depletion is not known, because this ion is not included in routine electrolyte testing in many clinics or hospitals (Whang *et al.*, 1994).

Gastrointestinal Disorders

Gastrointestinal disorders (Table III) may lead to magnesium depletion in various ways. The Mg content of upper intestinal tract fluids is approximately 1 mEq/L. Vomiting and nasogastric suction therefore may contribute to Mg depletion. The Mg content of diarrheal fluids is much higher (up to 15 mEq/L), and consequently Mg depletion

TABLE III Causes of Magnesium Deficiency

1. Gastrointestinal Disorders
   a. Prolonged nasogastric suction/vomiting
   b. Acute and chronic diarrhea
   c. Intestinal and biliary fistulas
   d. Malabsorption syndromes
   e. Extensive bowel resection or bypass
   f. Acute hemorrhagic pancreatitis
   g. Protein-calorie malnutrition
   h. Primary intestinal hypomagnesemia (neonatal)
2. Renal Loss
   a. Chronic parenteral fluid therapy
   b. Osmotic diuresis (glucose, urea, manitol)
   c. Hypercalcemia
   d. Alcohol
   e. Diuretics (furosemide, ethacrynic acid)
   f. Aminoglycosides
   g. Cisplatin
   h. Cyclosporin
   i. Amphotericin B
   j. Pentamidine
   k. Tacolimus
   l. Metabolic acidosis
   m. Renal disorders with Mg wasting
   n. Primary renal hypomagnesemia
3. Endocrine and Metabolic Disorders
   a. Diabetes mellitus (glycosuria)
   b. Phosphate depletion
   c. Primary hyperparathyroidism (hypercalcemia)
   d. Hypoparathyroidism (hypercalciuria, hypercalcemia owing to overtreatment with vitamin D)
   e. Primary aldosteronism
   f. Hungry bone syndrome
   g. Chronic renal disease
   h. Excessive lactatation

is common in acute and chronic diarrhea, regional enteritis, ulcerative colitis, and intestinal and biliary fistulas (Thoren, 1963; LaSala *et al.*, 1985; Nyhlin *et al.*, 1985). Malabsorption syndromes towing to nontropical sprue, radiation injury resulting from therapy for disorders such as carcinoma of the cervix, and intestinal lymphangiectasia may also result in Mg deficiency (Booth *et al.*, 1963; Goldman *et al.*, 1962; Habtezion *et al.*, 2002). Steatorrhea and resection or bypass of the small bowel, particularly the ileum, often results in intestinal Mg loss or malabsorption (Dyckner *et al.*, 1982; Van Gaal *et al.*, 1987). Lastly, acute severe pancreatitis is associated with hypomagnesemia, which may be owing to the clinical problem causing the pancreatitis, such as alcoholism, or to saponification of Mg in necrotic parapancreatic fat (Weir *et al.*, 1975; Ryzen and Rude, 1990). A primary defect in intestinal Mg absorption, which presents early in life with hypomagnesemia, hypocalcemia, and seizures, has been described as an autosomal recessive disorder linked to chromosome 9q22 as discussed earlier (Walder *et al.*, 1997; Schlingmann *et al.*, 2002; Schlingmann *et al.*, 2004), a disorder appears to be caused by mutations in TRPM6, which expresses a protein involved with active intestinal Mg transport.

Renal Disorders

Excessive excretion of Mg into the urine may be the basis of Mg depletion (Table III). Renal Mg reabsorption is proportional to tubular fluid flow as well as to sodium and calcium excretion. Therefore, chronic parenteral fluid therapy, particularly with saline, and volume expansion states such as primary aldosteronism, and hypercalciuric states may result in Mg depletion (Massry *et al.*, 1967; Coburn, 1970; McNair *et al.*, 1982). Hypercalcemia have been shown to decrease renal Mg reabsorption probably mediated by calcium binding to the calcium-sensing receptor in the thick ascending limb of Henle and decreasing transepithelial voltage and is probably the cause of renal Mg wasting and hypomagnesemia observed in many hypercalcemic states (Brown and Hebert, 1995; Cole and Quamme, 2000). Osmotic diuresis because of glucosuria will result in urinary Mg wasting (McNair *et al.*, 1982).

Many pharmaceutical drugs may cause renal Mg wasting and Mg depletion. The major site of renal Mg reabsorption is at the loop of Henle, therefore diuretics such as furosemide and ethacrynic acid have been shown to result in marked Mg wasting (Quamme, 1981). Aminoglycosides have been shown to cause a reversible renal lesion that results in hypermagnesuria and hypomagnesemia (Zaloga *et al.*, 1984; Kes and Reiner, 1990). Similarly, amphotericin B therapy has been reported to result in renal Mg wasting. Other renal Mg-wasting agents include cisplatin, cyclosporin, tacrolimus, and pentamidine (Meyer and Madias, 1994). A rising blood alcohol level has been associated with hypermagnesuria and is one factor contributing to Mg depletion in chronic alcoholism. Metabolic acidosis because of diabetic ketoacidosis, starvation, or alcoholism may also result in renal Mg wasting (Lau *et al.*, 1987).

Several renal Mg wasting disorders have been described that may be genetic or sporadic as discussed earlier in renal Mg metabolism (Konrad *et al.*, 2004; Meij *et al.*, 2002). Gitelman's syndrome (familial hypokalemia-hypomagnesemia syndrome) is an autosomal recessive disorder owing to a genetic defect of the thiazide-sensitive NaCl cotransporter gene on chromosome 16 (Simon *et al.*, 1996). Recently, mutations in the tight-junction gene Claudin 19 have been associated with renal Mg wasting and renal failure and ocular abnormalities (Konrad *et al.*, 2006). Mutations of other cation channels, TMPR6, TMPR7, and TMPR9, have also recently been associated with renal Mg wasting (Schmitz *et al.*, 2003; Schlingmann *et al.*, 2002; Schlingmann *et al.*, 2004). Other undefined genetic defects also exist (Kantorovich *et al.*, 2002).

Hypomagnesemia may accompany a number of other disorders (for review see Rude, 1996). Phosphate depletion has been shown experimentally to result in urinary Mg wasting and hypomagnesemia. Hypomagnesemia may also accompany the "hungry bone" syndrome, a phase of rapid bone mineral accretion in subjects with hyperparathyroidism or hyperthyroidism following surgical treatment. Finally, chronic renal tubular, glomerular, or interstitial diseases may be associated with renal Mg wasting. Rarely, excessive lactation may result in hypomagnesemia.

Diabetes Mellitus

Special consideration must be given to diabetes mellitus. It is the most common disorder associated with magnesium deficiency (McNair *et al.*, 1982; Rude, 1996; Song *et al.*, 2004; Lopez-Ridaura *et al.*, 2004). It is generally thought that the mechanism for magnesium depletion in diabetics is owing to renal magnesium wasting secondary to osmotic diuresis generated by hyperglycosuria. Dietary magnesium intake, however, falls below the RDA in diabetics. Magnesium deficiency has been reported to result in impaired insulin secretion as well as insulin resistance (Song *et al.*, 2004; Lopez-Ridaura *et al.*, 2004). The mechanism is unclear but may be because of abnormal glucose metabolism as magnesium is a cofactor in several enzymes in this cycle. In addition, magnesium depletion may decrease tyrosine kinase activity at the insulin receptor and magnesium may influence insulin secretion by the beta cell. Diabetics given magnesium therapy appear to have improved diabetes control. Recently, two studies have reported that the incidence of type-2 diabetes is significantly greater in people on a lower magnesium diet (Song *et al.*, 2004; Lopez-Ridaura *et al.*, 2004). Magnesium status should therefore be assessed in patients with diabetes mellitus as a vicious cycle may occur; diabetes out of control leading to magnesium loss and the subsequent magnesium deficiency resulting in impaired insulin secretion and action and worsening diabetes control.

ROLE OF MAGNESIUM IN BONE AND MINERAL HOMEOSTASIS

Because of the prevalence of Mg in both cells and bone, as well as its critical need for numerous biological processes in the body, it is not surprising that this mineral plays a profound role in bone and mineral homeostasis. Our understanding of the role of magnesium has developed principally through observations of the effect of Mg depletion in both humans and animals. Mg influences the formation and/or secretion of hormones that regulate skeletal homeostasis and the effect of these hormones on bone. Mg can also directly affect bone cell function as well as influence hydroxyapatite crystal formation and growth. These areas are discussed later and are outlined in Table IV.

Parathyroid Hormone Secretion

Calcium is the major regulator of PTH secretion. Mg, however, modulates PTH secretion in a manner similar to calcium. A number of *in vitro* and *in vivo* studies have demonstrated that acute elevations of Mg inhibit PTH secretion, whereas an acute reduction stimulates PTH secretion (Sherwood *et al.*, 1970; Cholst *et al.,* 1984; Ferment *et al.,* 1987; Toffaletti *et al.,* 1991, Rude, 1994; Rude and Shils, 2006). These data suggest that Mg could be a physiologic regulator of PTH secretion. Although early investigations indicated that Mg was equipotent to calcium in its effect on parathyroid gland function (Sherwood *et al.,* 1970), more recent studies demonstrated that Mg has approximately 30 to 50% the effect of calcium on either stimulating or inhibiting PTH secretion (Wallace and Scarpa, 1982; Ferment *et al.,* 1987; Toffaletti *et al.,* 1991; Rude, 1994). The finding in humans that a 5% (0.03 mM) decrease in serum ultrafilterable Mg did not result in any detectable change in intact serum PTH concentration while a 5.5% (.07 mM) decrease in ionized calcium resulted in a 400% increase in serum PTH supports this concept (Toffaletti *et al.,* 1991).

The inhibitory effects of Mg on PTH secretion may be dependent on the extracellular calcium concentration (Brown *et al.,* 1984). At physiological calcium and Mg concentrations, these divalent cations were found to be relatively equipotent at inhibiting PTH secretion from dispersed bovine parathyroid cells (Brown *et al.,* 1984). At a low calcium concentration (0.5 mM), however, a threefold greater Mg concentration was required for similar PTH inhibition. Altering the Mg concentration did not diminish the ability of calcium to inhibit PTH secretion. Differences

TABLE IV Effect of Mg Depletion on Bone and Mineral Metabolism

	Effect	Potential mechanism(s)
1.	Decreased PTH secretion	Altered phosphoinositol activity Decreased adenylate cyclase activity
2.	Decreased PTH action	Decreased adenylate cyclase activity Altered phosphoinositol activity
3.	Decreased serum $1,25(OH)_2D$	Decreased serum PTH Renal PTH resistance (decrease in 1α-hydroxylase activity)
4.	Impaired vitamin D metabolism and action	Decreased $1,25(OH)_2D$ formation Decreased intestinal epithelial cell and osteoblast activity Skeletal resistance to vitamin D
5.	Impaired bone growth/osteoporosis	Decreased PTH and $1,25(OH)_2D$ formation and action Decreased effect of insulin and IGF-1 Direct effect to decrease bone cell activity
6.	Increased bone resorption/osteoporosis	Increased cytokine production Increased substance P, TNFα, IL-1, IL-6 Increased RANKL, decreased OPG
7.	Altered hydroxyapatite crystal formation	Impaired calcium binding to hydroxyapatite Directly alter crystal growth

have also been noted in the effect of Mg and calcium on the biosynthesis of PTH *in vitro*. Changes in calcium over the range of 0 to 3.0 mM resulted in increased PTH synthesis (Hamilton *et al.*, 1971; Lee and Roth, 1975), whereas changes in Mg over the range of 0 to 1.7 mM had no effect.

The effect of Mg on PTH secretion appears to act through the Ca^{2+}-sensing receptor, which mediates the control of extracellular calcium on PTH secretion (Brown *et al.*, 1993). Mg^{2+} was shown to bind to this receptor, but with much less efficiency than Ca^{2+} (Hebert, 1996). Mg may also regulate calcium transport into the cell through other ion channels (Miki *et al.*, 1997). Acute changes in the serum Mg concentration may therefore modulate PTH secretion and should be considered in the evaluation of the determination of serum PTH concentrations.

Mg Depletion and Parathyroid Gland Function

While acute changes in extracellular Mg concentrations will influence PTH secretion qualitatively similar to calcium, it is clear that Mg deficiency markedly perturbs mineral homeostasis (Rude *et al.*, 1976; Rude, 1994; Rude and Shils, 2006). Hypocalcemia is a prominent manifestation of Mg deficiency in humans (Rude *et al.*, 1976; Rude, 1994; Rude and Shils, 2006), as well as in most other species (Shils, 1980; Anast and Forte, 1983; Rude and Shils, 2006). In humans, Mg deficiency must become moderate to severe before symptomatic hypocalcemia develops. A positive correlation has been found between serum Mg and calcium concentrations in hypocalcemic hypomagnesemic patients (Rude *et al.*, 1976). Mg therapy alone restored serum calcium concentrations to normal in these patients within days (Rude *et al.*, 1976). Calcium and/or vitamin D therapy will not correct the hypocalcemia (Rude *et al.*, 1976; Rude, 1994). Even mild degrees of Mg depletion, however, may result in a significant fall in the serum calcium concentration, as demonstrated in experimental human Mg depletion (Fatemi *et al.*, 1991). One major factor resulting in the fall in serum calcium is impaired parathyroid gland function. Low Mg in the media of parathyroid cell cultures impairs PTH release in response to a low media calcium concentration (Targovnik *et al.*, 1971). Determination of serum PTH concentrations in hypocalcemic hypomagnesemic patients has shown heterogeneous results. The majority of patients have low or normal serum PTH levels (Anast *et al.*, 1972; Suh *et al.*, 1973; Chase and Slatopolsky, 1974; Rude *et al.*, 1976; Rude *et al.*, 1978). Normal serum PTH concentrations are thought to be inappropriately low in the presence of hypocalcemia. Therefore, a state of hypoparathyroidism exists in most hypocalcemic Mg-deficient patients. Some patients, however, have elevated levels of PTH in the serum (Rude *et al.*, 1976, 1978; Allgrove *et al.*, 1984). The administration of Mg will result in an immediate rise in the serum PTH concentration regardless of the basal PTH level (Anast *et al.*, 1972; Rude *et al.*, 1976, Rude *et al.*, 1978). As shown in (Fig. 7), 10 mEq of Mg administered intravenously over 1 minute caused an immediate marked rise in serum PTH in patients with low, normal, or elevated basal serum PTH concentrations. This is distinctly different from the effect of an Mg injection in normal subjects where, as discussed earlier, Mg will cause an inhibition of PTH secretion (Cholst *et al.*, 1984; Fatemi *et al.*, 1991). The serum PTH concentration will gradually fall to normal within several days of therapy with return of the serum calcium concentration to normal (Anast *et al.*, 1972; Rude *et al.*, 1976; Rude *et al.*, 1978). The impairment in PTH secretion appears to occur early in Mg depletion. Normal human subjects placed experimentally on a low Mg diet for only 3 weeks showed similar but not as marked changes in the serum PTH concentrations (Fatemi *et al.*, 1991) in which there was a fall in both serum calcium and PTH concentrations in 20 of 26 subjects at the end of the dietary Mg deprivation period. The administration of intravenous Mg at the end of this Mg depletion period resulted in a significant rise in the serum PTH concentration qualitatively similar to that observed in hypocalcemic

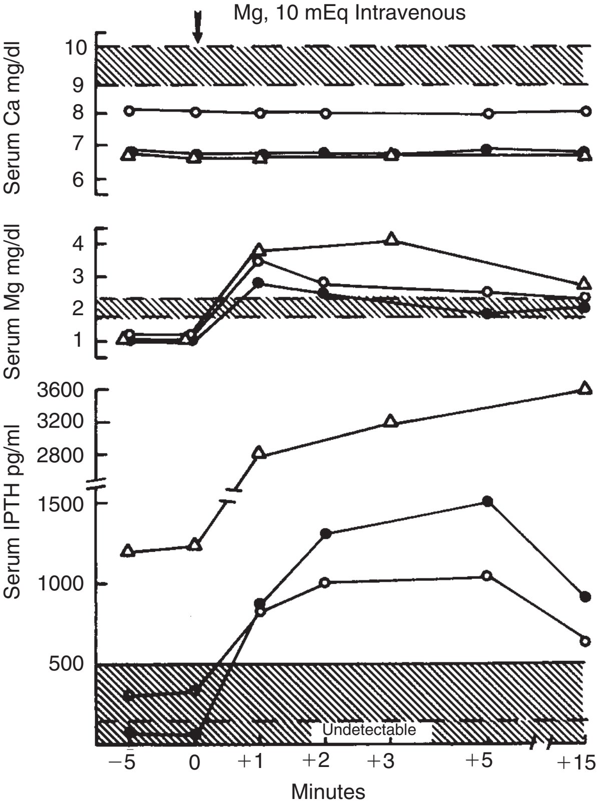

FIGURE 7 The effect of an IV injection of 10 mEq Mg on serum concentrations of calcium, magnesium, and immunoreactive parathyroid hormone (IPTH) in hypocalcemic magnesium-deficient patients with undetectable (•), normal (O), or elevated (Δ) levels of IPTH. Shaded areas represent the range of normal of each assay. The broken line for the IPTH assay represents the level of detectability. The magnesium injection resulted in a marked rise in PTH secretion within 1 minute in all three patients (Rude *et al.*, 1978).

Mg-depleted patients shown in Fig. 7, whereas a similar Mg injection suppressed PTH secretion prior to the low Mg diet. In this study, as with hypocalcemia hypomagnesemic patients, some subjects had elevations in the serum PTH concentration. The heterogeneous serum PTH values may be explained on the severity of Mg depletion. As the serum Mg concentration falls, the parathyroid gland will react normally with an increase in PTH secretion. As intracellular Mg depletion develops, however, the ability of the parathyroid to secrete PTH is impaired, resulting in a fall in serum PTH levels with a resultant fall in the serum calcium concentration. This concept is supported by the observation that the change in serum PTH in experimental human Mg depletion is correlated positively with the fall in red blood cell intracellular-free Mg^{2+} (Fatemi *et al.,* 1991). A slight fall in red blood cell Mg^{2+} resulted in an increase in PTH. However, a greater decrease in red blood cell Mg^{2+} correlated with a progressive fall in serum PTH concentrations.

It is conceivable that either PTH synthesis and/or PTH secretion may be affected. However, as the *in vitro* biosynthesis of PTH requires approximately 45 minutes (Hamilton *et al.,* 1971), the immediate rise in PTH following the administration of intravenous magnesium to Mg-deficient patients strongly suggests that the defect is in PTH secretion.

Mg Depletion and Parathyroid Hormone Action

The previous discussion strongly supports the notion that impairment in the secretion of PTH in Mg deficiency is a major contributing factor in the hypocalcemia. However, the presence of normal or elevated serum concentrations of PTH in the face of hypocalcemia (Rude *et al.,* 1976; Rude *et al.,* 1978; Rude, 1994) suggests that there may also be end-organ resistance to PTH action. In hypocalcemic Mg-deficient patients treated with Mg, the serum calcium concentration does not rise appreciably within the first 24 hours, despite elevated serum PTH concentrations (Rude *et al.,* 1976; Rude, 1994), which also suggests skeletal resistance to PTH because exogenous PTH administered to hypoparathyroid patients causes a rise in the serum calcium within 24 hours (Bethune *et al.,* 1968). Clinical studies have reported resistance to exogenous PTH in hypocalcemic Mg-deficient patients (Estep *et al.,* 1969; Woodard *et al.,* 1972; Rude *et al.,* 1976; Rude, 1994; Mihara *et al.,* 1995; Klein and Herndon, 1998; Sahota *et al.,* 2006). In one study, parathyroid hormone did not result in elevation in the serum calcium concentration or urinary hydroxyproline excretion in hypocalcemic hypomagnesemic patients as shown in (Fig. 8) (Estep *et al.,* 1969). Following Mg repletion, however, a clear response to PTH was observed. PTH has also been shown to have a reduced calcemic effect in Mg-deficient animals (MacManus *et al.,* 1971; Levi *et al.,* 1974; Forbes and Parker, 1980). The ability of PTH to resorb bone *in vitro* is also diminished greatly in the presence of low media Mg (Raisz and Niemann, 1969). In one study of isolated perfused femur in the dog, the ability of PTH to simulate an increase in the venous cyclic AMP was impaired during perfusion with low Mg fluid, suggesting skeletal PTH resistance (Freitag *et al.,* 1979). Not all studies have shown skeletal resistance to PTH, however (Salet *et al.,* 1966; Stromme *et al.,* 1969; Suh *et al.,* 1973, Chase and Slatopolsky, 1974). It appears likely that skeletal PTH resistance may be observed in patients with more severe degrees of Mg depletion. Patients in whom a normal calcemic response to PTH was demonstrated were in subjects who had been on recent Mg therapy (Salet *et al.,* 1966; Stromme *et al.,* 1969; Suh *et al.,* 1973, Chase and Slatopolsky, 1974). Patients who have been found to be resistant to PTH have, in general, not had prior Mg administration (Estep *et al.,* 1969; Woodard *et al.,* 1972; Rude

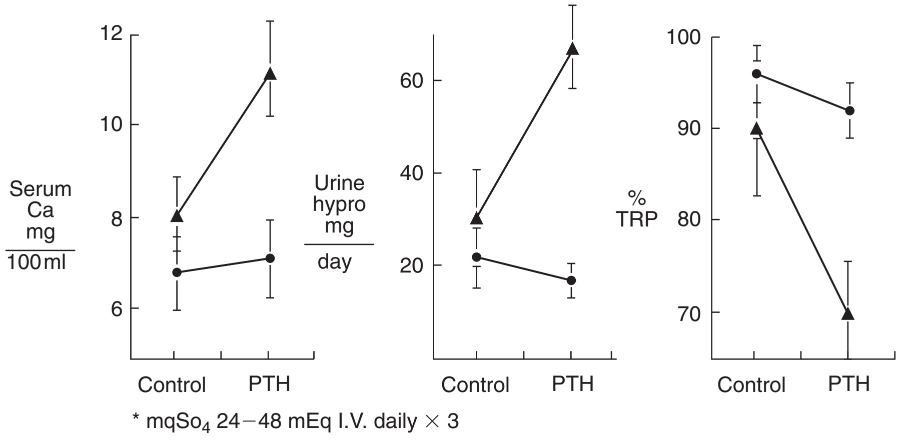

FIGURE 8 Mean and standard deviations of serum calcium concentration and urinary hydroxyproline and phosphate excretion in hypocalcemic magnesium-deficient patients before (•) and after (▲) 3 days of parenteral magnesium therapy (Estep *et al.,* 1969).

et al., 1976; Rude, 1994). Consistent with this notion is that in the Mg-depleted rat, normal responses to PTH were observed when the serum Mg concentration was 0.95 mg/dl (Hahn *et al.,* 1972); however, in another study, rats with a mean serum Mg of 0.46 mg/dl were refractory to PTH (MacManus *et al.,* 1971). In addition, a longitudinal study of Mg deficiency in dogs demonstrated a progressive decline in responsiveness to PTH with increasing degrees of Mg depletion (Levi *et al.,* 1974).

Calcium release from the skeleton also appears to be dependent on physicochemical processes as well as cellular activity (Pak and Diller, 1969; MacManus and Heaton, 1970). Low Mg will result in a decrease in calcium release from bone (Pak and Diller, 1969; MacManus and Heaton, 1970) and may be another mechanism for hypocalcemia in Mg deficiency.

The renal response to PTH has also been assessed by determining the urinary excretion of cyclic AMP and/or phosphate (Figs. 8 and 9) in response to exogenous PTH. In some patients, a normal effect of PTH on urinary phosphate and cyclic AMP excretion has been noted (Anast *et al.,* 1972; Suh *et al.,* 1973; Chase and Slatopolsky, 1974). In general, these were the same subjects in which a normal calcemic effect was also seen (Anast *et al.,* 1972; Suh *et al.,* 1973; Chase and Slatopolsky, 1974). In other studies, with more severely Mg-depleted patients, an impaired response to PTH has been observed (Estep *et al.,* 1969; Rude *et al.,* 1976; Medalle and Waterhouse, 1973; Rude, 1994; Mihara *et al.,* 1995). A decrease in urinary cyclic AMP excretion in response to PTH has also been described in the Mg-deficient dog and rat (Levi *et al.,* 1974; Forbes and Parker, 1980).

Mechanism of Impaired Mineral Homeostasis in Mg Depletion

The mechanism for impaired PTH secretion and action in Mg deficiency remains unclear. It has been suggested that there may be a defect in the second messenger systems in Mg depletion. PTH is thought to exert its biologic effects through the intermediary action of cyclic AMP (Bitensky *et al.,* 1973; Neer, 1995). Adenylate cyclase has been universally found to require Mg for cyclic AMP generation, both as a component of the substrate (Mg-ATP) and as an obligatory activator of enzyme activity (Northup *et al.,* 1982). There appears to be two Mg^{2+}-binding sites within the adenylate cyclase complex: one resides on the catalytic subunit and the other on the guanine nucleotide regulatory protein, Ns (Cech *et al.,* 1980; Maguire, 1984). The requisite role that Mg^{2+} plays in adenylate cyclase function suggests that factors that would limit the availability of Mg^{2+} to this enzyme could have significant effects on the cyclic nucleotide metabolism of a cell and hence overall cellular function. It is clear that some patients with severe Mg deficiency have a reduced urinary excretion of cyclic AMP in response to exogenously administered PTH (Rude *et al.,* 1976). In addition, PTH was shown to have a blunted effect in causing a rise in cyclic AMP from isolated perfused tibiae in Mg-deficient dogs (Freitag *et al.,* 1979). These observations correspond well with the impaired calcemic and phosphaturic effects of PTH in Mg-deficient patients and animals as discussed earlier.

While Mg^{2+} is stimulatory for adenylate cyclase, Ca^{2+} may inhibit or activate enzyme activity (Sunahara *et al.,* 1996). Nine isoforms of adenylate cyclase have been identified whose activities are modulated by both Mg^{2+} and Ca^{2+} (Sunahara *et al.,* 1996). In plasma membranes from parathyroid, renal cortex, and bone cells, Ca^{2+} will competitively inhibit Mg^{2+}-activated adenylate cyclase activity (Rude, 1983; Rude, 1985; Oldham *et al.,* 1984). In parathyroid plasma membranes, at a Mg^{2+} concentration of 4 mM, Ca^{2+} was found to inhibit adenylate cyclase in a bimodal pattern described in terms of two calcium inhibition constants with K_i values of 1–2 and 200–400 μM (Oldham *et al.,* 1984). At a lower Mg^{2+} concentration (0.5 mM) the only adenylate cyclase activity expressed was that inhibitable by the high-affinity Ca^{2+}-binding site. With increasing Mg concentrations, the fraction of total adenylate cyclase activity subject to high-affinity calcium inhibition became progressively less. Thus, the ambient Mg^{2+} concentrations can markedly affect the susceptibility of this enzyme to the inhibitory effects of Ca^{2+}. Total intracellular calcium has been observed to rise during Mg depletion (George and Heaton, 1975; Ryan and Ryan, 1979). Mg is not only important for the operation of Mg^{2+}, Ca^{2+}-dependent ATPase, but may also be countertransported during the uptake and release of calcium through calcium channels (Romani and Scarpa, 1992; Romani, 2006). The combination of higher intracellular Ca^{2+} and increased sensitivity

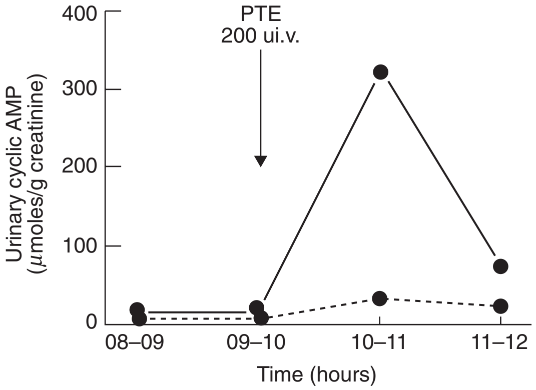

FIGURE 9 The effect of an IV injection of 200 units of parathyroid extract on the excretion of urinary cyclic AMP in a magnesium-deficient patient before (• --- •) and after (• — •) 4 days of magnesium therapy. Urine was collected for four consecutive 1-hour periods, two before and two after the PTE injection. Although Mg-deficient, the patient had a minimal rise in urinary cyclic AMP in response to PTH, but following Mg therapy the response was normal (Rude *et al.,* 1976).

to Ca^{2+} inhibition because of Mg depletion could explain the defective PTH secretion in Mg deficiency. An increase in the release of intracellular Ca^{2+} via the phosphoinositol system is also possible, as discussed later. A similar relationship between Mg^{2+} and Ca^{2+} was described for adenylate cyclase obtained from bone (Rude, 1985). Ca^{2+} caused a competitive inhibition of Mg^{2+}-activated skeletal adenylate cyclase with a high-affinity Ca^{2+}-binding site with a K_iCa of 1–2 μM. Lowering the Mg^{2+} concentration increased overall Ca^{2+} inhibition. Thus, a fall in the intracellular Mg^{2+} concentration would render the adenylate cyclase enzyme more susceptible to inhibition by the prevailing intracellular Ca^{2+} concentrations and may be a mechanism by which both PTH secretion and PTH end-organ action are compared in Mg deficiency.

Adenylate cyclase is a widely distributed enzyme in the body, and if the hypothesis just given were true, the secretion and action of other hormones mediated by adenylate cyclase might also exhibit impaired activity in Mg deficiency. This has not been found to be true, as the actions of ACTH, TRH, GnRH, and glucagon are normal in Mg depletion (Cohan *et al.,* 1982). Prior investigations have suggested that Mg affinity for adenylate cyclase is higher (lower K_aMg) in liver, adrenal, and pituitary than in parathyroid (for reviews, see Rude and Oldham, 1985; Rude, 1994). In one study, investigation of K_aMg and K_iCa in tissues from one species (guinea pig) demonstrated that under agonist stimulation, the K_aMg from liver is less than thyroid less than kidney, which equals bone, and the K_iCa^{2+} for liver greater than renal greater than kidney also equals bone (Rude and Oldham, 1985). These data suggest that adenylate cyclase regulation by divalent cations varies from tissue to tissue and may explain the greater propensity for disturbed mineral homeostasis in Mg deficiency.

Although cyclic AMP is an important mediator of PTH action, current studies do not suggest an important role in mediating Ca^{2+}-regulated PTH secretion (Brown, 1991; Dunlay and Hruska, 1990). PTH has been shown to activate the phospholipase C second messenger system (Dunlay and Hruska, 1990). PTH activation of phospholipase C leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol-1,4,5-triphosphate (IP_3) and diacylglycerol. IP_3 binds to specific receptors on intracellular organelles (endoplasmic reticulum, calciosomes), leading to an acute transient rise in cytosolic Ca^{2+} with a subsequent activation of calmodulin-dependent protein kinases. Diacylglycerol activates protein kinase C. Mg depletion could perturb this system via several mechanisms. First, an Mg^{2+}-dependent guanine nucleotide-regulating protein is also involved in the activation of phospholipase C (Babich *et al.,* 1989; Litosch, 1991). Mg^{2+} has also been shown to be a noncompetitive inhibitor of IP_3-induced Ca^{2+} release (Volpe *et al.,* 1990). A reduction of Mg^{2+} from 300 to 30 μM increased Ca^{2+} release in response to IP_3 by two- to threefold in mitochondrial membranes obtained from canine cerebellum (Volpe *et al.,* 1990). The Mg concentration required for a half-maximal inhibition of IP_3-induced Ca^{2+} release was 70 μU. In these same studies, Mg^{2+} was also found to inhibit IP_3 binding to its receptor. Mg, at a concentration of 500 μM, decreased maximal IP_3 binding threefold (IC_{50} 200 μM) (Volpe *et al.,* 1990). These Mg^{2+} concentrations are well within the estimated physiologic intracellular range (200–500 μ*M*) and therefore Mg^{2+} may be an important physiological regulator of the phospholipase C second messenger system. These effects may be related to Mg deficiency-induced activation of the α-subunit of the Ca-sensing receptor (Quitterer *et al.,* 2001; Vetter and Lohse, 2002).

The effect of Mg depletion on cellular function in terms of the second messenger systems is most complex, potentially involving substrate availability, G protein activity, release and sensitivity to intracellular Ca^{2+}, and phospholipid metabolism.

Mg Depletion and Vitamin D Metabolism and Action

Mg may also be important in vitamin D metabolism and/or action. Patients with hypoparathyroidism, malabsorption syndromes, and rickets have been reported to be resistant to therapeutic doses of vitamin D until Mg was administered simultaneously (for review, see Rude, 1994). Patients with hypocalcemia and Mg deficiency have also been reported to be resistant to pharmacological doses of vitamin D (Medalle *et al.,* 1976; Leicht *et al.,* 1990), 1α hydroxyvitamin D (Ralston *et al.,* 1983; Selby *et al.,* 1984) and 1,25-dihydroxyvitamin D (Graber and Schulman, 1986). Similarly, an impaired calcemic response to vitamin D has been found in Mg-deficient rats (Lifshitz *et al.,* 1967), lambs (McAleese and Forbes, 1959), and calves (Smith, 1958).

The exact nature of altered vitamin D metabolism and/or action in Mg deficiency is unclear. Intestinal calcium transport in animal models of Mg deficiency has been found to be reduced in some (Higuchi and Lukert, 1974) but not all (Coburn *et al.,* 1975) studies. Calcium malabsorption was associated with low serum levels of 25-hydroxyvitamin D in one study (Lifshitz *et al.,* 1967), but not in another (Coburn *et al.,* 1975), suggesting that Mg deficiency may impair intestinal calcium absorption by more than one mechanism. Patients with Mg deficiency and hypocalcemia frequently have low serum concentrations of 25-hydroxyvitamin D (Rude *et al.,* 1985; Fuss *et al.,* 1989) and therefore nutritional vitamin D deficiency may be one factor. Therapy with vitamin D, however, results in high serum levels of 25-hydroxyvitamin D without correction of the hypocalcemia (Medalle *et al.,* 1976), suggesting that the vitamin D nutrition is not the major reason. In addition, conversion of radiolabeled vitamin D to 25-hydroxyvitamin D was found to be normal in three

Mg-deficient patients (Lukert, 1980). Serum concentrations of 1,25-dihydroxyvitamin D have also been found to be low or low normal in most hypocalcemic Mg-deficient patients (Rude *et al.,* 1985; Fuss *et al.,* 1989; Leicht *et al.,* 1992; Rude and Shils, 2006). Mg-deficient diabetic children, when given a low calcium diet, did not exhibit the expected normal rise in serum 1,25-dihydroxyvitamin D or PTH (Saggese *et al.,* 1988); the response returned to normal following Mg therapy (Saggese *et al.,* 1991). Because PTH is a major trophic for 1,25-dihydroxyvitamin D formation, the low serum PTH concentrations could explain the low 1,25-dihydroxyvitamin D levels. In support of this is the finding that some hypocalcemic Mg-deficient patients treated with Mg have a rise in serum 1,25-dihydroxyvitamin D to high normal or to frankly elevated levels, as shown in (Fig. 10) (Rude *et al.,* 1985). Most patients, however, do not have a significant rise within 1 week after institution of Mg therapy, despite a rise in serum PTH and normalization of the serum calcium concentration (Fig. 10) (Rude *et al.,* 1985). These data suggest that Mg deficiency in humans also impairs the ability of the kidney to synthesize 1,25-dihydroxyvitamin D. This is supported by the observation that the ability of exogenous administration of 1–34 human PTH to normal subjects after 3 weeks of experimental Mg depletion resulted in a significantly lower rise in serum 1,25-dihydroxyvitamin D concentrations than before institution of the diet (Fatemi *et al.,* 1991). It appears, therefore, that the renal synthesis of 1,25-dihydroxyvitamin D is sensitive to Mg depletion. Although Mg is known to support 25-hydroxy-1α-hydroxylase *in vitro* (Fisco and Traba, 1992), the exact Mg requirement for this enzymatic process is not known. Bone-specific binding sites for 1,2-dihydroxyvitamin D have also been described to be reduced in Mg deficiency (Risco and Traba, 2004).

The association of Mg deficiency with impaired vitamin D metabolism and action therefore may be because of several factors, including vitamin D deficiency (Rude *et al.,* 1985; Carpenter, 1988; Fuss *et al.,* 1989; Leicht and Biro, 1992) and a decrease in PTH secretion (Anast *et al.,* 1972; Suh *et al.,* 1973; Chase and Slatopolsky, 1974; Rude *et al.,* 1976; Rude *et al.,* 1978), as well as a direct effect of Mg depletion on the ability of the kidney to synthesize 1,25-dihydroxyvitamin D (Rude *et al.,* 1985; Fuss *et al.,* 1989; Fatemi *et al.,* 1991). Osteoporotic patients with a blunted response to PTH exhibit Mg deficiency (Sahota *et al.,* 2006). In addition, Mg deficiency may directly impair intestinal calcium absorption (Higuchi and Lukert, 1974; Rude *et al.,* 1976; Rude *et al.,* 1985). Skeletal resistance to vitamin D and its metabolites may also play an important role (Lifshitz *et al.,* 1967; Ralston *et al.,* 1983; Selby *et al.,* 1984; Graber and Schulman, 1986). It is clear, however, that the restoration of normal serum 1,25-dihydroxyvitamin D concentrations is not required for normalization of the serum calcium level (Fig. 9). Most Mg-deficient patients who receive Mg therapy exhibit an immediate rise in PTH, followed by normalization of the serum calcium prior to any change in serum 1,25-dihydroxyvitamin D concentrations (Rude *et al.,* 1985; Fuss *et al.,* 1989). An overall view of the effect of Mg depletion on calcium metabolism is shown in Fig. 11.

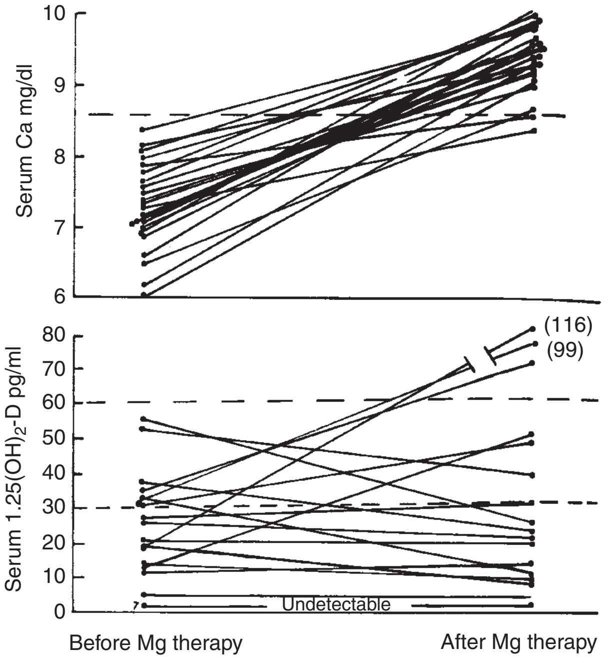

FIGURE 10 Serum concentrations of calcium and 1,25-diydroxyvitamin D in hypocalcemic magnesium-deficient patients before and after 5–8 days of parenteral magnesium therapy. The broken line represents the upper and lower limits of normal for serum 1,25-dihydroxyvitamin D and the lower limit of normal for the serum calcium (Rude *et al.,* 1985).

MAGNESIUM DEPLETION: SKELETAL GROWTH AND OSTEOPOROSIS

Women with postmenopausal osteoporosis have decreased nutrition markers, suggesting that osteoporosis is associated with nutritional deficiencies (Rico *et al.,* 1993; Ames, 2006). A low calcium intake is one of these nutritional factors (Rico *et al.,* 1993) and predicts bone mineral density and fracture rate (Dawson-Hughes *et al.,* 1990; Chapuy *et al.,* 1992). A large segment of our population also has low dietary Mg intake (Morgan *et al.,* 1985; Marier, 1986; Cleveland *et al.,* 1994). Mg deficiency, when severe, will disturb calcium homeostasis markedly, resulting in impaired PTH secretion and PTH end organ resistance, leading to hypocalcemia (Rude, 1998). Mg exists in macronutrient quantities in bone, and long-term, mild-to-moderate dietary Mg deficiency has been implicated as a risk factor for osteoporosis (Sojka and Weaver, 1995; Cohen and Kitzes, 1981).

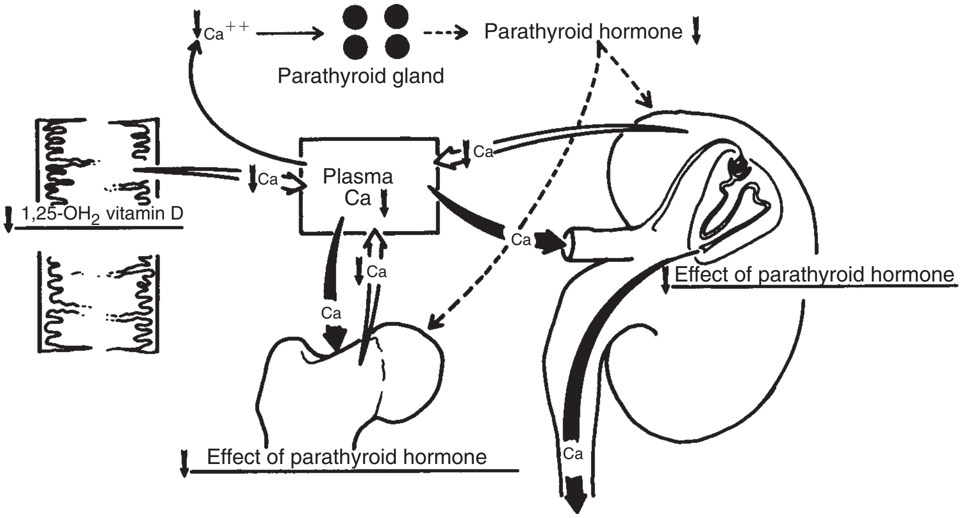

FIGURE 11 Disturbance of calcium metabolism during magnesium deficiency. Hypocalcemia is caused by a decrease in PTH secretion, as well as renal and skeletal resistance to the action of PTH. Low serum concentrations of 1,25-dihydroxyvitamin D may result in reduced intestinal calcium absorption (Rude and Oldham, 1990).

Epidemiological Studies

Epidemiological studies have provided a major link associating dietary Mg inadequacy to osteoporosis. One cross-sectional study assessed the effect of dietary nutrients on appendicular (radius, ulna, and heel) bone mineral density (BMD) in a large group of Japanese Americans living in Hawaii (Yano *et al.,* 1985). In 1,208 males (ages 61–81), whose mean Mg intake was 238±111 mg/day, no correlation of Mg intake with BMD was observed at any site. In a subgroup of 259 of these subjects who took Mg supplements, however (mean Mg intake of 381 mg/day), BMD was correlated positively with Mg intake at one or more skeletal sites. In 912 females (ages 43–80) whose Mg intake was 191±36 mg/day, a positive correlation with BMD was also observed. In contrast to males, no correlation with BMD was found in females who took Mg supplements (total Mg intake of 321 mg/day). In a smaller study of women ages 35–65 (17 premenopausal, mean Mg intake 243±44 mg/day; 67 postmenopausal, mean Mg intake 249±68 mg/day) in which BMD was measured in the distal forearm, no cross-sectional correlation was observed with Mg intake in either group (Freudenheim *et al.,* 1986). Longitudinal observation over 4 years, however, demonstrated that loss of bone mass was related inversely to Mg intake in premenopausal women ($p < 0.05$) and had a similar trend in the postmenopausal group ($p < 0.085$). In another cross-sectional study, a positive correlation of BMD of the forearm (but not femur or spine) was found in a larger group of 89 premenopausal women (age 37.8±0.8; Mg intake of 243±9 mg/day), but no similar correlation was found in 71 recently menopausal women ages 58.9±0.9 (Mg intake of 253±11 mg/day) (Angus *et al.,* 1988). In contrast, a study of 194 older postmenopausal women (ages 69–97; mean Mg intake of 288 mg/day) demonstrated a significant positive correlation with BMD of the forearm (Tranquilli *et al.,* 1994).

Other studies have concentrated on BMD of the axial skeleton. Sixty-six premenopausal women (ages 28–39) whose Mg intake was 289±73 mg/day had a significant relationship between dietary Mg intake and rate of change of BMD of the lumbar spine and total body calcium over a 1-year period (Houtkooper *et al.,* 1995). A cross-sectional study that combined 175 premenopausal and postmenopausal women ages 28–74 (mean Mg intake was 262±70 mg/day) found no correlation with BMD at the lumbar spine, femoral neck, or total body calcium (Michaelsson *et al.,* 1995). In a study of 994 premenopausal women ages 45–49, whose Mg intake was 311±85 mg/day, New *et al.* (1997) found a significant correlation of BMD of the lumbar spine with Mg intake. A significant difference was also observed in lumbar spine BMD between the highest and the lowest quartiles of dietary Mg intake. A report by this same group in a study of 65 pre- and postmenopausal women ages 45–55 again found higher bone mass of the forearm (but not femoral neck or hip) in subjects consuming a Mg intake of 326±90 mg/day (New *et al.,* 2000). Women with a high childhood intake of fruits (Mg and potassium) did have higher femoral neck BMD than those on a lower fruit intake, however. Another cross-sectional study assessed Mg intake in older males and females (ages 69–97) (345 males and 562 females), as well as a 2-year longitudinal study of a subset of these subjects (229 males and 399 females) (Tucker *et al.,* 1999). In the cross-sectional analysis in males, Mg intake (300±110 mg/day) was correlated with BMD of the radius and hip. In the 4-year longitudinal study of these subjects, a positive inverse relationship between bone loss of the hip and Mg intake was observed. A positive cross-sectional correlation of BMD of the hip was also observed in females (Mg intake of 288±106 mg/day), but not in the longitudinal assessment. Dietary Mg intake in a large population of non-Hispanic white males and females from the NHANES III database was also found by multiple regression analysis to predict BMD at several sites in the proximal femur (Carpenter *et al.,* 2000). Most recently, Mg intake was significantly correlated with whole body and hip BMD in elderly white females and males (Ryder *et al.*, 2005).

In a study of younger individuals, the effect of dietary Mg intake of preadolescent girls (ages 9–11) on bone mass/quality in these young women was evaluated at age 18–19 (Wang *et al.,* 1999). Ultrasound determination of bone mass of the calcaneus in 35 African American women (Mg intake 237±83 mg/day) and in 26 Caucasian women (Mg intake 240±61 mg/day) was performed. Mg intake was related positively to quantitative ultrasound properties of bone, suggesting that this nutrient was important in skeletal growth and development. Bounds *et al.* (2005) reported that bone mineral content was significantly related to Mg intake at age 8, including longitudinal intake from age 2 to 8.

In summary, these epidemiological studies link dietary Mg intake to bone mass. Exceptions appear to include women in the early postmenopausal period in which the effect of acute sex steroid deficiency may mask the effect of dietary factors such as Mg. In addition, diets deplete in Mg are usually deficient in other nutrients, which affect bone mass as well. Therefore, further investigations are needed to provide a firm relationship of dietary Mg inadequacy with osteoporosis.

Bone Turnover

In two of the epidemiological studies cited earlier, markers of bone turnover were determined. In one, where no correlation was found between BMD and dietary Mg intake, serum osteocalcin did not correlate with Mg (or any other nutrient) intake (Michaelsson *et al.,* 1995). New *et al.* (2000) also found that serum osteocalcin was not associated with the dietary intake of Mg or other nutrients. Mg intake, however, was significantly negatively correlated with the urinary excretion of pyridinoline and deoxypyridinoline, suggesting that a low Mg diet was associated with increased bone resorption (New *et al.,* 2000).

The affect of short-term administration of Mg on bone turnover in young normal subjects has been conflicting. Magnesium, 360 mg per day, was administered for 30 days to 12 normal males age 27 (mean dietary intake prior to supplementation was 312 mg/day) and markers of bone formation (serum osteocalcin and C terminus of type I procollagen) and bone resorption (type I collagen telopeptide) were compared with 12 age-matched controls (Dimai *et al.,* 1998). Markers of both formation and resorption were suppressed significantly however, only during the first 5–10 days of the study. A similar trial of 26 females ages 20–28 in a double-blind, placebo-controlled, randomized crossover design has been reported (Doyle *et al.,* 1999). Magnesium, 240 mg/day, or placebo was administered for 28 days (mean dietary Mg intake was 271 mg/day prior to and during the study). No effect of Mg supplementation was observed on serum osteocalcin, bone-specific alkaline phosphatase, or urinary pyridinoline and deoxypyridinoline excretion.

Mg Status in Osteoporosis

Significant correlation between serum Mg and bone metabolism has been observed. Gur *et al.* (2002) demonstrated lower serum values of Mg in 70 osteoporotic subjects compared to 30 nonosteoporotic postmenopausal women. In a similar study involving 20 perimenopausal and 53 postmenopausal women, women with severe osteoporosis had significantly lower ionized Mg levels (Brodowski, 2002). This was also confirmed in another study (Saito *et al.*, 2004). In a study of 168 patients with Crohn's disease, higher serum Mg predicted higher BMD at the femur (Habtezion *et al.*, 2002). A study of a family with primary hypomagnesemia owing to renal Mg wasting demonstrated significant reductions in serum and lymphocyte Mg concentrations; affected members had significantly reduced BMD at the lumbar spine and proximal femur (Kantorovich *et al.*, 2002). Mg is principally an intracellular cation, therefore serum Mg concentration may not reflect Mg status. Researchers have employed Mg tolerance testing, red blood cell Mg, and skeletal Mg content. A small group of 15 osteoporotic subjects (10 female, 5 male) ages 70–85 (the presence or absence of osteoporosis was determined by radiographic features) was compared to 10 control nonosteoporotic subjects (Cohen and Kitzes, 1983). Both groups had normal serum Mg concentrations, which were not significantly different from each other. The Mg tolerance test, however, revealed a significantly greater retention in the osteoporotic patients (38%) as compared to 10% in the control subjects, suggesting Mg deficiency. In a second study by this group, 12 younger women ages 55–65 with osteoporosis (as determined by x-ray) had significantly lower serum Mg concentrations than 10 control subjects; however, no difference in the Mg tolerance test was observed (Cohen *et al.,* 1983). Red blood cell Mg was found to be significantly lower in 10 postmenopausal women who had at least one vertebral fracture as compared to 10 subjects with degenerative osteoarthritis; however, no difference in plasma Mg was found (Reginster *et al.,* 1985). In a second report, 10 postmenopausal women ages

68.9±9 with vertebral crush fracture were compared to 10 nonosteoporotic women ages 67.2±6 years (Reginster, 1989). In comparison to the 10 controls, the osteoporotic subjects had a significantly lower serum Mg, but no difference was noted in red blood cell Mg.

The majority of body Mg (50–60%) resides in the skeleton, and skeletal Mg reflects Mg status. In the two studies cited earlier in which an Mg deficit was suggested by either Mg tolerance testing or low serum Mg concentration, the Mg content of iliac crest trabecular bone was reduced significantly in osteoporotic patients (Cohen and Kitzes, 1983; Cohen *et al.,* 1983). Two additional studies also found a lower bone Mg content in elderly osteoporotic patients (Manicourt *et al.,* 1981; Milachowski *et al.,* 1981). However, no difference in bone Mg content between osteoporotic subjects and bone obtained from cadavers was found (Reginster, 1989). Another study found no difference between patients with osteoporosis and control subjects in cortical bone (Basle *et al.,* 1990), while two studies reported higher bone Mg content in osteoporosis (Basle *et al.,* 1990; Burnell *et al.,* 1982).

In summary, Mg status has been assessed in very few osteoporotic patients. Low serum and red blood cell Mg concentrations, as well as a high retention of parenterally administered Mg, suggest an Mg deficit; however, these results are not consistent from one study to another. Similarly, although a low skeletal Mg content has been observed in some studies, others have found normal or even high Mg content. Larger scale studies are needed.

Effect of Mg Therapy in Osteoporosis

The effect of dietary Mg supplementation on bone mass in patients with osteoporosis has not been studied extensively. Administration of 600 mg of Mg per day to 19 patients over 6–12 months (Abraham, 1991) was reported to increase BMD of the calcaneus (11%) compared to a 0.7% rise in 7 control subjects. All subjects were postmenopausal (ages 42–75) and on sex steroid replacement therapy. Subjects who received Mg also received 500 mg of calcium per day, as well as many other dietary supplements, making if difficult to conclude if Mg alone was the sole reason for the increase in bone mass. In a retrospective study, Mg (200 mg per day) given to 6 postmenopausal women (mean age 59) was observed to have a small nonsignificant 1.6% rise in bone density of the lumbar spine; no change was seen in the femur (Eisinger and Clairet, 1993). Stendig-Lindberg *et al.* (1993) conducted a 2-year trial in which 31 postmenopausal osteoporotic women were administered 250 mg Mg per day, increasing to a maximum of 750 mg per day for 6 months depending on tolerance. All subjects were given 250 mg Mg per day from months 6 to 24. Twenty-three age-matched subjects served as controls. At 1 year there was a significant 2.8% increase in bone density of the distal radius. Twenty-two of the 31 subjects had an increase in bone density while 5 did not change. Three subjects that showed a decrease in bone density had primary hyperparathyroidism and one underwent a thyroidectomy. No significant effect of Mg supplementation was shown at 2 years, although only 10 subjects completed the trial. In a small uncontrolled trial, a significant increase in bone density of the proximal femur and lumbar spine in celiac sprue patients who received approximately 575 mg Mg per day for 2 years was reported (Rude and Olerich, 1996). These subjects had demonstrated evidence of reduced free Mg in red blood cells and peripheral lymphocytes. Recently, Carpenter *et al.* (2006) administered 300 mg Mg to girls ages 8–14 over 1 year and noted a significantly greater increase in hip bone mineral content in the Mg-treated group.

In summary, the effect of Mg supplements on bone mass has generally led to an increase in bone mineral density, although study design limits useful information. Larger, long-term, placebo-controlled, double-blind investigations are required.

Osteoporosis in Patients at Risk for Mg Deficiency

Osteoporosis may occur with greater than usual frequency in certain populations in which Mg depletion is also common. These may include diabetes mellitus (Levin *et al.,* 1976; McNair *et al.,* 1979, McNair *et al.,* 1981; Hui *et al.,* 1985; Saggese *et al.,* 1988; Krakauer *et al.,* 1995), chronic alcoholism (Bikle *et al.,* 1985; Lindholm *et al.,* 1991; Peris *et al.,* 1992), and malabsorption syndromes (Molteni *et al.,* 1990; Mora *et al.,* 1993). Changes in bone and mineral metabolism in patients with diabetes mellitus and alcoholism are surprisingly similar to those in Mg depletion as discussed earlier. Serum PTH and/or 1,25(OH)$_2$D concentrations have been found to be reduced in both human and animal studies (McNair *et al.,* 1979; Hough *et al.,* 1981; Imura *et al.,* 1985; Ishida *et al.,* 1985; Nyomba *et al.,* 1986; Saggese *et al.,* 1988; Verhaeghe *et al.,* 1990). A prospective study of pregnant diabetic women demonstrated a fall in serum 1,25(OH)$_2$D during pregnancy rather than the expected rise observed in normal women (Kuoppala, 1988). These subjects were also found to have reduced serum Mg concentrations. Diabetic children, with reduced serum Mg and calcium concentrations and low bone mineral content, were shown to have an impaired rise in serum PTH and 1,25(OH)$_2$D in response to a low calcium diet; this defect normalized following Mg repletion (Saggese *et al.,* 1988, Saggese *et al.,* 1991). Similar observations were found in the diabetic rat (Welsh and Weaver, 1988). Duodenal calcium absorption has also been reported to be low in diabetic rats (Nyomba *et al.,* 1989; Verhaeghe *et al.,* 1990). The calcium malabsorption may be because of low serum

$1,25(OH)_2D$, as duodenal calbindin D_{9K} has been found to be reduced (Nyomba *et al.*, 1989; Verhaeghe *et al.*, 1990).

The reduction in bone mass in diabetes mellitus and alcoholism also appears to be related to a decrease in bone formation, similar to what is observed in experimental Mg depletion (see later; section 6). A histomorphometric study of bone has shown decreased bone formation, bone turnover, osteoid, and osteoblast number (Tamayo *et al.*, 1981; Goodman and Hori, 1984; Verhaeghe *et al.*, 1990; Hough *et al.*, 1981; Bouillon, 1991). Reduced bone turnover is supported by the finding that serum osteocalcin, a marker of osteoblast activity, is low in humans (Pietschmann *et al.*, 1988; Rico *et al.*, 1989) and in rats (Ishida *et al.*, 1988; Verhaeghe *et al.*, 1990). Patients with vitamin D insufficiency and osteoporosis were shown to have a blunted PTH response that was related to Mg deficiency (Sahota *et al.*, 2006).

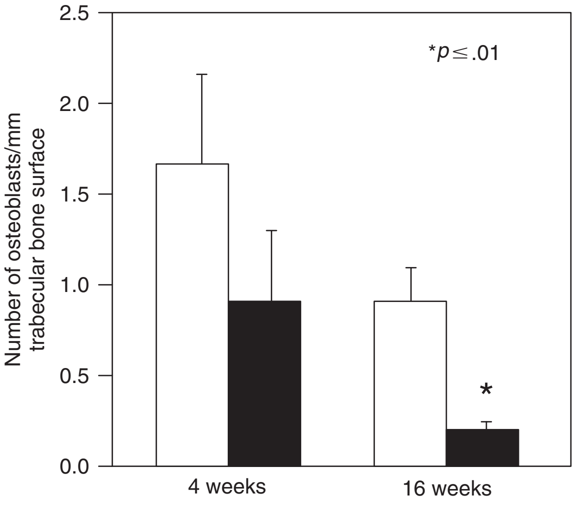

FIGURE 12 After 16 weeks of magnesium deficiency in the rat (solid bars), the osteoblast number was reduced significantly compared to controls (open bars) (Rude *et al.*, 1999) $^*p < 0.01$.

Magnesium Depletion and Osteoporosis: Experimental Animal Models

The effect of dietary Mg depletion on bone and mineral homeostasis in animals has been studied since the 1940s. Most studies have been performed in the rat. Dietary restriction has usually been severe, ranging from 0.2 to 8 mg per 100 chow (normal = 50–70 mg/100 g). A universal observation has been a decrease in growth of the whole body as well as the skeleton (Lai *et al.*, 1975; McCoy *et al.*, 1979; Mirra *et al.*, 1982; Carpenter *et al.*, 1992; Boskey *et al.*, 1992; Kenny *et al.*, 1994; Gruber *et al.*, 1994). The epiphyseal and diaphyseal growth plate is characterized by thinning and a decrease in the number and organization of chrondrocytes (Mirra *et al.*, 1982). Osteoblastic bone formation has been observed by quantitative histomorphometry to be reduced, as shown in (Fig. 12) (Carpenter *et al.*, 1992; Gruber *et al.*, 1994, Rude *et al.*, 1999). Serum and bone alkaline phosphatase (Mirra *et al.*, 1982; Loveless and Heaton, 1976; Lai *et al.*, 1975), serum and bone osteocalcin (Boskey *et al.*, 1992; Carpenter *et al.*, 1992; Creedon *et al.*, 1999), and bone osteocalcin mRNA (Carpenter *et al.*, 1992; Creedon *et al.*, 1999) have been reduced, suggesting a decrease in osteoblastic function. This is supported by an observed decrease in collagen formation and sulfation of glycosaminoglycans (Trowbridge and Seltzer, 1967). A decrease in tetracycline labeling has also suggested impaired mineralization (Carpenter *et al.*, 1992; Jones *et al.*, 1980). Dietary calcium supplementation in the Mg-depleted rat will not prevent loss of trabecular bone; indeed, a high calcium intake suppressed bone formation even more (Matsuzaki and Miwa, 2006). Data on osteoclast function have been conflicting. A decrease in urinary hydroxyproline (MacManus and Heaton, 1969) and dexoypyridinoline (Creedon *et al.*, 1999) has suggested a decrease in bone resorption; however, Rude *et al.* (1999) reported an increase in the number and activity of osteoclasts in the Mg-deficient rat, as shown in (Fig. 13). Bone from Mg-deficient rats has been described as brittle and fragile (Lai *et al.*, 1975; Duckworth *et al.*, 1940). Biomechanical testing has directly demonstrated skeletal fragility in both rat and pig (Boskey *et al.*, 1992; Kenny *et al.*, 1992; Miller *et al.*, 1965; Heroux *et al.*, 1975; Smith and Nisbet, 1968). Osteoporosis has been observed to occur in dietary Mg depletion by 6 weeks or longer (Boskey *et al.*, 1992; Carpenter *et al.*, 1992; Rude *et al.*, 1999; Heroux *et al.*, 1975; Smith and Nisbet, 1968), as shown in (Fig. 14). Bone implants into Mg-deficient rats have also shown osteoporosis in the implanted bone (Belanger *et al.*, 1975; Schwartz and Reddi, 1979). A high calcium diet will not affect this Mg deficiency-induced reduction of bone mass (Matsuzaki *et al.*, 2005). The effect of higher than the recommended dietary Mg intake on mineral metabolism in the rat has been reported (Toba *et al.*, 2000). In this study, increasing dietary Mg from 48 to 118 mg/100 g chow resulted in a decrease in bone resorption and an increase in bone strength in ovariectomized rats. No loss of BMD was observed, suggesting a beneficial effect of Mg in acute sex steroid deficiency.

These severe degrees of Mg deficiency are unlikely to commonly occur in the human population. We have recently reported less severe degrees of Mg restriction in the rat (10%, 25%, and 50% of the recommended nutrient requirement) and also observed detrimental effect on bone (Rude *et al.*, 2004; Rude *et al.*, 2005; Rude *et al.*, 2006) including reduced trabecular bone, decreased bone formation, and increased bone resorption. The effect of an Mg diet at 10% of the recommended nutrient requirement on trabecular bone is demonstrated in Fig. 14. These inadequate dietary Mg levels are present in segments of the human population as discussed earlier and implicate Mg deficiency as a possible risk factor for osteoporosis.

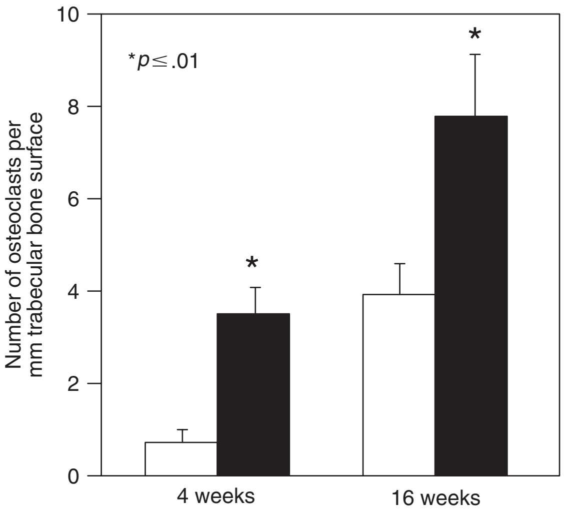

FIGURE 13 After 4 and 16 weeks of magnesium deficiency in the rat (solid bars), the osteoclast number was elevated significantly compared to controls (open bars) (Rude *et al.*, 1999) $^{*}p < 0.01$.

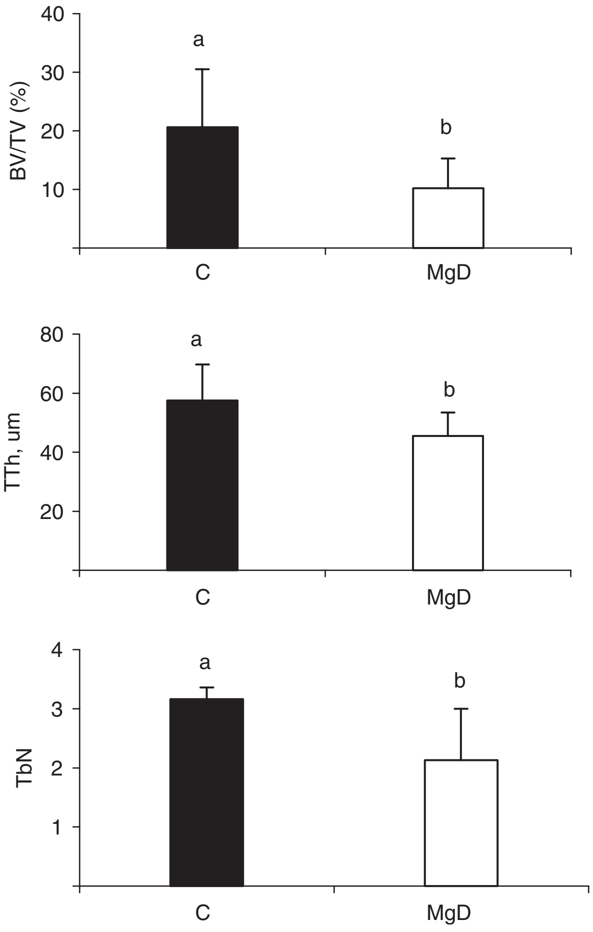

FIGURE 14 Effect of a low Mg diet at 10% of nutrient requirement on trabecular bone volume (BV/TB), mean trabecular width (TTh), and trabecular number (TbN) in rats fed Control (C) or low magnesium (MgD) diets as determined by microcomputed tomography. Values are means ± SD, $n = 7$. Bars without a common letter differ ($p < 0.01$). (Rude *et al.*, 2004).

Possible Mechanisms for Mg Deficiency-Induced Osteoporosis

Several potential mechanisms may account for a decrease in bone mass in Mg deficiency. Mg is mitogenic for bone cell growth, which may directly result in a decrease in bone formation (Liu *et al.*, 1988). Mg also affects crystal formation; a lack of Mg results in a larger, more perfect crystal, which may affect bone strength, as discussed later (Cohen and Kitzes, 1983).

Mg deficiency can perturb calcium homeostasis and result in a fall in both serum PTH and 1,25(OH)$_2$D as discussed earlier (Rude *et al.*, 1978; Fatemi *et al.*, 1991). Because 1,25(OH)$_2$D stimulates osteoblast activity (Azria, 1989) and the synthesis of osteocalcin and procollagen (Francheschi *et al.*, 1988), decreased formation of 1,25(OH)$_2$D may be a major cause of decreased bone formation, such as that observed in experimental Mg deficiency (Heroux *et al.*, 1975; Jones *et al.*, 1980; Kenney *et al.*, 1994). A decrease in bone-specific binding sites for 1,25(OH)$_2$-D has also been described, which could account for vitamin D resistance (Risco and Traba, 2004). Similarly, PTH has been demonstrated to be trophic for bone (Marcus, 1994) and therefore impaired PTH secretion or PTH skeletal resistance may result in osteoporosis.

Because insulin promotes amino acid incorporation into bone (Hahn *et al.*, 1971), stimulates collagen production (Wettenhall *et al.*, 1969), and increases nucleotide synthesis by osteoblasts (Peck and Messinger, 1970), insulin deficiency or resistance may alter osteoblast function in diabetes. However, insulin also causes an increase in intracellular Mg, and because Mg has been shown to be trophic for the osteoblast (Liu *et al.*, 1988), insulin deficiency may result in intracellular Mg depletion and impaired osteoblast activity. Serum IGF-1 levels have also been observed to be low in the Mg-deficient rat, which could affect skeletal growth (Dorup *et al.*, 1991).

Although the explanation just given may explain low bone formation, it does not explain the observation of an increase in osteoclast bone resorption. Each year about 25% of trabecular bone is resorbed and replaced in human adults, whereas only 3% of cortical bone undergoes remodeling (Papanicolou *et al.*, 1998). This suggests that the rate of locally controlled bone remodeling is important in the development of osteoporosis. Mg has been shown to inhibit the N-methyl-D-aspartate (NMDA) receptor (McIntosh, 1993). Activation of the NMDA receptor induces the release of neurotransmitters, such as substance P (McIntosh, 1993). Reduction of extracellular Mg lowers the threshold level of excitatory amino acids (i.e., glutamate) necessary to activate this receptor. In one study, dietary Mg deficiency produced raised serum levels of neuropeptides such as substance P and calcitonin gene related protein in rodents (Weglicki *et al.*, 1996a). This neurogenic response is followed by release of proinflammatory cytokines, such as TNFα, IL-1β, IL-6, by T lymphocytes during the first week of dietary Mg depletion (Weglicki *et al.*, 1996a; Weglicki *et al.*, 1996b; Kramer *et al.*, 1997). Many of the actions of substance P are mediated through the neurokinin 1 (NK-1) receptor. Elevated plasma cytokines and the inflammatory cardiac lesions observed in Mg-deficient rats have been shown to be prevented by administration of a NK-1 receptor antagonist (Weglicki *et al.*, 1996a; Weglicki *et al.*, 1996b; Kramer *et al.*, 1997). Studies have demonstrated that there are nerve fibers containing a number of neuropeptides including substance P in bone (Lerner, 2000). Substance P has also been shown to increase the release of IL-1β and IL-6 by bone marrow cells (Rameshwar *et al.*, 1994). These cytokines, which are systemically released as well as locally produced in the bone microenvironment, are known to stimulate the recruitment and activity of osteoclasts and increase bone resorption (Miyaura *et al.*, 1995; Nanes, 2003). Increased amount of substance P, TNFα, and IL-1β have been found in Mg-depleted mice (Rude *et al.*, 2003) and/or rats (Rude *et al.*, 2004; Rude *et al.*, 2005; Rude *et al.*, 2006). These cytokines could contribute to an increase in osteoclastic bone resorption and explain the uncoupling of bone formation and bone resorption observed in the rat. Increased production of these cytokines has been implicated in the development of sex steroid deficiency or postmenopausal osteoporosis (Pacifici, 1996; Manolagas *et al.*, 1995). Evidence also suggests that inducible nitric oxide synthetase is stimulated by these cytokines and may mediate localized bone destruction associated with metabolic bone diseases (Ralston, 1994; Brandi *et al.*, 1995). Mg-deficient rodents have increased free radical formation, which may affect the cytokine cascade and influence skeletal metabolism (Kramer *et al.*, 1997). These cytokines could contribute to an increase in osteoclastic bone resorption and explain the uncoupling of bone formation and bone resorption observed in the rat (Rude *et al.*, 1999; Rude *et al.*, 2004; Rude *et al.*, 2005; Rude *et al.*, 2006). The final pathway of osteoclastogenesis has been proposed to involve three constituents of a cytokine system: receptor activator of nuclear factor kB ligand (RANKL); its receptor, receptor activator of nuclear factor kB (RANK); and its soluble decoy receptor, osteoprotegerin (OPG) (Hofbauer and Heufelder, 2000; Manolagas, 2000). RANKL is a membrane-bound, cytokine-like molecule that is expressed in preosteoblastic cells. It stimulates the differentiation, survival, and fusion of osteoclastic precursor cells to activate mature RANK expressed in hematopoietic osteoclast progenitors, and serves as an essential factor for osteoclastic differentiation and activation. RANKL binds to RANK with high affinity and this interaction is essential for osteoclastogenesis. OPG is expressed in a variety of cell types, however, in bone it is mainly produced by cells of osteoblastic lineage. OPG has very potent inhibitory effects on osteoclast formation. It acts like a decoy receptor and blocks the RANKL/RANK interaction (Hofbauer and Heufelder, 2000). The relative presence of RANKL and OPG therefore dictates osteoclast bone resorption activity. Osteoclasts can be formed or activated in a RANKL and/or a RANKL-independent mechanism by TNFα (Hofbauer and Heufelder, 2001; Horowitz *et al.*, 2001; Nanes, 2003). The immunohistochemical presence of these two cytokines in Mg-deficient vs. control animals was examined and a decrease in OPG and an increase in RANKL were observed (Rude *et al.*, 2005). Whether or not these mechanisms for Mg-induced bone loss are valid for suboptimal chronic dietary Mg deficit in human osteoporosis is unknown.

Magnesium and Mineral Formation

Mg may also independently influence bone mineral formation. In *in vitro* studies, Mg has been shown to bind to the surface of hydroxyapatite crystals and to retard the nucleation and growth of hydroxyapatite and its precrystalline intermediate, amorphorous calcium phosphate (Blumenthal *et al.*, 1977; Bigi *et al.*, 1992; Sojka and Weaver, 1995). Mg has also been demonstrated to compete with calcium for the same absorption site on hydroxyapatite (Aoba *et al.*, 1992). Therefore, surface-limited Mg may play a role in modulating crystal growth in the mineralization process.

In vivo studies have demonstrated that as the Mg content of bone decreases, the hydroxyapatite crystal size increases, whereas high Mg content results in smaller crystals. Rats fed excess Mg have smaller mineral crystal in their bone than control pair-fed animals (Burnell *et al.*, 1986). In contrast, Mg-deficient rats have a significant increase in hydroxyapatite crystal size (Boskey *et al.*, 1992). Clinical studies are also consistent with this effect of Mg on crystal formation. A crystallinity index determined by infrared spectrophotometry has shown larger and more perfect bone mineral crystals along with decreased bone Mg in bone samples obtained from patients with diabetes mellitus, postmenopausal osteoporosis, and alcoholic osteoporosis (Blumenthal *et al.*, 1977; Cohen and Kitzes, 1981; Cohen *et al.*, 1983; Sojka and Weaver, 1995). These conditions are known to have a high incidence of Mg depletion. In contrast, uremic patients, characterized by high serum Mg levels, have smaller, less perfect crystals and high bone Mg (Blumenthal *et al.*, 1977; Cohen and Kitzes, 1981; Cohen *et al.*, 1983; Sojka and Weaver, 1995). An inverse correlation was found to exist between bone Mg and crystallinity index. The effect of these findings on crystallization in terms of bone strength and bone metabolism has yet to be elucidated.

Mg may also have another indirect effect on crystallization by influencing both osteocalcin formation and osteocalcin binding to hydroxyapatite. Osteocalcin has been shown to inhibit the conversion of brushite to hydroxyapatite and the nucleation of mineral formation (Wians *et al.*, 1990). Therefore, the decrease in osteocalcin production, as suggested by decreased serum and bone osteocalcin, in Mg depletion may influence mineralization. However, Mg has also been demonstrated to inhibit the binding of osteocalcin to hydroxyapatite by reducing the number of available hydroxyapatite-binding sites for osteocalcin (Wians *et al.*, 1983). Maximal inhibition occurred at 1.5 mM Mg, which is within the physiologically relevant concentration range.

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Chapter 25

Metals in Bone: Aluminum, Boron, Cadmium, Chromium, Lanthanum, Lead, Silicon, and Strontium

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INTRODUCTION

The mineral phase of bone, made up principally of calcium and phosphate, acts as a sink for metals in the blood that circulates to the skeleton. Uptake by bone mineral is a function of the metal's plasma concentration, its affinity for the bone mineral, and its effect on the extracellular matrix. It is also a function of the degree of mineralization of the skeleton. Bone lead content, for example, was found to be higher in persons who were on a low calcium intake and presumably had lower bone calcium than in individuals on high calcium intake (Hernandez-Avila *et al.*, 1996). If, in addition, the metal interacts with bone cells, their metabolism may be affected and this in turn may alter osteoblast and osteoclast function. Fluoride and bisphosphonates are examples of compounds that become part of the bone mineral, but also have an inhibitory effect on osteoclasts.

Once a metal becomes incorporated in the bone mineral, its return to the circulation depends on how much and how long the metal is associated with the bone mineral surface and on the rate of osteoclast-mediated bone turnover. Metals deposited or exchanged with other elements on the surface of the bone mineral tend to be exchanged rapidly. As additional bone mineral is deposited, the opportunity for isoionic or heteroionic exchange diminishes and osteoclastic resorption becomes the dominant process responsible for the metal's reentry into the circulation. There it is subject to the stochastic processes of loss from the circulation via excretion (urine and stool), redeposition in bone, or soft-tissue uptake. Excretion typically accounts for only a small fraction of the loss from the circulation, but constitutes the only significant route to clear the organism of the metal load. Bone turnover is greater in trabecular than in cortical bone and differs in regions of the skeleton. Metal is taken up in the skeletal regions with the highest turnover rate, whereas retention is highest in regions with lowest turnover. Bone may therefore be thought of not only as a metal reservoir, but as the storage site of body burdens.

Conditions that affect the rate of bone turnover may alter uptake of a given mineral from the circulation. For example, fetal bone formation and calcification are at a maximum during the third trimester of pregnancy, when deposition of maternal bone mineral in the fetal skeleton becomes important (Franklin *et al.*, 1997). This is even more so with a low-birth-weight newborn (Gonzalez-Cossio *et al.*, 1997). Similarly, end-stage renal failure patients with secondary hyperparathyroidism mobilize lead or lanthanum at a significantly greater rate than do healthy persons. This rate is dramatically decreased following parathyroidectomy (Kessler *et al.*, 1999).

The time-dependent increase in the concentration of a bone-seeking metal is due to continued input, as true, for example, in smelter workers, but the increase in the blood will be far less steep and may seem to reach an equilibrium. This is why measuring the level of a metal on the bone surface (Farias, 1998) may be a better approach to estimating the body burden.

ALUMINUM

Interest in the interaction between aluminum (Al) and bone was stimulated as a result of the observation that patients with renal dystrophy accumulated aluminum in their skeleton in quantities that tended to exceed those accumulated by patients with comparable rates of bone turnover, as in hyperparathyroidism (Goodman and Duarte, 1991). Moreover, all accumulation was markedly enhanced as a result of long-term dialysis, leading to bone disease characterized by impaired mineralization and diminished bone cell activity (Goodman and Duarte, 1991). Bone disease

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of this type has also been described in individuals with chronic renal disease who are not on dialysis (O'Brien *et al.,* 1990) and in persons receiving total parenteral nutrition (Klein *et al.*, 1982), as well as in aluminum welders (Elinder *et al.*, 1991). In patients with chronic renal failure, Al poisoning may lead to one of three disorders: aluminum-induced bone disease, microcytic anemia, and encephalopathy (Hellstrom *et al.*, 2006).

Aluminum is the third most common element in the crust of the earth, is its most abundant metal, and has found wide application in a variety of industries (Hellstrom *et al.*, 2006). Aluminum is present in many foods and food containers.

The majority of healthy subjects have Al plasma concentrations that approximate 2 μg/liter (Sharp *et al.*, 1993), a concentration that reflects the Al content of the diet. Al retention is said to be 4% in healthy subjects with normal renal function (Hellstrom *et al.,* 2006). With age, the bone content of Al increases, as it does with increased Al input into the body (Klein, 2005). Al is a contaminant of solutions used for intravenous support of hospitalized or ambulatory patients (Klein, 1995) and is a major constituent of many commercial food containers. As with all bone-seeking metals, as the input of Al from the environment continues over a lifetime, the plasma Al concentration will rise, though relatively slowly, but bone content is proportional to the accumulation rate. Therefore the increase in bone content of Al will be much steeper than would appear on the basis of the time-dependent rise in plasma Al concentrations. This is undoubtedly the reason why Kausz and colleagues (1999) concluded that a patient's plasma Al level does not predict well the presence of aluminum bone disease.

The effect of greater Al input from the food and the environment has not been explored systematically, nor have differences due to gender (Sharp *et al.*, 1993) or metabolism (absorption, excretion and bone turnover). Al accumulation in bone increases not only due to increased or longer-lasting intake, as in old compared to young persons, but also due to decreased capacity for excretion, as in renal osteodystrophy. Bone accumulation of Al can also increase when turnover is diminished, as in diabetes mellitus (Pei *et al.*, 1993). At sufficiently high concentrations, as may occur in newly laid bone or at bone mineral surfaces, Al inhibits mineralization and acts on bone cells, although it has been difficult to separate physicochemical from biological effects (Goodman and Duarte, 1991).

Aluminum appears to enter skeletal tissue by the same routes as calcium. It inhibits hydroxyapatite formation *in vitro* (Blumenthal and Posner, 1984). Data on the *in vivo* effect of Al on bone mineralization point to the overall toxicity of Al. Al concentrations that interfere with calcification *in vivo* are bound to be higher than when Al is added to bone cells in culture. Bouglé *et al.* (1998) report that in low-birth-weight infants mineral density and mineral content of the lumbar spine decreased significantly as serum Al levels increased. This was not true for full-term infants. One explanation may be that in low-birth-weight infants there is less bone mineral than in full-term infants. Therefore the amount of Al that interferes with initiation and progression of bone mineralization in the low-birth-weight newborn is too small to have the same effect in the full-term neonate. Similarly, patients with end-stage renal disease (ESRD) undergoing dialysis have less bone than comparable healthy adults and their Al body burden is relatively greater than in healthy controls. Moreover, as pointed out earlier, they may, as a result of undergoing dialysis, be receiving a greater Al load, so that their bone Al load is greater both relatively and absolutely than that of healthy subjects.

Bone uptake, as stated previously, is proportional to the Al concentration of the plasma. However, once Al is taken up by bone, it is the relative distribution of surface to deep bone Al and the rate of transfer from surface to interior that will determine what remains in that bone site. Cancellous bone, constituting about 20% of the skeletal mass in normal individuals, but perhaps less in patients undergoing dialysis, turns over faster than cortical bone (Marshall *et al.,* 1973). For this reason, the Al content of cancellous bone may be smaller than that of cortical bone. This has been reported for strontium (Boivin *et al.,* 1996).

The plasma Al concentration at any time reflects Al ingestion, the fraction of ingested Al that is absorbed, and the rate of excretion, as well as the amount of Al that is released by osteolysis. High bone levels, to be sure, indicate high prior exposure, but single plasma or bone analyses are unlikely to provide information on Al toxicity. This statement is illustrated by the report of Suzuki *et al.* (1995), who found significant bone accumulation of Al in patients on chronic hemodialysis, even though the water and dialysis fluid provided over the preceding decade had contained less than 10 μg Al per liter. Goodman and Duarte (1991) point out that Al may have to reach concentrations of 30 to 40 mg Al/kg dry bone before affecting bone structure and function. That concentration is equivalent to 1% to 2% of the calcium content of bone (Widdowson and Dickerson, 1964), whereas the Al concentration in bone of normal subjects is only 5–7 mg/kg dry bone (Hodsman *et al.*, 1982). Similarly, Hellstrom *et al.* (2006) report no significant association between the Al content of bone and the bone mineral density or content of the femoral neck. The authors conclude that the Al content of the skeleton over a lifetime has no effect on the incidence of osteoporosis. A higher than normal Al intake at the time bone mass begins to decrease, typically in the fifth decade, may, however, enhance the development of osteoporosis.

In addition to inhibiting the formation of calcium hydroxyapatite, detectable only at high rates of Al entry into the skeleton, Al also interferes with the formation of calcified and uncalcified nodules in primary cultures of neonatal mouse calvarial cells (Sprague *et al.*, 1993). Those nodules are specific for osteoblastic calvarial cells. Bellows and colleagues (1995) have shown that Al inhibits *in vitro* initiation and progression of osteoid nodule mineralization. Working with long-term rat calvarial cell cultures, these investigators showed that Al initially accelerated the rate of osteoprogenitor cell differentiation. Al also initially accelerated the formation of osteoid nodules, at the same time inhibiting mineralization. Ultimately, however, Al exerted toxic effects, with nodules and matrix disintegrating by days 17 to 19 of the cultures (Bellows *et al.*, 1999). Kidder *et al.* (1993) have reported that Al suppressed proliferation of marrow fibroblast-like stromal cells and of calvarial osteoblasts (cf. Sprague *et al.*, 1993). Interestingly, in more mature, confluent cultures, Al addition stimulated DNA synthesis and collagen production even in the absence of 1,25-dihydroxycholecalciferol (Kidder *et al.*, 1993). It is uncertain whether fluoride inhibits Al accumulation in rat bone (Ittel *et al.*, 1993) by interfering with binding to the osteoclasts, by blocking Al from substituting for calcium in hydroxyapatite, or whether Al does both. Iwasaki *et al.* (2006) point out that uncalcified osteoid covers the bone surface in osteomalacia, a condition that can also be induced by Al accumulation.

Goodman and Duarte (1991) state that the amount of surface stainable aluminum is the best available indicator of aluminum toxicity and estimate that when surface levels of Al exceed 30%, bone formation and/or mineralization are adversely affected. *In vivo* neutron activation is the current method for measuring Al accumulation in the body noninvasively. Pejovic-Milic and colleagues (2005) have published a neutron activation method with a detection limit of 0.24 mg Al, superior to the method at Brookhaven National Laboratory. Both methods use thermalized neutrons.

Al toxicity that results from Al accumulation is a well-known complication of patients in chronic renal failure. Chelators are the treatment of choice for ridding the body of metals. In the case of Al toxicity, desferrioxamine treatment and elimination of all exogenous Al sources are indicated, with careful attention paid to avoid or minimize side affects, including infections (D'Haese *et al.*, 1996). Two experimental studies are of interest in this connection: Haynes and colleagues (2004) report that aluminum ingestion of eucalypt leaves, part of the natural diet of koalas, small mammals native to Australia, is associated with a high incidence of renal failure in these animals. Baydar *et al.* (2005) report that rats fed folic acid in high-Al diets for 8 weeks had lower Al accumulation in bone and in a variety of soft tissues.

Specific mechanisms by which Al acts on bone and other cells have not been elucidated. Jeffery *et al.* (1996) discuss possible effects on cell signaling, mechanisms by which Al inhibits hemoglobin synthesis, and effects of Al on PTH secretion. These reviewers also list a series of recommendations for further study of the multiple actions of Al in the mammalian organism.

One topic that has received attention is the result of incorporating Al in a total metal-to-metal hip replacement. Grubl and colleagues (2006) report that 1 year after hip replacement, there was no change in the serum Al levels of 13 patients. Inasmuch as the rates at which plasma levels of metals increase with metal input are relatively modest, it is not surprising that plasma levels of Al did not increase significantly in a year. No bone measurements were made by these investigators. .

Lima *et al.* (2006), who studied the effect of a metal alloy made of zinc, aluminum, and copper—Zinalco—implanted into bone, found all three metals diffused into tissue, thereby "promoting nonhomogeneous bone." Cointry and colleagues (2005) studied the effect of Al accumulation in rat cortical bone and concluded that Al intoxication (27 mg Al/day for 26 weeks) significantly reduced the load bone can sustain and the postyield fraction of that load. Treatment reduced mineralization of the cortical bone and had a negative impact on bone stiffness and on the yield stress of the bone.

Notwithstanding the deleterious effects of high-level deposits of Al in bone, most reports of healthy aging subjects have found no demonstrable effect of long-term normal Al intake (Hellstrom *et al.*, 2006). This is not true for individuals with diminished renal function or patients on dialysis. For example, in a study by London *et al.* (2004) of arterial calcification in 58 patients with ESRD on hemodialysis, patients with elevated arterial calcification scores had low numbers of osteoclasts, smaller osteoblast surfaces, and a high percentage of aluminum-stained surfaces. Thus there seems to exist a relationship between low bone turnover and adynamic bone disease, on one hand, and arterial calcification, on the other. For this reason it is important for clinicians to remain alert for potential Al effects when dealing with patients with diminished renal function—not uncommon among the elderly—or patients with renal disease.

BORON

It is uncertain whether boron, B, an essential element for many plant species, is essential for mammals. However, as reported by Nielsen and Hunt (cited by King *et al.*, 1991), a low-B diet appears to exacerbate the effects of vitamin D deficiency in chicks, B supplementation reducing the effects of vitamin D deficiency. It was therefore suggested that B may play a role in bone metabolism. Moreover, Nielsen *et al.* (1987) had reported that increasing the dietary intake of B from 0.25 to 3.25 mg/day in postmenopausal women increased plasma estradiol and testosterone concentrations and decreased urinary calcium output.

A more extensive study on the effect of dietary B supplementation (5 ppm per day for 5 weeks) on the action of estrogen in ovariectomized rats, carried out by Sheng *et al.* (2001), found that B alone had no effect on various bone parameters in these animals, but when the ovariectomized rats were treated with estrogen and B, the addition of B significantly enhanced the beneficial effects of estrogen on bone quality.

For this reason, B may play a role in postmenopausal osteoporosis. This possibility was investigated by Beattie and Peace (1993), who studied six postmenopausal women volunteers on a metabolic ward. Each subject took two levels of B, 0.33 and 3.33 mg/day, for 3 weeks. B had no effect on mineral, steroid, or urinary pyridinium crosslink excretion, the last a measure of collagen turnover. As the subjects shifted from the low- to the high-B diet, Ca absorption and urinary Ca excretion increased. The stimulation of calcium metabolism may have been the result of supplementation with an adequate amount of B. However, this does not exclude an action of B on some pathway other than calcium. This inference is confirmed by a study of Armstrong and colleagues (2002), who reported that in young pigs on a low-B diet that was supplemented with B, the bending moment of femurs was improved, but that B supplementation to a diet with a normal B content (6.7 mg B/kg diet) had no effect. In 2001 Armstrong and Spears found that increasing B from 5 to 15 mg/kg diet increased growth and bone strength in barrows (male pigs), but it is not clear whether the higher dietary B concentration represents a nutritionally adequate level. Finally, Armstrong and colleagues, a year later, studied the effect of B addition at 5 mg/kg to a diet for gilts (young sows) that is otherwise low in B and found that B addition had beneficial effects on reproduction and bone characteristics. It thus appears that some basic dietary content of B is needed for normal metabolism, including that of bone, but neither the precise amount nor the mechanism of action—whether direct or indirect—has been established.

Hegsted and colleagues (1991) studied the effect of B addition on vitamin D deficiency in rats. They placed weanling rats, 21 days of age, on a vitamin D–deficient diet, and 12 weeks later, when both the B-supplemented and B-deficient rats were hypocalcemic, the supplemented groups had higher net calcium absorption and were in somewhat more positive balance. However, there were no effects on soft-tissue calcium levels and none on a variety of bone parameters (bone mineral density and length of femur, bone and ash weight, bone Ca, Mg, and P). Hypocalcemia, which can be brought about in 2 to 3 weeks when the rats are on a low-calcium diet, takes much longer to develop and is less severe when calcium intake has been high (Bronner and Freund, 1975). It is also uncertain whether these animals developed genuine vitamin D deficiency, because their intestinal calbindin D_{9k} content, the molecular measure of vitamin D deficiency (Bronner and Freund, 1975), had not been determined.

In a careful study of the effect of B on chick nutrition, Hunt and colleagues (1994) found that B addition modified the effects of vitamin D deficiency and proposed that the plasma B level is homeostatically regulated. The effects on vitamin D_3 deficiency were minor and the inference concerning B homeostasis was not based on rigorous experimentation, because urinary B output was not measured. Conceivably a zero intake of B may aggravate metabolic defects due to vitamin D deficiency, but B is so widely distributed in nature that a genuine B deficiency can probably be achieved only under strict laboratory conditions.

Chapin *et al.* (1997), utilizing young adult male rats, have studied the effect of increasing B intake, in the form of boric acid, from 0 to 9000 ppm boric acid for 9 weeks. They found that bone B had increased in all treated animals and that even though within 1 week of the cessation of feeding B in the diet, serum and urine B values had dropped to normal, bone retained its B level for as long as 32 weeks after cessation of the B diet. The only change in bone these investigators found was a 5% to 10% increase in vertebral resistance to crush force. The authors point out that these increases occurred at exposure levels that were "substantially below those that were previously reported to be toxic."

Probably the most conservative conclusion from the dietary effect of B, whether on bone metabolism alone, on the metabolism of Ca and P, or on overall body function, including reproduction, is that some dietary level of B is needed for full expression of body function, but that supplementation beyond that level probably has no effect.

Finally, a novel B polymerization technique applied to a material for craniofacial bone repair has been shown to be sufficiently biocompatible to indicate a potential for use of the material (Gough *et al.*, 2003).

CADMIUM

Cadmium intoxication, whether acute or chronic, is principally the result of heavy metal mining, i.e., for lead, zinc, or copper, with Cd often not the object of the mining process, but constituting a contaminant. Cd mining, as in certain areas of Belgium, and Cd smelting also constitute major sources of Cd and lead to Cd toxicity in exposed workers. The most dramatic and attention-drawing incident of Cd poisoning occurred in Japan during the latter part of World War II, although the nature of the disease, which became known as the itai-itai disease, and its relationship to Cd poisoning were not fully understood until the 1960s (Nogawa, 1981). *Itai* is Japanese for the exclamation "ouch," associated with tenderness and pain to the touch. The main symptoms of this disease were osteomalacia in postmenopausal women, traced to a high Cd content of rice grown in certain areas whose irrigation water came from a river that had become severely contaminated with Cd because of upstream mining (Nogawa, 1981; Tsuchiya, 1981).

The three organ systems that are principally affected by Cd poisoning are the respiratory system, implicated particularly in acute poisoning due to Cd contamination of dust, the kidney, and the skeleton. Principal renal symptoms are proteinuria, glycosuria and microglobulinuria. Osteomalacia and osteoporosis are the skeletal symptoms that have been identified in patients with itai-itai disease and in others that have had a chronic low-dose exposure for a long time (Tsuchiya, 1981). Uriu and colleagues (2000) have shown that "chronic Cd exposure exacerbated the uncoupling between bone formation and resorption in ovariectomized rats." Their findings thus add weight to the reported bone effects of chronic Cd exposure, including decreased mechanical strength. In a similar earlier study, Hiratsuka *et al.* (1997) also showed that chronic Cd intoxication caused osteomalacic lesions in ovariectomized rats. In individuals with skeletal symptoms, calcium deficiency aggravated the disease, and high doses of vitamin D, leading to increased calcium absorption, overcame or minimized the symptoms (Nogawa, 1981).

The effect of Cd is very much a function of the dose taken in Cd appears to have an effect on epithelial cells in the intestine (Hietanen, 1981) and to react with bone cells. It causes diminished calcium absorption and increased calcium loss from bone (Wilson and Bhattacharyya, 1997). It appears to bind to cells as well as to proteins, causing cell desquamation in the intestine and changes in cell-to-cell binding in the kidney. The latter leads to direct or indirect interference with the hydroxylation of 25-hydroxyvitamin D_3 (Kjellstrom, 1992), so that the biosynthesis of the intestinal and renal calbindins is diminished (Kimura, 1981; Sagawara, 1974). This in turn leads to a diminution of the active, transcellular transport of calcium in the duodenum (Bronner *et al.*, 1986), and to diminished active reabsorption of calcium in the distal convoluted tubule (Bronner, 1989, 1991). Moreover, Cd binds to calbindin 9kD, displacing Ca^{2+} (Fullmer and Wasserman, 1977), so that active calcium transport is interfered with. A direct linear relationship exists between Cd intake and calcium excretion in the urine (Nogawa, 1981), doubtless due to Cd-induced damage of the tight junctions of the renal tubule. As a result, less calcium is reabsorbed in the renal distal tubule and calciuria results. Thus, Cd input induces Ca loss.

The effect of Cd on bone is a dual one: direct interaction with bone cells, diminishing their ability to mineralize (Miyahara *et al.*, 1988), inhibiting procollagen C-proteinases (Hojima *et al.*, 1994), thereby preventing collagen self-assembly in the extracellular matrix and effectively decreasing collagen production (Miyahara *et al.*, 1988). Cd may also affect gene expression. Regunathan and coworkers (2003) developed an *in vivo* model for cadmium-induced bone loss. In this model mice excrete bone mineral in the feces after Cd gavage. Using microarray to evaluate gene changes in bone cells, the authors hypothesize on the basis of their results that Cd stimulates demineralization via a P38 MARK pathway that activates osteoclasts.

Brzoska and colleagues have in recent years carried out extensive studies in a rat model of human exposure to Cd (Brzoska *et al.*, 2005a, 2005b, and added self-citations in these papers). Their overall results, qualitatively similar to what has been reported by others (see earlier discussion), indicate that low-level Cd exposure affects the mineral and biomechanical properties of growing bone, with collagen and glycosoaminoglycan affected. Thus the mechanical weakness of the femur, the bone studied most extensively by the Polish group, can be attributed to a decrease in the cross-linking of collagen, leading to increased collagen solubility (Galicka *et al.*, 2004), to diminished regulation of fibril assembly, diminished calcium supply, and, as discussed in the next paragraph, to inhibition of hydroxyapatite nucleation and growth. Brzoska *et al.* also observed that male rats were less susceptible than female rats to the effects of Cd. A possible explanation, as also for Al (cf. previous section), is that the female skeleton is smaller than that of males and therefore the amount of Cd ingested during the experimental period is proportionately greater in the female than the male.

Blumenthal and colleagues (1995) report that Cd has an inhibitory effect on hydroxyapatite formation *in vitro* and suggest that "the interference of Cd with mineralization can be partially explained by its inhibitory effect on hydroxyapatite nucleation and growth." This would be in addition to any direct effect of Cd on bone cell function. Thus, Long (1997) has reported that the concentration of 200 to 500 mM Cd in the culture medium caused changes in cell morphology and a decrease in osteoblast and osteoclast number and in alkaline phosphatase activity. All of these changes are likely to contribute to diminished collagen production and impaired mineralization. A second, indirect effect of Cd is to accelerate bone turnover, particularly bone resorption (Chang *et al.*, 1981), a result of the induced calcium deficiency. It is uncertain whether the increase is due to stimulation of parathyroid hormone release in response to hypocalcemia.

Shank and Vitter Vitter (1981) have calculated that if Cd is administered five times, with intervals of 48 hours between each administration, then 48 hours after the last dose the liver content would account for about two thirds of the dose and the kidney for 7% to 8%. It is not surprising, therefore, that bone effects are manifested only with chronic exposure, as a result of Cd acting directly on bone cells, modulating hydroxyapatite formation, as well as inducing changes in calcium metabolism. Because these changes are similar to those of calcium and vitamin D deficiency, bone effects resulting from Cd accumulation are aggravated by conditions that intensify or aggravate calcium needs.

It is not surprising therefore that Cd may be a risk factor for osteoporosis (Jarup *et al.*, 1998; Kazantzis, 2004), a reason for studies like those by Brzoska and colleagues.

CHROMIUM

The element chromium, Cr, belongs to the first series of the transition elements, occurs in several oxidation states, with the trivalent being the most stable (Mertz, 1969). Cr is thought to be an essential micronutrient. It appears essential for optimal glucose utilization and its deficiency "can be a cause of or an aggravating factor in the glucose intolerance of infants" that suffer from protein-calorie insufficiency or have non-insulin-dependent diabetes (Hambidge, 1974). Its role in skeletal metabolism is largely unexplored, with interest stimulated by the increasing use, especially in elderly adults, of metal-containing prostheses, where Cr constitutes part of the alloy. For example, Berry *et al.* (1993) have reported that extensive osteolysis occurred around an aseptic, well-fixed, stable, uncemented total knee prosthesis and concluded that debris resulting from wear, in the form of polyethylene, metal, or both, may be responsible for the breakdown of bone. For this reason, Sankaramanivel *et al.* (2006), on the hypothesis that chromium interferes with bone remodeling, treated rats with chromium intraperitoneally for 5 days and found, as expected, significant accumulation of chromium in the skeleton and reduced activity of alkaline phosphatase and tartrate-resistant acid phosphatase, with changes in bone formation rate and structure that suggested an alteration in bone turnover. There was also an unexplained rise in serum phosphate. Dunstan *et al.* (2005) studied urine and blood levels of various metals, including Cr, in patients who had metal-on-metal or metal-on-polyethylene articulations *in situ* for over 30 years. Urinary excretion of Cr was significantly elevated in all arthroplasty groups, compared to controls, with a tendency for the urinary output to increase more in the metal-on-metal groups, particularly those with loose articulation. In patients with loose metal-on-metal articulations there was a huge increase in the urinary cobalt output, cobalt being part of the metal prosthesis. It seems logical to infer that more body fluids can enter loose than tight articulations and cause more metal to be dissolved. This inference is supported by the report of Chojnacka (2005), who found that animal bones are an efficient sorbent for Cr, with sorption a function of Cr concentration.

Kinetic analyses of the distribution of Cr in the body have shown (Onkelinx, 1977) that in rats, about 40% of the ^{51}Cr that is lost out of the central compartments flows to a "sink", consisting of various soft tissues and bone. Total bone content is some 2.5 times higher than that of all other tissues after 262h, i.e. when uptake approaches a plateau. DoCauto *et al.* (1995) did a kinetic study in humans and found that the compartment with slowest turnover, presumably similar to the "sink" in the rat study (Onkelinx, 1977), reached a near-plateau of about 35% of the injected dose of Cr(III) between days 7 and 58 after dose administration; thereafter this compartment began to empty out, so that by day 248 it contained only 17% of the dose. In the rat study (Onkelinx, 1977), the bone gained Cr with time, whereas the other tissues either lost or held on to their Cr. Thomann *et al.* (1994) have identified a "major storage compartment" in rats that received Cr in their drinking water for 6 weeks and were studied 140 days later, a period during which they no longer received Cr. The half-life of Cr in that storage compartment, made up of bone, skin, hair, and muscle, was in excess of 100 days. Their study thus confirms the essential findings reported by Onkelinx (1977). Thomann *et al.* (1994) suggest, as can also be inferred from the Onkelinx (1977) study, that the storage compartment functions to maintain "elevated body burdens and tissue concentrations of Cr." In other words, Cr, as true for other metals stored in bone, is released from bone mineral by ion exchange and by osteoclast-mediated resorption Cr and thus constitutes a Cr reservoir.

Cr deficiency or Cr excess leads to bone changes. Deficiency is difficult to produce in the laboratory and is unlikely to be encountered in humans, even though low intakes in the elderly have been associated with glucose intolerance (Hambidge, 1974). The association of high local metal concentrations including Cr with failed joint prostheses has been reported (James *et al.,* 1993; Dunstan *et al.,* 2005), but the specifics of how Cr excess acts on bone cells and tissues have not. A report by Gralak and colleagues (2002) may be of interest in this connection. These investigators found that a semipurified diet containing 10% soybean led to lower bone content of Cr in rats fed the diet for 28 days than in controls fed the diet without soybean.

LANTHANUM

Lanthanum, La, atomic weight 138.09, is a rare-earth element with chemical affinities similar to those of Ba, Sr, and Ca. It occurs naturally at low levels in drinking water and some foods. It is of interest because in recent years lanthanum carbonate has been studied as a possible replacement for calcium-based phosphate binders, which, for many years, have constituted the principal treatment for lowering the plasma phosphate of hyperphosphatemic patients with ESRD. The reason for replacing calcium-based phosphate binders is that the high intake of calcium has in some patients led to hypercalcemia and cardiovascular calcification, with an increase in the risk of death from cardiovascular disease. Whereas calcium absorption even at high intakes exceeds 10% (Bronner, 1991), La absorption is extremely low, less than 0.002% (Bronner *et al.,* submitted). As La enters the circulation, it is deposited in the liver during the first pass, is endocytosed into liver cell cytosomes and then appears to be transported directly to the bile (Yang *et al.,* 2006). The remainder of the La in circulation is either excreted in the urine or deposited in bone, both at the bone surface and in the bone interior, with the rate of La movement from surface to bone much slower than in the case of Ca (Bronner *et al.,* submitted). With continued high intake of La, as in hyperphosphatemic ESRD patients, bone becomes the major site of La in the body. Yet, the total quantity of La accumulated in bone as a result of ingesting 3 g elemental La daily for a decade is predicted, on the basis of a four-compartment model based on experimental findings, to be less than 70 mg, with about 90% in deep bone, the mineral surface of the skeleton accounting for some 9% (Bronner *et al.,* submitted).

When La intake stopped, La was lost from bone at a rate of 13% per annum in a dialysis population (Bronner *et al.,* submitted). This rate of loss, due to ionic exchange at the bone surface and osteoclast-mediated bone resorption leading to bone turnover, is much higher than that estimated for healthy adults (Marshall *et al.,* 1973). Hyperphosphatemic ESRD patients tend to have high bone turnover because of secondary hyperparathyroidism. Also, La tends to accumulate at the bone surface; this increases the opportunity for bone surface La to exchange with the body's fluid compartments. Specific differences notwithstanding, the overall fate of La in the body is similar to that of calcium.

LEAD

Lead (Pb) contamination of the environment, largely due to the widespread use of Pb compounds in paints and in gasoline, has become a major public health problem. Although the use of Pb-containing gasoline has been severely restricted in the United States, and although paints, both exterior and interior, are now formulated without Pb in many countries, Pb contamination continues to remain an important problem throughout the world. As is true for many trace elements, the major body site of Pb is the skeleton (Aufderheide and Wittmers, 1992). Even if Pb ingestion or inhalation is stopped, Pb from bone continues to enter the circulation as bone turns over (Durbin, 1992). Consequently the skeleton is the major deposit site of Pb and is also the principal source of endogenous Pb (Berglund *et al.*, 2000). Rust *et al.* (1999) have reported that even when removal of Pb paint reduces a child's exposure by 50%, the blood level of Pb may decrease only 25%. This can be explained by the fact that Pb absorbed from the diet and released from bone turnover are the two major sources of Pb in the circulation. Bone turnover is a continuing process, at an average rate of 3.6% per year in healthy adults (Marshall *et al.,* 1973) and perhaps at twice that rate in children. It is Pb that enters soft tissues from the circulation that constitutes the principal health hazard.

It may be obvious that greater bone turnover leads to greater Pb release. A telling illustration is the report by Hac and Krechniak (1996), who showed that after cessation of Pb exposure, the accumulated Pb content of rat hair declined very rapidly to the preexposure level, whereas in that same period only one third of the accumulated bone Pb was lost, hair turning over much faster than bone. Moreover, as indicated earlier, a portion of resorbed bone that enters the circulation reenters bone (Manton, 1985).

The importance of bone as a reservoir for Pb and the effects of bone turnover have been illustrated in several reports. Stack, in his review (1990), points out that the bone Pb level is a positive, linear function of the blood level. Even when the blood Pb concentration reaches a plateau, bone Pb will continue to go up, although ultimately the bone Pb level will also plateau (Stack, 1990). Gulson *et al.* (1998) have shown that the blood Pb concentration in women who breast-fed their infants was significantly higher than during their pregnancy. Breast milk calcium is partly of skeletal origin (Bronner, 1960), and women who breast-feed have higher bone turnover.

The importance of bone turnover is exemplified by the report that Pb is a risk factor for hypertension (Houston and Johnson, 1999; Hu *et al.*, 1996). A possible explanation may be that higher calcium intakes favor lower blood pressure (McCarron *et al.*, 1989) and that Pb in bone, in replacing calcium, lowers, if only slightly, the blood calcium level. This in turn may affect blood pressure by lowering angiotension release or by an indirect effect on the vasculature. This inference derives support from deCastro and Medley (1997), who, on the basis of blood pressure and blood Pb measurements in high school students, suggested a possible association between chronic bone Pb accumulation and later adolescent hypertension. This has been also observed in older adults. Working with a community-based cohort of 964 men and women, aged 50 to 70 years, Martin *et al.* (2006) report that the blood lead level was a strong and consistent predictor of systolic and diastolic blood pressure, whether or not the analysis was adjusted for race/ethnicity and socioeconomic status. Moreover, this was true for recent doses of lead and their acute effect on blood pressure, as well as for cumulative doses from chronic lead exposure.

To study health effects of Pb, blood is sampled and plasma is analyzed, with inductively coupled plasma/mass spectrometry currently the most reliable procedure. Timchalk and colleagues (2006) gave rats oral doses of Pb by gavage and observed that Pb saliva concentrations correlated well with plasma concentrations. Because saliva sampling is less invasive than blood sampling, this may be a useful approach to epidemiological studies of Pb.

When Pb is ingested, it largely follows the routes of calcium. It binds to calbindin (CaBP 9kD) in the duodenum; therefore vitamin D enhances Pb absorption (Fullmer, 1992). In situations of calcium deficiency, when intestinal (calbindin levels are high, Pb absorption is enhanced (Fullmer, 1992). On high calcium intakes, most of the calcium and therefore, presumably, most of the Pb is absorbed by the paracellular route, largely in the ileum (Marcus and Lengemann, 1962; Pansu *et al.*, 1993; Duflos *et al.*, 1995). Pb that has entered the body fluids leaves these via the urine, via the intestine and by entry into bone. Figure 1 is a model of the rates of Pb entry to and return from the two major bone compartments, cortical and trabecular, and

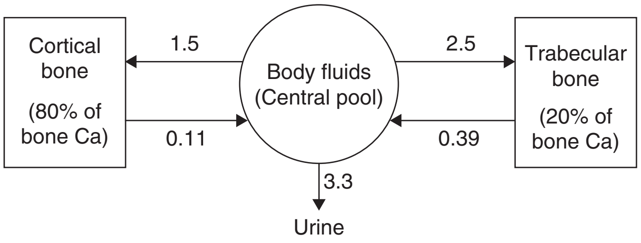

FIGURE 1 Model of how the adult body handles lead. The arrows refer to turnover rates per year. Based on data of chronically exposed lead workers (Christofferson *et al.*, 1987).

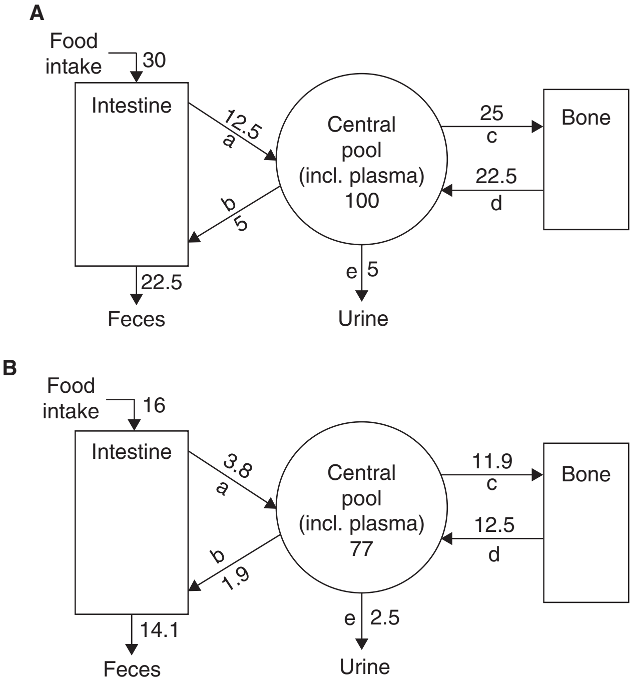

FIGURE 2 Models of calcium metabolism in a 14–year-old girl (**A**) and a 62–year-old postmenopausal woman (**B**). The arrows refer to flows, mmol Ca day^{-1}; the units for the central pools are mmol Ca (from Bronner, 1994).

of the loss rate via urine. The model, derived from findings in chronically exposed Pb workers, does not take into account losses of endogenous Pb in the stool; O'Flaherty (1993) states that the ratio for urinary Pb clearance to that of endogenous Pb in the feces varies from 1:1 to 3:1. O'Flaherty (1993) also states that Pb behaves like calcium in movements into, within, and out of bone. If one assumes that Pb in general behaves like calcium, one can calculate from. Figure 2 that when Pb leaves the body pool, it has a 70% chance of entering bone, whereas the corresponding figure from Fig. 1 would be 55%, neglecting endogenous fecal output. Also, according to Figure 1, nearly two thirds of the Pb flow would be to cortical bone and a little over one third to trabecular bone. However, trabecular bone turns over faster than cortical bone (Marshall *et al.*, 1973); therefore, more than one third of Pb reentering the circulation from bone would be of trabecular origin. This may be the reason why menopausal and postmenopausal women, whose loss of bone is largely trabecular, have higher blood levels of Pb than premenopausal women with a comparable exposure history (Potula and Kaye, 2006; Potula *et al.*, 2006). Potula and Kaye (2006) call attention to the fact that in menopausal women who have received hormone replacement therapy, blood and bone Pb levels were lower than in untreated menopausal women. This can be attributed to the higher bone turnover of untreated menopausal women, inasmuch as hormone therapy after menopause reduces bone loss (Lindsay *et al.*, 1976, quoted by Potula and Kaye, 2006). Also, in the population of women who had been exposed to lead as a result of working in a lead smelter, women whose menopause was induced tended to have lower Pb levels than women who had had a natural menopause (Potula and Kaye, 2006). Women whose menopause is induced tend to be younger than the typical, menopausal woman; their period of bone loss is longer and their Pb blood levels should be higher. However, a much larger percentage of women with induced menopause were on hormone replacement therapy than women with a natural menopause and as a result the Pb levels of the women with natural menopause were higher.

Rabinowitz *et al.* (1976) did a kinetic analysis of Pb, given as a stable isotope tracer, in five healthy male volunteers and found that 54% to 78% of the Pb leaving the blood per day was excreted in the urine, and that the body pool of Pb consisted of three compartments, with the third and largest assigned to bone. Pb in the first compartment had a mean life of 36±5 days, of 30 to 55 days in compartment 2, and a much longer life (10^4 days) in compartment 3. Pb absorption was calculated to vary from 6.5% to 13.7%, averaging 10%.

O'Flaherty (1991) has listed the various routes by which Pb, like calcium, enters bone, i.e., exchange with calcium in the bone mineral, and accretion, i.e., the net transfer into a single microscopic volume of bone, by either increase in volume (apposition) or mineralization (increase in density). The end result is that some 90% of the body burden of Pb accumulates in bone (Aufderheide and Wittmers, 1992). Because the surface uptake or binding of calcium and therefore presumably of Pb to the bone mineral is the dominant process of entry into bone (Bronner and Stein, 1992), the bone surface tends to have the highest Pb content. Ultimately, some of the surface Pb is lost by removal or by being "buried" by newly deposited bone mineral. For this reason, the microdistribution of Pb in bone is of importance; that is why bone surface content of Pb has been considered more important for evaluating the body Pb burden than total bone content. How this can and should be evaluated is controversial (Jones *et al.*, 1992).

Some studies have reported on the effect of Pb addition on cultured bone cells. Schanne *et al.* (1989) found that Pb concentrations in the culture medium of 5 and 25 μM increased the intracellular free calcium ion concentration in ROS 17/2.8 cells, classified as osteoblastic osteosarcoma cells, and speculated that Pb toxicity may be mediated by disturbances of intracellular [Ca^{2+}]. However, it seems unlikely that raising the intracellular [Ca^{2+}] from 0.13 to 0.25 μM would significantly impair the cell's ability to function. In a later paper these authors (Schanne *et al.*, 1992) report that the addition of Pb to a culture of ROS 17/2.8 cells interfered with a 1,25-$(OH)_2$-D_3-induced increase in intracellular Ca^{2+}. Somewhat similar observations have been reported by Long and Rosen (1994). Klein and Wiren (1993), studying the same cell line, found that Pb concentrations between 2 and 200 μM had no effect on cell number, DNA, or protein synthesis. However, Pb addition caused a decrease in mRNA concentrations of alkaline phosphatase, of type 1 procollagen, and of osteocalcin.

Hicks *et al.* (1996) have reported that sublethal doses of Pb in isolated chick chondrocytes caused suppression of alkaline phosphatase, as well as of type II and type X collagen expression, and a decrease in thymidine incorporation. The authors suggest that Pb may inhibit endochondral bone formation. Gonzalez-Risla and colleagues (1997) found that Pb exposure inhibited development of the cartilage growth plate of rats and suggest this may be one cause of the adverse effects of Pb exposure on skeletal development. Another cause may involve disruption of mineralization during growth (Hamilton and O'Flaherty, 1995).

Dowd *et al.* (1994) have reported that submicromolar concentrations of free Pb compete with Ca^{2+} binding to osteocalcin. Because Pb^{2+} inhibits osteocalcin binding to hydroxyapatite, enough osteocalcin may become inactivated to affect bone mineral dynamics. A related observation was made by Sauk *et al.* (1992), who found that Pb addition inhibited the release of osteonectin/SPARC by ROS 17/2.8 cells and lowered the cellular content of osteonectin/SPARC mRNA. How these *in vitro* effects relate to the *in vivo* effects of Pb poisoning is not known.

Miyahara *et al.* (1994) reported that Pb may induce the formation of osteoclast-like cells by increasing the intracellular concentrations of Ca^{2+} and cAMP. Conceivably therefore, Pb alters Ca channels in a way that allows increased inflow of extracellular Ca^{2+}. Those Pb-sensitive channels—which also permit increased Pb inflow (Schanne *et al.*, 1989)—appear therefore to be located in both osteoclasts and osteoblasts. Carmouche *et al.* (2005) exposed mice to varying concentrations of Pb, but found no effect on osteoclasts isolated from the exposed animals. However, even low Pb doses affected the rate of fracture healing, and high doses induced fibrous nonunions by inhibiting the progression of endochondral ossification.

In a recent review, Holtz and colleagues (2007) summarized the effects of Pb on osteoblasts, indicating (see also above) that Pb inhibits synthesis of collagen type I, secretion of osteonectin, alkaline phosphatase activity, and adversely affects osteoblast proliferation. The authors suggest that "the mechanism by which lead has its effects on bone formation may involve a decrease in the pool of phosphorylated Smads [smoxprotein, drosophila] in exposed cells."

In the same review, Holtz *et al.* (2007) discuss the effects of Pb on articular chondrocytes. The metal seems to release these cells from an arrested state and allows them to progress along a hypertrophic pathway, which in turn leads to matrix degradation, mineralization and apoptosis, processes that in articular cartilage bring about arthritis.

The overall effect of Pb poisoning on bone metabolism is not clear. Koo *et al.* (1991) failed to find significant changes in vitamin D metabolism, bone mineral content, and Ca and P_i plasma concentrations in children of adequate nutritional status who had been chronically exposed to low to moderate Pb levels. Needleman and colleagues (1996) conclude from their study of the relationship between body Pb burden and social adjustment in public school children that high bone Pb levels are associated with attention deficit, aggression and delinquency and that these effects follow a developmental course. Indeed, it is the overall effect of raised blood levels of Pb on development and behavior of children that led to the advocacy of Pb removal from the environment and to the policy of minimizing Pb contamination.

Whatever the direct effect of Pb on bone, the skeleton clearly constitutes the major Pb store in the body. For example, when rats were fed a marginal zinc diet that leads to retarded bone growth and diminished bone mass, bone lead concentration was enhanced, compared to that of controls (Jamieson *et al.*, 2006). It is apparent, therefore, that skeletal Pb content determines overall exposure and risk.

Lead removal from bone, as of other metal contaminants, is best accomplished by chelators, typically administered systemically. The best way to prevent Pb accumulation is to eliminate it from the immediate environment, such as by making paint or gasoline lead-free. Another approach is to prevent or diminish Pb absorption. That it can be achieved is claimed on the basis of rat studies, with the aid of calcium alginate and calcium pectate. Both of these compounds appear to act by binding exogenous or endogenous lead in the intestine and therefore may find use in preventing and treating lead poisoning (Khotimchenko *et al.*, 2006).

SILICON

Silicon, Si, atomic number 14, atomic weight 28.0, belongs to periodic group IVb and is classified as part of the carbon family, which includes germanium, tin, and Pb (Moeller, 1952). Carlisle (1986) has listed the Si concentration in various soft tissues of rats and monkeys as varying typically between 1 and 2 μg/g wet weight; in people the Si concentration appears to be about one order of magnitude higher. Connective tissues tend to have a high Si content, mainly because Si is an integral component of the glycosaminoglycans and their protein complexes that contribute to the tissue structures (Carlisle, 1986).

In 1972, Schwarz and Milne reported that the addition of 50 mg Si/100 g diet increased the growth rate of rats by some 30%, and Carlisle, in the same year, found a comparable increase in the daily weight gain of chicks. Carlisle (1986) has reported that the addition of Si hastened mineralization in weanling rats and that Si deficiency in chicks (Carlisle, 1972) led to abnormally shaped bones. Over the past decades, there have been many reports that have or have not verified the initial findings (see Carlisle, 1986, for a review of reports through mid-1980). Thus, Seaborn and Nielsen (1994) have reported that when Si, as sodium metasilicate, was fed to weanling rats at the rate of 25 μg/g fresh diet, the decreased Ca and Mg content of the femur found in the control animals was reversed. In these experiments, it proved possible to substitute germanium for Si, but germanium did not replace Si in other effects that result from Si deficiency. On the other hand, Eliot and Edwards (1991) concluded on the basis of 16-day experiments with broiler chicks that "dietary silicon supplementation has no effect on growth and skeletal development." Eisinger and Clairet (1993) analyzed bone mineral density in 53 women with osteoporosis and found that in eight subjects Si supplementation induced a statistically significant increase in femoral bone mineral density.

Rico and colleagues (2000) studied the effect of 30-day Si supplementation in ovariectomized rats and showed that supplementation overcame the losses of bone mass in the 5th lumbar vertebra and in the femur found in the ovariectomized controls. The authors concluded that Si "may have a potential therapeutic application in the treatment of involutive osteoporosis."

In the same year, DeAza and colleagues (2000) showed that a compound made of alpha-$CaSiO_3$, called pseudowollastonite, is integrated into the structure of living bone tissue through a dissolution–precipitation–transformation mechanism, with osteoblasts migrating to the interface and colonizing the bone surface.

One reason for interest in possible Si effects on bone is that granules of special glasses have been used to repair bone defects in the dental field (Gatti and Zaffe, 1991a). These vitreous materials contain Si, as do materials used to complete suturectomy for the treatment of craniosynostosis (Antikainen, 1993). In the case of the granules, analyses of the embedded jaws showed (Gatti and Zaffe, 1991b) that Si had diffused into the surrounding tissue. There also was no osteoinduction, but it is not clear whether this was caused by Si or the procedure.

Lai *et al.* (2002) implanted bioactive glass granules in the tibiae of rabbits and determined the biopathway of the Si released from the bioactive glass. As expected, the urinary output of the rabbits with implants was significantly higher than that of the controls and the amount of silicon excreted matched that implanted after 24 weeks. The authors conclude that the resorbed Si is excreted in the urine in a soluble and harmless form.

Gorustovich and colleagues (2002), in a somewhat similar study in rats, implanted titanium and bioactive glass particles and killed the animals at 14, 30, and 60 days. They found increased bone thickness in the peri-implant of the Ti/bioglass group, as compared to the group with Ti implants only, suggesting an increase in medullary bone formation in the group with Si-containing bioglass.

Seaborn and Nielsen (2002a), having shown in an earlier study that Si deprivation decreases the collagen concentration in 9-week-old rats (2002b), showed that hydroxyproline was significantly diminished in tibiae from Si-deficient as compared to Si-supplemented animals. They also found that collagen formation from proline in bone and other sites was corrected by Si addition to the diet, concluding that Si is a nutrient of concern in wound healing and bone formation.

Porter and colleagues (2003) have shown that incorporation of silicate into hydroxyapatite increases the rate of bone apposition to bioceramic implants of hydroxyapatite. It does so by increasing dissolution from the implanted hydroxyapatite, thereby increasing the in vivo bioactivity over an implant that consists of pure hydroxyapatite. Similarly, Pabbruwe *et al.* (2004) demonstrated that doping a bioinert ceramic with small amounts of Si can significantly improve tissue ingrowth and osteogenesis. In a later paper, Porter *et al.* (2006) studied in greater detail the structure of the bond between bone and Si-containing bioceramic implants and found that organized, mineralized collagen fibrils had grown into the strut porosity at the interface between the Si-containing implant and the surface of the surrounding bone. Porter (2006) has reviewed the role played by Si in bone mineralization and formation and applied this knowledge and the findings of the studies by her and her colleagues (Porter *et al.*, 2003, 2006) to the interaction between bone and Si-containing ceramic implants and on their performance.

STRONTIUM

Strontium, Sr, like calcium, is a periodic group IIa element and, although not very abundant, constituting only 0.03% of the igneous rocks of the earth, is usually classified as a "familiar element" because of the existence of readily available natural sources (Moeller, 1952). It is not an essential element, and interest in Sr metabolism stems from the fact that ^{90}Sr "is an abundant and potentially hazardous by-product of nuclear fission" (Underwood, 1977). Although tests of nuclear explosions have largely ceased, the fact that Sr behaves metabolically much like calcium has helped maintain interest in this element (Blumsohn *et al.*, 1994; Kollenkirchen, 1995).

In a detailed formal study (Bronner *et al.*, 1963) it was found that although Sr and Ca followed the same metabolic pathway qualitatively, there were significant quantitative differences in how the body handled these two elements. The major difference was in the urine, with the fraction of Sr excreted in the urine three times that of Ca, on the average. In the stool, the fecal loss of endogenous Sr was greater than that of Ca, but only moderately so (by ~10%). In terms of the fraction of injected isotope that was calculated to reach bone, there was no difference on the average, but the ratio "varied from patient to patient and was not consistent in a given patient." Blumsohn *et al.* (1994) have reexplored the relationship between Sr and Ca absorption in patients with osteoporosis and with chronic renal failure. Sr absorption was approximately half that of Ca, but the time course of the two was similar, when evaluated by deconvolution. Stable Sr is less expensive than stable Ca, but differences in absorption between the two elements are sufficiently great that measuring calcium absorption with a calcium isotope seems more meaningful. Treatment with 1,25-$(OH)_2D_3$ stimulated Sr absorption more than Ca absorption. A possible explanation is that Sr binds more tightly than Ca^{2+} to the newly induced calbindin (Fullmer and Wasserman, 1977).

Fed in large amounts, Sr has long been known to cause rickets in experimental animals (Lehnerdt, 1910, quoted by Neufeld and Boskey, 1994). In Turkish children growing up in regions where the soil Sr content was greater than 350 ppm, the incidence of rickets was nearly twice that in children from regions where the Sr content was lower (Ozgur *et al.*, 1996). Strontium interferes with intestinal calcium absorption and synthesis of 1,25-$(OH)_2D_3$ (Omdahl and DeLuca, 1972) and interferes with mineralization (Sobel and Hanok, 1952), apparently via direct action on bone cells (Neufeld and Boskey, 1994), although the nature of this action is not clear. One way in which bone formation may be interfered with by Sr is to delay the natural progression of osteoid to bone, i.e., at the stage when cartilage is converted to bone. This interference is consistent with the greater accumulation of complexed acidic phospholipids in Sr-fed rats or in mesenchymal cell micromass cultures (Neufeld and Boskey, 1994). Davis *et al.* (2000) report that Sr becomes associated with the collagen matrix produced in cell culture. It would be interesting to know to what extent the inhibitory effect of Sr can be attributed to displacing Ca^{2+} in other calcium-mediated processes.

There have been reports that nontoxic amounts of Sr may be beneficial in osteoporosis (Storey, 1961, and McCaslin and James, 1981, quoted by Morohashi *et al.* 1994; Brandi, 1993) and in rats, where 0.19% $SrCl_2$ in the diet stimulated bone formation (as evaluated histomorphometrically) and raised the trabecular calcified bone volume by 10% (Marie *et al.* 1985). In 1993, Marie *et al.* reported that an organic distrontium salt, S12911, now known as strontium ranelate, inhibited the increase in bone resorption in ovariectomized rats, without reducing bone formation. It is unclear, however, how much of this effect is due to Sr. In mice, 0.27% $SrCl_2$ in the diet increased the osteoid surface, but had no effect on trabecular calcified bone volume (Marie and Hott, 1985). Grynpas *et al.* (1996) fed 0.2% Sr to 28-day-old rats consuming a 0.5% Ca and 0.5% P diet and found that the number of bone-forming sites and the vertebral bone volume had increased by 17% compared with controls. No detectable adverse effects on mineralization, mineral profile, or mineral chemistry were observed in the Sr-fed animals. Similarly, Morohashi *et al.* (1994) found no harmful effects when rats were fed 0.05% or 0.10% Sr in a semisynthetic vitamin D–deficient diet, whereas 0.5% Sr depressed bone calcium content and the bone calcium deposition rate. In these studies, the limiting Sr concentration was 175 μM; beyond that level, calcium metabolism was depressed. However, at the lower Sr intakes, there also was no beneficial effect of Sr intake. It would thus seem that as Sr replaces Ca, the metabolism of calcium is depressed, with high Sr intakes leading to rickets and poor bone formation and mineralization.

In recent years, strontium ranelate (Fig. 3) has been prescribed in Europe for treatment of osteoporosis. In monkeys, the compound did not induce acute or subchronic toxic effects on the gastric mucosa (Fisch *et al.*, 2006). Absorption of the cation, ranelate, is said to be nil or very low, with the strontium anion absorbed, presumably mostly paracellularly, even though Sr binds tightly to the calcium-binding protein, CaBP 9kD, which effects transcellular calcium transport in the duodenum and proximal jejunum (Bronner and Pansu, 1999). At high calcium intakes, as

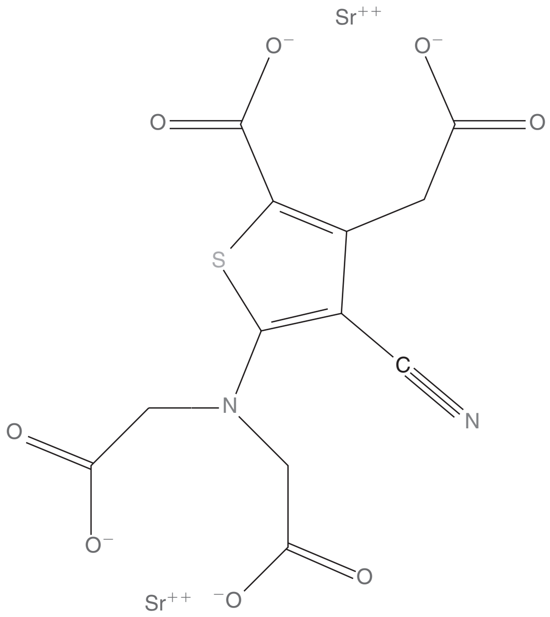

FIGURE 3 The structure of strontium ranelate. (http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=6918182).

recommended to patients with osteoporosis, most calcium and presumably most strontium is absorbed in the ileum (Bronner and Pansu, 1999), where transport is paracellular.

Meunier and colleagues (2004) studied 1442 patients with osteoporosis and history of at least one vertebral fracture, half of whom received 2 g daily of strontium ranelate, the other half receiving a placebo and serving as controls. The group on strontium had a lower risk of fracture at the end of 1 and 3 years; their bone mineral density was higher, as was their bone-specific alkaline phosphatase, an index of bone formation, whereas their urinary output of C-telopeptide, a marker of bone resorption, was diminished. The mechanism of action of strontium under these conditions is not known. Strontium competes with calcium for bone deposition, but only modestly. In monkeys that had been placed on increasing levels of Sr intake, only one in 10 calcium ions in the bone crystal was replaced by strontium in the group that had been fed the highest level of Sr ranelate (Boivin *et al.,* 1996). Conceivably, strontium in quantities that do not lead to strontium rickets, by competing with calcium, may cause an increase in bone formation, being initially deposited in new bone and especially in trabecular bone, which turns over faster than cortical bone (Marshall *et al.,* 1973). Normally this would lead to higher bone turnover and therefore higher bone resorption. This was not true in the Meunier *et al.* study. Marie and colleagues, in an earlier review (2001), suggested that Sr may act directly on osteoclasts, inhibiting recruitment and activity, without exerting toxic effects. At the time of the review, fewer studies had dealt with bone formation, but Marie *et al.* suggest preosteoblast replication and collagen synthesis provide the mechanism for stimulating osteoblast activity.

Recommendations for the therapeutic use of strontium ranelate are mixed. A long editorial in *Joint Bone Spine* (Naveau, 2004), after reviewing experimental data, the pharmacology and therapeutic trials, concludes that “if preliminary clinical results are confirmed by ongoing studies [strontium ranelate] should prove a useful addition to the armamentarium for osteoporosis.” The editorial does point out that bone mineral density evaluations following Sr therapy tend to overestimate the benefit, inasmuch as Sr incorporation in bone mineral increases the attenuation of x-rays, resulting in an overestimate of bone mineral content, with the overestimate proportional to the bone Sr content (see also Blake and Fogelman, 2005).

An unsigned article in *Revue Prescrire* (Anonymous, 2005) points out that Sr was first used to treat osteoporosis in the 1950s, and that it was then abandoned because its use leads to osteomalacia in animals and dialysis patients, at least when bone contains more than 5% Sr. The article warns about the use of Sr in patients more than 80 years of age, as they often have moderate renal failure. It quotes assessments in Norway, Germany, Sweden, and Finland, which call attention to the risks of venous thrombosis and neurological disorders, with the German assessment stating “that strontium ranelate has a negative risk-benefit balance.” The article concludes that “women with osteoporosis can do without strontium . . . avoiding the poorly documented risks associated with strontium.”

An interesting use of Sr has been in the radiotherapy with ^{89}Sr of painful bone metastases (Robinson *et al.,* 1995; Pons *et al.,* 1997; Papatheophanis, 1997). Such treatment has been more widely reported for pain relief in patients with prostatic cancer, but has also found application in patients with breast cancer (Pons *et al.*, 1997). Immediately following the injection of $^{89}SrCl_2$, patients experienced a flare reaction of pain. Thereafter pain relief lasted on the average 6 months, with treatment effectively repeatable for another 6 months (Pons *et al.*, 1997). As yet there is no indication that ^{89}Sr is tumoricidal.

The promotion of strontium led Cheung *et al.* (2005) to develop a strontium-containing bioactive cement and compare it with the more conventional cements of polymethylacrylate and hydroxyapatite. Cements are used to treat osteoporotic spine fractures. A good cement has a relatively low setting temperature, exhibits good osteointegration, and matches the stiffness of osteoporotic bone. The Sr-containing cement had a relatively low setting temperature and adequate compressive and bending strengths, with bending strength and modulus fairly close to those of human cancellous bone. The report concludes that the Sr-containing bioactive cement is appropriate for vertebroplasty and kyphoplasty. The authors attribute this to the local delivery of Sr, but it is not clear whether the same formula using calcium—not necessarily as hydroxyapatite—would not be as or even more effective.

CONCLUDING REMARKS

All metals discussed in this chapter enter the skeleton, but in nearly all reports there is no clear distinction between cellular effects and those due to accumulation in the bone mineral. Significant accumulation of a given metal may alter mineral characteristics, but the details of these changes have yet to be established. Furthermore, study of the mechanisms of metal accumulation—heteroionic exchange and/or deposition and inclusion in the bone crystals—may help us learn more about mineralization. Similarly, understanding the qualitative and quantitative effects of metal addition to bone cell cultures may reveal mechanisms of cellular action. It is also important to explore the possible toxic effects of metals used in prostheses. Thus Wang *et al.* (1996) found a metal-induced increase in the release of bone cytokines and wondered whether this led to the osteolysis they observed, an event that can severely compromise the outcome of joint arthroplasty. On the other hand, Pohl and colleagues (2000) and also Piatelli *et al.* (1998) found no impairment of periodontal healing following the insertion of titanium implants. Studies of the use of aluminum oxide in bioceramics have generally found no major biocompatibility problems (Chang *et al.,* 1996; Piatelli *et al.*, 1996, 1998; Shinzato *et al.*, 1999; Okada *et al.*, 2000), but truly long-term results are still outstanding. Clearly the study of metals in bone continues to provide ample research opportunities to all with an interest in a given metal or the biology of bone.

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Chapter 26

Biology of the Extracellular Ca^{2+}-Sensing Receptor

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INTRODUCTION

Complex, free-living terrestrial organisms, such as humans, maintain their level of extracellular ionized calcium (Ca_o^{2+}) within a narrow range of about 1.1 to 1.3 m*M* (Bringhurst *et al.*, 1998; Brown, 1991). This near constancy of Ca_o^{2+} ensures that Ca^{2+} ions are available for their extracellular roles, including serving as a cofactor for clotting factors, adhesion molecules, and other proteins and controlling neuronal excitability (Brown, 1991). Moreover, calcium and phosphate salts form the mineral phase of bone, which affords a rigid framework that protects vital bodily structures and permits locomotion and other movements. The skeleton also provides a nearly inexhaustible reservoir of these ions when their availability in the diet is insufficient for the body's needs (Bringhurst *et al.*, 1998).

In contrast to Ca_o^{2+}, the cytosolic-free calcium concentration (Ca_i^{2+}) has a basal level—about 100 n*M*—that is nearly 10,000-fold lower (Berridge *et al.*, 2003). Ca_i^{2+}, however, can increase 10-fold or more when cells are stimulated by extracellular signals acting on their respective cell surface receptors as a result of influx of Ca^{2+} and/or its release from intracellular stores (Berridge *et al.*, 2003). Ca_i^{2+} plays central roles in regulating cellular processes as varied as muscular contraction, cellular motility, differentiation and proliferation, hormonal secretion, and apoptosis (Berridge *et al.*, 2003). Because all intracellular Ca_o^{2+} ultimately originates from that present in the extracellular fluids (ECF), maintaining near constancy of Ca_o^{2+} also ensures that this ion is available for its myriad intracellular roles.

The level of Ca_o^{2+} is maintained by a homeostatic mechanism in mammals that comprises the parathyroid glands, calcitonin (CT)-secreting C cells, kidney, bone, and intestine (Bringhurst *et al.*, 1998; Brown, 1991). Key elements of this system are cells that are capable of sensing small deviations in Ca_o^{2+} from its usual level and responding in ways that normalize it (Brown, 1991). Calcium ions have long been known to traverse the plasma membrane through various types of ion channels and other transport mechanisms (Berridge *et al.*, 2003); however, the actual mechanism by which Ca_o^{2+} was "sensed" remained an enigma for many years. This chapter provides an update on our present understanding of the process of Ca_o^{2+} sensing, which has increased greatly over the past 10 to 15 years, especially as it relates to the mechanisms that maintain Ca_o^{2+} homeostasis.

It is becoming increasingly clear that Ca_o^{2+} serves as a versatile extracellular first messenger—in many instances acting via the Ca^{2+}-sensing receptor (CaR)—that controls numerous physiological processes beyond those governing Ca_o^{2+} homeostasis (for review, see Tfelt-Hansen and Brown, 2005). Although it is beyond the scope of this chapter to review the "nonhomeostatic" roles of the CaR in detail, an emerging body of evidence supports the concept that the receptor participates in important interactions between the system regulating Ca_o^{2+} metabolism and other homeostatic systems (i.e., that controlling water metabolism). These interactions may be crucial for the successful adaptation of complex life forms to the terrestrial environment. This chapter will likewise address these recently emerging homeostatic relationships.

CLONING OF THE CaR

Just 15 years ago, the concept that there was a specific Ca_o^{2+}-sensing "receptor" was only supported by indirect evidence derived from studies of a limited number of Ca_o^{2+}-sensing cells, particularly parathyroid cells (Brown, 1991; Juhlin *et al.*, 1987; Nemeth and Scarpa, 1987; Shoback *et al.*, 1988). It was necessary, therefore, in devising a strategy for cloning the putative receptor to employ an approach that detected its Ca_o^{2+}-sensing

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activity using a bioassay—namely, expression cloning in *Xenopus laevis* oocytes. Racke *et al.* (1993) and Shoback and co-workers (Chen *et al.*, 1994) both demonstrated that *X. laevis* oocytes became responsive to $\mathrm{Ca}_{\mathrm{o}}^{2+}$-sensing receptor agonists after they were injected with messenger RNA (mRNA) extracted from bovine parathyroid glands. Brown *et al.* (1993) were then able to utilize this strategy to isolate a full-length, functional clone of the $\mathrm{Ca}_{\mathrm{o}}^{2+}$-sensing receptor. The use of conventional, hybridization-based approaches subsequently permitted the cloning of cDNAs coding for CaRs from human parathyroid (Garrett *et al.*, 1995b) and kidney (Aida *et al.*, 1995b), rat kidney (Riccardi *et al.*, 1995), brain (namely striatum; Ruat *et al.*, 1995), and C cell (Garrett *et al.*, 1995c); rabbit kidney (Butters *et al.*, 1997); and chicken parathyroid (Diaz *et al.*, 1997; reviewed in Brown *et al.*, 1999). All exhibit very similar predicted structures and represent tissue and species homologues of the same ancestral gene.

PREDICTED STRUCTURE OF THE CAR AND ITS RELATIONSHIPS TO OTHER G PROTEIN-COUPLED RECEPTORS

The topology of the CaR protein predicted from its nucleotide sequence is illustrated in Figure 1. It has three principal structural domains, which include (a) a large, ~600-amino-acid extracellular amino-terminal domain (ECD), (b) a "serpentine" seven membrane-spanning motif that is characteristic of the superfamily of G protein-coupled receptors (GPCRs), and (c) a sizable carboxyl-terminal (C-) tail of about 200 amino acids. Several GPCRs have been identified that share striking topological similarities with the CaR, particularly in their respective, large ECDs, as well as a modest (20−30%) overall degree of amino acid identity. These structurally related GPCRs are designated family C receptors (Kolakowski, 1994; Brauner-Osborne *et al.*, 2007). The mammalian family C receptors include

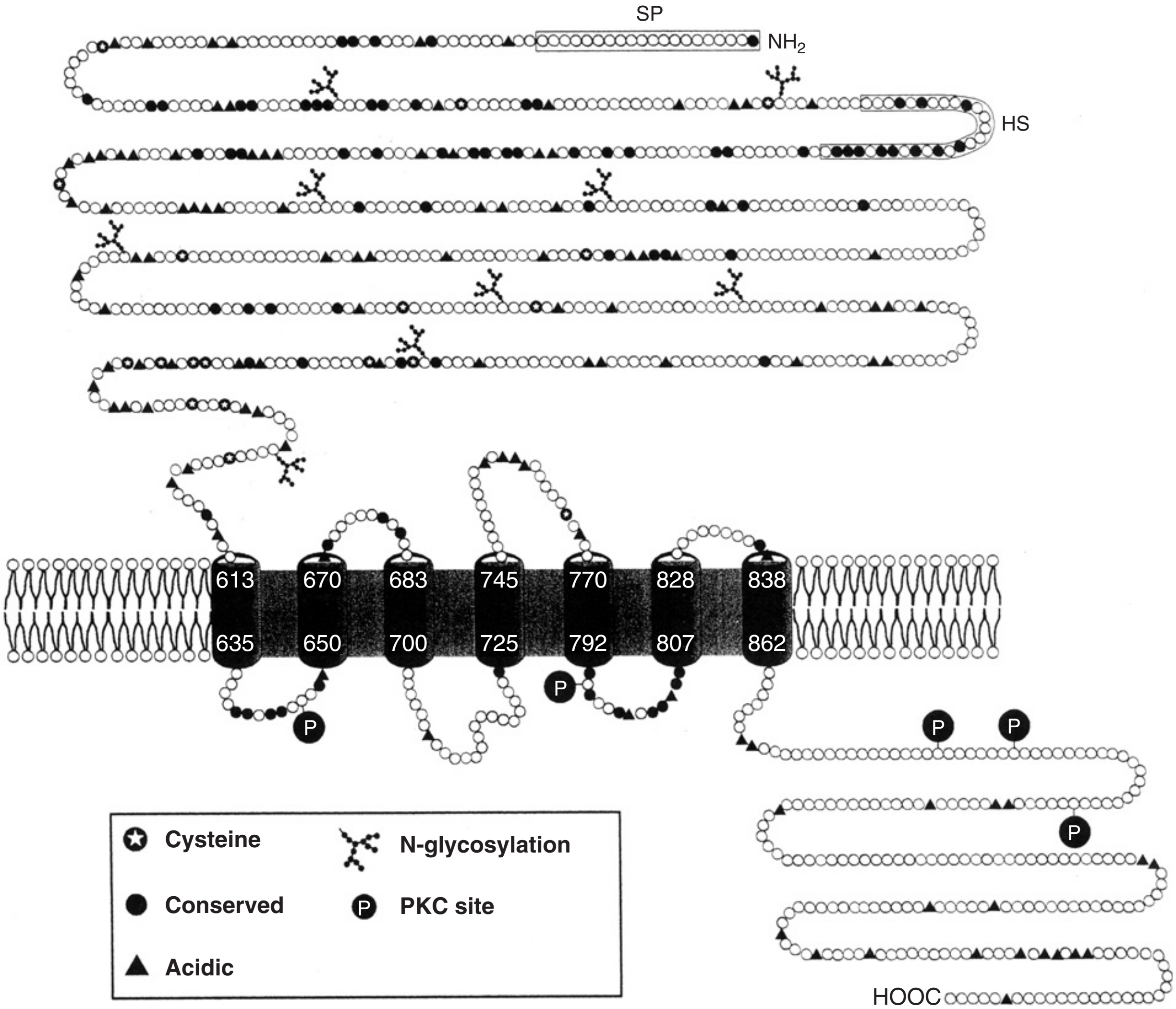

FIGURE 1 Predicted structure of the CaR (see text for additional details). SP, signal peptide; HS, hydrophobic segment. Reproduced with permission from Brown *et al.* (1993).

eight metabotropic glutamate receptors (mGluRs), two GABA$_B$ receptors, the CaR, 3 taste receptors (T1R1-T1R3), the V2R pheromone receptors, and a promiscuous amino acid-sensing receptor, GPRC6A (Christiansen *et al.*, 2007), that may also serve as a second Ca_o^{2+}-sensing receptor (see later discussion; Pi *et al.*, 2005).

mGluRs are activated by glutamate, the major excitatory neurotransmitter in the central nervous system (CNS; Nakanishi, 1994). The GABA$_B$ receptors are GPCRs that recognize γ-aminobutyric acid (GABA), the principal inhibitory neurotransmitter of the CNS (Kaupmann *et al.*, 1997; Ng *et al.*, 1999). The putative pheromone receptors reside solely in neurons of the vomeronasal organ in rodents (VNO; Matsunami and Buck, 1997), which controls instinctual behavior, via input from environmental pheromones. The taste receptors enable us to taste sweet substances (and artificial sweeteners) as well as so-called "umami" (i.e., the taste of monosodium glutamate; Hoon *et al.*, 1999; Xu *et al.*, 2004).

Therefore, all of the family C GPCRs share the property of having small molecules as their ligands that provide environmental cues (i.e., pheromones) or serve as extracellular messengers within the CNS (e.g., glutamate or GABA) or in bodily fluids more generally (i.e., Ca_o^{2+}, amino acids). As detailed later, ligands of the CaR and the other family C GPCRs are thought to bind principally to their respective ECDs. In contrast, most other GPCRs binding small ligands, such as epinephrine or dopamine, have binding sites within their TMDs and/or extracellular loops (ECLs). The ligand-binding capacity of the family C ECDs probably has its origin in a family of extracellular binding proteins in bacteria (Felder *et al.*, 1999), the so-called periplasmic binding proteins (PBPs). These serve as receptors for a wide variety of small ligands present in the environment, including ions (including Mg_o^{2+}, but apparently not Ca_o^{2+}), amino acids, and other nutrients (Tam and Saier, 1993). PBPs promote bacterial chemotaxis toward these environmental nutrients and other substances and facilitate their cellular uptake by activating specific transport systems in the cell membrane (Tam and Saier, 1993).

The family C GPCRs, therefore, can be thought of as representing fusion proteins, which comprise an extracellular ligand-binding motif (the ECD) linked to a signal-transducing motif (the seven transmembrane domains) that couples the sensing of extracellular signals to intracellular signaling systems (i.e., G proteins and their associated second messenger pathways). It is of interest that some of the biological functions regulated by the CaR are the same as those controlled by the PBPs, namely chemotaxis (e.g., of monocytes toward elevated levels of Ca_o^{2+}; Sugimoto *et al.*, 1993) and cellular transport (i.e., of Ca_o^{2+} by CaR-regulated, Ca^{2+}-permeable channels; Chang *et al.*, 1995). Moreover, as described later, the CaR binds not only Ca_o^{2+} but also additional ligands, including amino acids (Conigrave *et al.*, 2000), which further supports its evolutionary and functional relationships to the other family C GPCRs and, ultimately, to PBPs.

BIOCHEMICAL PROPERTIES OF THE CaR

Studies using chimeric receptors comprising the ECD of the CaR coupled to the TMDs and C tail of the mGluRs (and vice versa) have demonstrated the existence of key binding sites for Ca_o^{2+} within the CaR's ECD (Brauner-Osborne *et al.*, 1999; Silve *et al.*, 2005), although even a receptor lacking the entire ECD still has the capacity to respond to polyvalent cations (Hu and Spiegel, 2003). Studies have indicated that specific residues within the ECD (e.g., Ser147 and Ser170) may be involved, directly or indirectly, in the binding of Ca_o^{2+} (Silve *et al.*, 2005; Huang *et al.*, 2007b). Given the apparent "positive cooperativity" of the CaR and the resultant steep slope of the curve describing its activation by various polycationic agonists (e.g., Ca_o^{2+} and Mg_o^{2+}; Brown, 1991), however, it is likely that the CaR binds several calcium ions. This positive cooperativity is essential to ensure that the CaR responds over the narrow range of Ca_o^{2+} regulating, for instance, PTH secretion. This cooperativity could result, at least in part, from the presence of Ca_o^{2+}-binding sites on both of the individual ECDs within the dimeric CaR (see later discussion) as well as the existence of more than one binding site within each receptor monomer. Further work is needed, therefore, in defining more precisely the identity of the CaR's Ca_o^{2+}-binding site(s). Eventual solution of the three-dimensional structure of the receptor's ECD by x-ray crystallography will no doubt illuminate how the CaR binds Ca_o^{2+} and its other agonists and modulators.

As noted earlier, the CaR resides on the cell surface primarily in the form of a dimer (Bai *et al.*, 1998a; Ward *et al.*, 1998). CaR monomers within the dimeric receptor are linked by disulfide bonds within their ECDs that involve the cysteines at amino acid positions 129 and 131 (Ray *et al.*, 1999). Moreover, functional interactions can occur between the monomeric subunits of the dimeric CaR because two individually inactive CaRs that harbor inactivating mutations in different functional domains (e.g., the ECD and C tail) can reconstitute substantial biological activity when they heterodimerize after cotransfection in human embryonic kidney (HEK293) cells (Bai *et al.*, 1999). Therefore, even though the individual CaRs lack biological activity, they "complement" one another's defects through a mechanism that must involve intermolecular interactions to form a partially active heterodimer. In addition to forming homodimers with itself, the CaR can also heterodimerize with the mGluRs (Gama *et al.*, 2001)as well as the GABA$_B$ receptors (Cheng *et al.*, 2006). In the latter case, heterodimerization changes the cell surface expression as well as the functional properties of the CaR (Cheng *et al.*, 2006).

The ECD of the CaR on the cell surface is N-glycosylated extensively with complex carbohydrates (Bai *et al.*, 1996). Eight of the predicted N-glycosylation sites in the ECD of the human CaR are glycosylated (Ray *et al.*, 1998). Disrupting four or five of these sites decreases the cell surface expression of the receptor by 50% to 90%. Therefore, glycosylation of at least three sites is required for robust cell surface expression, although glycosylation per se does not appear to be critical for the capacity of the CaR to activate its intracellular signaling pathways (Ray *et al.*, 1998).

Within its intracellular loops and C tail, the human CaR harbors five predicted protein kinase C (PKC) and two predicted protein kinase A (PKA) phosphorylation sites (Bai *et al.*, 1998b; Garrett *et al.*, 1995b). Activation of PKC diminishes CaR-mediated stimulation of phospholipase C (PLC), and studies utilizing site-directed mutagenesis have demonstrated that phosphorylation of a single, key PKC site in the C tail of the CaR at Thr888 can account for most of the inhibitory effect of PKC on the function of the receptor (Bai *et al.*, 1998b; Davies *et al.*, 2007). Therefore, PKC-induced phosphorylation of the C tail may afford a means of conferring negative feedback regulation on the coupling of the receptor to PLC. That is, PLC-elicited activation of protein kinase C—through the ensuing phosphorylation of the CaR at Thr888—limits further activation of this pathway. The functional significance of the PKA sites is not fully elucidated, although one study found that PKA synergized with PKC in inhibiting CaR-mediated activation of PLC (Bosel *et al.*, 2003).

BINDING PARTNERS OF THE CaR AND THEIR BIOLOGICAL ROLES

A number of molecular binding partners of the CaR have been identified that provide insights into the molecular details of the receptor's trafficking to the cell surface, its signaling and other biological functions, and its desensitization and degradation. During its initial biosynthesis, the capacity of the CaR to successfully reach the cell surface depends, at least in some cells, on its capacity to bind to receptor activity-modifying proteins (RAMPs; Bouschet *et al.*, 2005). The CaR in parathyroid and likely in other cells resides within caveolae, flask-like invaginations of the plasma membrane (Kifor *et al.*, 1998), where it binds directly or indirectly to caveolin-1, a key structural protein within caveolae that binds to multiple other proteins, including signaling molecules such as G proteins (Williams and Lisanti, 2004). The C tail of the cell surface CaR interacts with filamin-A, an actin-binding protein that, like caveolin-1, serves a scaffolding function by binding multiple classes of proteins (Hjalm *et al.*, 2001); (Awata *et al.*, 2001). The CaR's capacity to activate MAPKs, such as ERK1/2 (see next section) depends, in part, on its physical interaction with filamin-A (and likely the latter's capacity to bind various MAPK components; Hjalm *et al.*, 2001; (Awata *et al.*, 2001). The CaR's capacity to activate the low-molecular-weight G protein, Rho, via the Rho guanine nucleotide exchange factor, RhoGEF, also depends on the receptor's binding to filamin-A (Pi *et al.*, 2002). The CaR's C tail has recently been shown to bind to two potassium channels, Kir4.1 and 4.2, an interaction that inhibits the activities of these channels (Huang *et al.*, 2007a). The CaR is noteworthy for the limited extent to which it is desensitized on repeated exposure to Ca_o^{2+} and other polycationic agonists, e.g., in parathyroid cells, which depends, in part, on its tethering to the actin-based cytoskeleton by filamin-A (Zhang and Breitwieser, 2005). However, it does desensitize in heterologous systems following its phosphorylation by (1) PKC, which reduces the CaR's capacity to activate PLC or (2) G protein-coupled receptor kinases (GRK) and subsequent binding of the phosphorylated receptor to the adapter protein, β-arrestin (Lorenz *et al.*, 2007). The latter decreases its capacity to activate G proteins. Although there is only limited information on the fate of the desensitized receptor, a portion of it can undergo proteasomal degradation after binding to the ubiquitin ligase, dorfin, and subsequent ubiquitination (Huang *et al.*, 2006).

INTRACELLULAR SIGNALING BY THE CaR

Activation of the CaR by its agonists stimulates the activities of phospholipases C, A_2 (PLA_2), and D (PLD) in bovine parathyroid cells and in HEK293 cells stably transfected with the human CaR (Kifor *et al.*, 1997). In most cells, CaR-evoked activation of PLC involves the participation of the pertussis toxin-insensitive G protein(s), $G_{q/11}$ (Brown and MacLeod, 2001), although in some it can take place through a pertussis toxin-sensitive pathway, most likely via one or more isoforms of the G_i subfamily of G proteins (Emanuel *et al.*, 1996). In bovine parathyroid cells and CaR-transfected HEK293 cells, CaR-mediated stimulation of PLA_2 and PLD occurs through PKC-dependent mechanisms, presumably via receptor-dependent activation of PLC (Kifor *et al.*, 1997), although some studies have shown that CaR-mediated activation of PLD involves $G_{12/13}$ (Huang *et al.*, 2004).

The high Ca_o^{2+}-induced, transient increase in Ca_i^{2+} in bovine parathyroid cells and CaR-transfected HEK293 cells likely results from activation of PLC (Kifor *et al.*, 1997) and resultant IP_3-mediated release of intracellular Ca^{2+} stores (Bai *et al.*, 1996). High Ca_o^{2+} also elicits sustained elevations in Ca_i^{2+} in both CaR-transfected HEK293 cells (Bai *et al.*, 1996) and bovine parathyroid cells (Brown, 1991), acting through an incompletely defined influx pathway(s) for Ca^{2+}. Via the patch-clamp technique, we have shown that the CaR activates a Ca_o^{2+}-permeable, nonselective cation channel (NCC) in

CaR-transfected HEK cells (Ye *et al.*, 1996). An NCC with similar properties is present in bovine parathyroid cells and is likewise activated by high Ca^{2+}—an effect presumably mediated by the CaR (Chang *et al.*, 1995). This latter NCC may participate in the high Ca_o^{2+}-induced, sustained elevation in Ca_i^{2+} in parathyroid cells (Brown *et al.*, 1990). Activation of the CaR by Ca_o^{2+} initiates oscillations in Ca_i^{2+} in several cell types (Breitwieser and Gama, 2001; Young and Rozengurt, 2002; De Luisi and Hofer, 2003), through a mechanism involving activation of PLC (Rey *et al.*, 2005) and the Ca^{2+}-permeable channel, TRPC1, and this process can be modulated by PKC (Young *et al.*, 2002). Amino acids stimulate a different pattern of oscillations (Young and Rozengurt, 2002), indicating that the nature of the CaR agonist can affect the signals generated by the receptor.

High Ca_o^{2+} reduces agonist-stimulated cAMP accumulation in bovine parathyroid cells markedly (Chen *et al.*, 1989; Gerbino *et al.*, 2005; Corbetta *et al.*, 2000), an action that is thought to involve inhibition of adenylate cyclase by one or more isoforms of G_i, as it is pertussis toxin-sensitive (Chen *et al.*, 1989). Other cells, however, can exhibit high Ca_o^{2+}-elicited diminution of cAMP accumulation that involves indirect pathways, including inhibition of a Ca_i^{2+}-inhibited isoform of adenylate cyclase due to the associated rise in Ca_i^{2+} (de Jesus Ferreira *et al.*, 1998) or by increasing the degradation of cAMP (Geibel *et al.*, 2006). The CaR also activates mitogen-activated protein kinases (MAPK) in several types of cells, including rat-1 fibroblasts (McNeil *et al.*, 1998b), ovarian surface cells (McNeil *et al.*, 1998a), and CaR-transfected but not non-transfected HEK293 cells (Kifor *et al.*, 2001). As is the case with other GPCRs, the CaR stimulates the activity of MAPK (e.g. extracellar signal regulated kinases 1and 2 and p38 MAPK) through both PKC- and tyrosine kinase-dependent pathways. The latter involves, in part, c-Src-like cytoplasmic tyrosine kinases. The PKC-dependent activation of MAPK is presumably downstream of G_q-mediated activation of PLC, whereas that involving tyrosine kinases may utilize the G_i-dependent pathway involving $\beta\gamma$ subunits released as a consequence of the activation of this G protein (Kifor *et al.*, 2001; McNeil *et al.*, 1998b).

THE CaR GENE AND ITS REGULATION

Relatively little is presently known regarding the structure of the CaR gene, especially its upstream regulatory regions and the factors controlling its expression. The human CaR gene is on the long arm of chromosome 3, as demonstrated by linkage analysis (Chou *et al.*, 1992), and in band 3q13.3−21, as documented by fluorescent *in situ* hybridization (Janicic *et al.*, 1995). The rat and mouse CaR genes reside on chromosomes 11 and 16, respectively (Janicic *et al.*, 1995). The CaR gene has seven exons: The first includes the upstream untranslated region, the next five encode various regions of the ECD, and the last encodes the rest of the CaR from its first TMD to the C terminus (Pearce *et al.*, 1995). Characterization of the upstream regulatory regions of the gene will be of great interest because expression of the CaR can change in a various physiologically relevant circumstances—some of which are delineated hereafter.

Several factors increase CaR expression. Both high Ca_o^{2+} and $1,25(OH)_2D_3$ upregulate expression of the gene in certain cell types. High Ca_o^{2+} increases expression of the CaR in ACTH-secreting, pituitary-derived AtT-20 cells (Emanuel *et al.*, 1996) as well as in avian (Yarden *et al.*, 2000) and human parathyroid glands (Mizobuchi *et al.*, 2004), whereas administration of $1,25(OH)_2D_3$ elevates expression of the CaR *in vivo* in kidney and parathyroid gland of the rat in some (Brown *et al.*, 1996; Canaff and Hendy, 2002) but not all studies (Rogers *et al.*, 1995). Interleukin-1β increases the level of CaR mRNA modestly in bovine parathyroid gland fragments (Nielsen *et al.*, 1997) as well as in rat parathyroid and kidney (Canaff and Hendy, 2005), which may contribute to the associated reduction in PTH secretion that was observed in the former study. In rat kidney, substantial upregulation of the CaR takes place during the perinatal and immediate postnatal period; the resultant higher level of expression of the receptor then persists throughout adulthood (Chattopadhyay *et al.*, 1996). There is likewise a developmental increase in expression of the CaR in rat brain. In contrast to that occurring in the kidney, however, the rise in the expression of the CaR in the brain occurs about a week postnatally (Chattopadhyay *et al.*, 1997). Moreover, the increase in CaR expression in the brain is only transient—it decreases severalfold approximately 2 weeks later and reaches a lower level that remains stable thereafter (Chattopadhyay *et al.*, 1997). The biological importance of these developmental changes in the expression of the receptor is currently unknown. The CaR is overexpressed in some cancers (Li *et al.*, 2005), and it has been suggested to be a marker of breast cancer cells that have a propensity for metastasis to bone (Mihai *et al.*, 2006) to act as a factor contributing to lytic bone disease in animal models of prostate cancer (Liao *et al.*, 2006).

In contrast, several instances have been defined in which CaR expression decreases. Calf parathyroid cells exhibit a rapid, marked (80−85%) reduction in expression of the receptor after they are placed in culture (Brown *et al.*, 1995; Mithal *et al.*, 1995), which is likely to be a major factor that contributes to the associated reduction in high Ca_o^{2+}-evoked inhibition of PTH secretion. The expression of the CaR in rat kidney decreases in a model of chronic renal insufficiency induced by subtotal nephrectomy (Mathias *et al.*, 1998). This latter reduction in CaR expression might contribute to the hypocalciuria that occurs in human renal insufficiency, as reduced renal CaR expression and/or activity increases tubular reabsorption of Ca^{2+} in humans with inactivating mutations of the receptor (Brown, 1999). Because 1,25(OH)$_2$D$_3$ upregulates renal CaR expression (Brown *et al.,* 1996; Canaff and Hendy, 2005), the reduction in CaR expression in the setting of impaired renal function could be the result, in part, of the concomitant decrease in circulating levels of 1,25(OH)$_2$D$_3$ (Bringhurst *et al.,* 1998). As in parathyroid cells, the CaR in several other cells types is thought to serve as an inhibitor of cell proliferation, e.g., in cells from the colonic crypts (Kallay *et al.,* 2003) and in keratinocytes (Tu *et al.,* 2004). Therefore, the reduced expression in some colon cancers may contribute to tumorigenesis (Kallay *et al.,* 2003; Bhagavathula *et al.,* 2005).

ROLES OF THE CaR IN TISSUES MAINTAINING Ca_o^{2+} HOMEOSTASIS

Parathyroid

The parathyroid glands of several species express high levels of CaR mRNA and protein, including those of humans (Kifor *et al.,* 1996), rats (Autry *et al.,* 1997), mice (Ho *et al.,* 1995), rabbits (Butters *et al.,* 1997), and chickens (Diaz *et al.,* 1997). Indeed, the level of CaR expression in the parathyroid chief cells is one of the highest, if not the highest, in the cells and tissues examined to date. Abundant evidence supports the importance of the CaR as the key mediator of the inhibitory action of elevated Ca_o^{2+} on PTH secretion. As noted earlier, the reduced CaR expression in bovine parathyroid cells maintained in culture is associated with a progressive loss of high Ca_o^{2+}-induced inhibition of PTH secretion (Brown et al., 1995; Mithal *et al.,* 1995). In addition, individuals with familial hypocalciuric hypercalcemia (FHH), who are heterozygous for inactivating mutations of the CaR gene (Brown, 1999), or mice that are heterozygous for targeted disruption of the CaR gene (Ho *et al.,* 1995) exhibit modest right shifts in their set points for Ca_o^{2+}-regulated PTH secretion (the level of high Ca_o^{2+} half-maximally inhibiting PTH release), indicative of "Ca_o^{2+} resistance" Moreover, humans and mice homozygous for loss of the normal CaR (Brown, 1999; Ho *et al.,* 1995) show much greater impairment of high Ca_o^{2+}-elicited suppression of PTH release, showing that that the "Ca_o^{2+} resistance" of the parathyroid is related inversely to the number of normally functioning CaR alleles. Therefore, the biochemical findings in mice in which the CaR has been "knocked out," as well as in humans with naturally occurring inactivating mutations of the CaR, prove the central role of the CaR in Ca_o^{2+}-regulated PTH release. Despite several decades of study, however, the principal intracellular signaling mechanism(s) through which the CaR inhibits PTH secretion remains enigmatic (for review, see Diaz *et al.,* 1998). However, recent evidence indicates that activation of the $G_{q/11}$ pathway is essential, since mice with knockout of both of these G proteins have severe hyperparathyroidism mimicking that present in mice homozygous for knockout of the CaR itself (Wettschureck *et al.,* 2007). By a series of poorly defined steps, the CaR eventually induces polymerization of the actin-based cytoskeleton in a way that may provide a physical impediment to the release of PTH-containing secretory vesicles (Quinn *et al.,* 2007).

Another aspect of parathyroid function that is likely to be CaR regulated is PTH gene expression. Garrett *et al.* (1995a) showed in preliminary studies that the "calcimimetic" CaR activator NPS R-568, which activates the receptor allosterically through a mechanism involving an increase in the apparent affinity of the CaR for Ca_o^{2+} (Nemeth *et al.,* 1998b), decreases the level of PTH mRNA in bovine parathyroid cells. More recent studies (Levi *et al.,* 2006) have documented that the same calcimimetic reduces the elevated level of preproPTH mRNA in rats with experimentally induced secondary hyperparathyroidism owing to subtotal nephrectomy. This effect is post-transcriptional and involves modulation of the binding of the transacting factor, AUF1, to preproPTH mRNA. Finally, the CaR, directly or indirectly, tonically inhibits the proliferation of parathyroid cells, because persons homozygous for inactivating mutations of the CaR (Brown, 1999) or mice homozygous for "knockout" of the CaR gene (Ho *et al.,* 1995) exhibit marked parathyroid cellular hyperplasia. Treatment of rats with experimentally induced renal impairment with calcimimetics prevents the parathyroid hyperplasia that would otherwise be anticipated to occur in this setting (Wada *et al.,* 1998; Colloton *et al.,* 2005), providing further evidence that activation of the CaR suppresses parathyroid cellular proliferation.

C Cells

Unlike the effect of Ca_o^{2+} on PTH release, elevating Ca_o^{2+} stimulates CT secretion—a response that conforms to the classical, positive relationship between Ca_o^{2+} and activation of exocytosis in most other hormone-secreting cells (Bringhurst *et al.,* 1998; Brown, 1991). This observation was one of several pieces of indirect evidence that the mechanism underlying Ca_o^{2+} sensing in C cells might differ in a fundamental way from that in parathyroid cells. More recent data, however, have demonstrated that rat, human, and rabbit C cells express the CaR (Butters *et al.,* 1997; Freichel *et al.,* 1996; Garrett *et al.,* 1995c). Moreover, cloning of the CaR from a rat C cell tumor cell line showed it to be identical to that expressed in rat kidney (Garrett *et al.,* 1995c). Available evidence, albeit limited, indicates that the CaR mediates the stimulatory effect of high Ca_o^{2+} on CT secretion; namely, there is a shift to the right in the relationship between the serum calcium concentration and CT levels in mice heterozygous for knockout of the CaR gene (Fudge and Kovacs, 2004). Tamir and co-workers have suggested that the following sequence of steps mediates CaR-stimulated CT secretion (McGehee *et al.,* 1997). High Ca_o^{2+} first stimulates phosphatidylcholine-specific PLC, which generates diacylglycerol, thereby activating an NCC through a PKC-dependent mechanism. The activated NCC then enhances cellular uptake of Na$^+$ and Ca^{2+}, producing cellular depolarization and consequent stimulation of voltage-dependent, principally L-type Ca^{2+} channels that elevate Ca$_i^{2+}$ and activate exocytosis of 5-hydroxytryptamine- and CT-containing secretory granules.

Kidney

In the kidney of the adult rat, the CaR is expressed along almost the entire nephron, with the highest levels of protein expression at the basolateral aspect of the epithelial cells of the cortical thick ascending limb (CTAL; Riccardi *et al.,* 1998). The CTAL plays a key role in the hormone (e.g., PTH-regulated reabsorption of divalent minerals (De Rouffignac and Quamme, 1994; Friedman and Gesek, 1995)). The CaR is also present on the basolateral membranes of the cells of the distal convoluted tubule (DCT), where Ca^{2+} reabsorption—similar to that in the CTAL—is stimulated by PTH. Further sites of renal CaR expression include the base of the microvilli of the proximal tubular brush border (Riccardi *et al.,* 1998), the basolateral surface of the tubular cells of the medullary thick ascending limb (MTAL; Riccardi *et al.,* 1998), and the luminal side of the epithelial cells of the inner medullary collecting duct (IMCD; Sands *et al.,* 1997). None of these latter three nephron segments play major roles in renal Ca$_o^{2+}$ handling, but the CaR expressed in them could conceivably modulate the handling of other solutes and/or water. As will be discussed in more detail later, the CaR in the CTAL, in addition to modulating reabsorption of Ca^{2+} and Mg^{2+}, also participates in controlling the renal handling of Na$^+$, K$^+$, and Cl$^-$ (Hebert *et al.,* 1997). Finally, the CaR expressed in the IMCD likely mediates the well-recognized inhibitory action of high Ca$_o^{2+}$ on vasopressin-evoked water reabsorption (Sands *et al.,* 1997, 1998), which is one cause of the defective urinary-concentrating ability in some patients with hypercalcemia (Brown, 1999; Brown *et al.,* 1999).

In the proximal tubule, activation of the CaR inhibits PTH-stimulated phosphaturia (Ba *et al.,* 2003) and increases expression of the vitamin D receptor (Maiti and Beckman, 2007), which could conceivably contribute to the direct, high calcium-induced lowering of 1,25(OH)$_2$D$_3$ levels in parathyroidectomized, PTH-infused rats (Weisinger *et al.,* 1989). The localization of the CaR on the basolateral membrane in the CTAL indicates that it might represent the mediator of the previously demonstrated inhibitory effect of high peritubular but not luminal levels of Ca$_o^{2+}$ on Ca^{2+} and Mg^{2+} reabsorption in perfused tubular segments from this portion of the nephron (De Rouffignac and Quamme, 1994). Figure 2 shows a schematic illustration

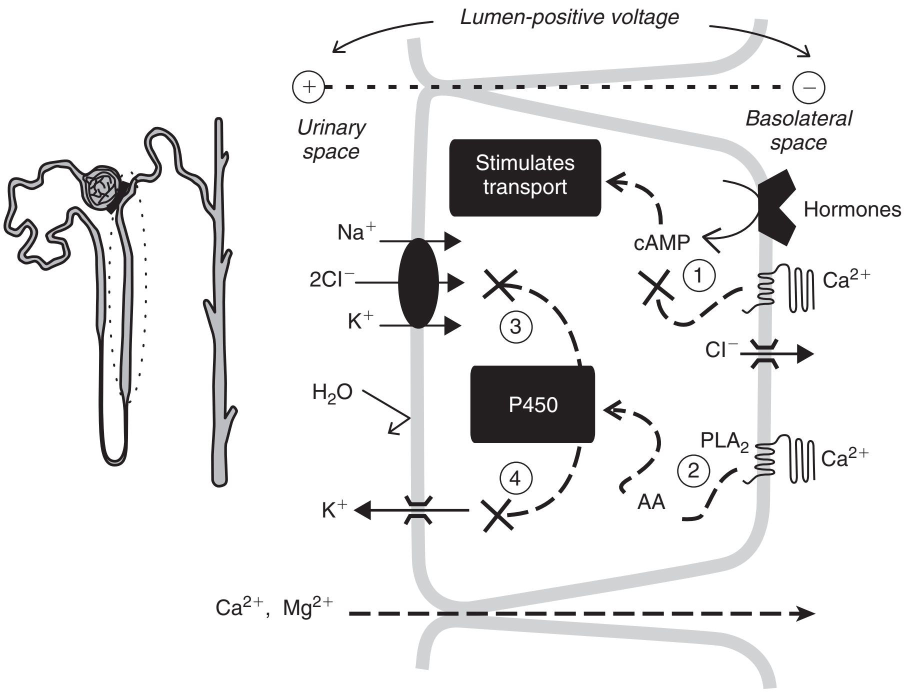

FIGURE 2 Possible mechanisms by which the CaR modulates intracellular second-messenger pathways and ionic transport in the CTAL. Hormones that elevate cAMP (i.e., PTH) stimulate paracellular Ca^{2+} and Mg^{2+} reabsorption by increasing the activities of the Na$^+$-K$^+$-2Cl$^-$ cotransporter and an apical K$^+$ channel and, therefore, increasing the magnitude of V_t. The CaR, which like the PTH receptor is on the basolateral membrane, inhibits PTH-stimulated adenylate cyclase and activates PLA$_2$ (2). This latter action increases free arachidonic acid, which is metabolized by the P450 pathway to an inhibitor of the apical K$^+$ channel (4) and, perhaps, the cotransporter (3). Actions of the CaR on both adenylate cyclase and PLA$_2$ will reduce V$_t$ and, therefore, diminish paracellular divalent cation transport. Reproduced with permission from Brown and Hebert (1997a).

of how the CaR may inhibit PTH-enhanced divalent cation reabsorption in the CTAL. As indicated in detail in Figure 2, the CaR acts in a "Lasix-like" fashion to diminish overall activity of the Na^+-K^+-$2Cl^-$ cotransporter, which generates the lumen-positive, transepithelial potential gradient that normally drives passive paracellular reabsorption of about 50% of NaCl and most of the Ca^{2+} and Mg^{2+} in this part of the nephron (Hebert *et al.,* 1997). Some studies, however, while demonstrating inhibition of calcium transport in CTAL by elevating peritubular Ca_o^{2+}, failed to show any concomitant reduction in NaCl or magnesium transport or in the transepithelial potential (DesFleurs *et al.,* 1998), or else have documented inhibitory effects of peritubular calcium on not only paracellular but also transepithelial Ca^{2+} transport (Motoyama and Friedman, 2002). Further studies are needed to resolve these differences. It is of interest that individuals with FHH manifest a markedly reduced ability to upregulate urinary excretion of Ca^{2+} despite their hypercalcemia—even when they have been rendered aparathyroid by total parathyroidectomy (Attie *et al*., 1983). Thus, the PTH-independent, excessive reabsorption of Ca^{2+} in FHH likely results, in part, from a decreased complement of normally functioning CaRs in the CTAL. This defect renders the tubule "resistant" to Ca_o^{2+} and reduces the normal, high Ca_o^{2+}-evoked hypercalciuria that occurs in this nephron segment (Brown, 1999). Thus, in normal persons, hypercalcemia-evoked hypercalciuria likely has two distinct CaR-mediated components: (1) suppression of PTH secretion and (2) direct inhibition of tubular reabsorption of Ca^{2+} in the CTAL.

It is not presently known whether the CaR modulates PTH-stimulated Ca^{2+} reabsorption in the DCT, which could potentially occur via inhibition of PTH-stimulated cAMP accumulation. In addition, extracellular Ca^{2+} has been shown to modulate the expression of key, vitamin D—inducible genes involved in transcellular Ca^{2+} transport in this nephron segment, namely, the apical uptake channel TRPV5, calbindin D and the basolateral calcium pump, PMCA2b, and exchanger, NCX1 (Thebault *et al.,* 2006). These changes take place both indirectly, by high Ca^{2+}-elicited changes in PTH secretion and pari passu $1{,}25(OH)_2D_3$ production, as well as by apparently direct renal actions of Ca_o^{2+} that are independent of $1{,}25(OH)_2D_3$. The contribution of these actions of Ca_o^{2+} to the function of DCT in tubular reabsorption of Ca^{2+} under normal circumstances is not currently known.

Intestine

The intestine is a key participant in maintaining Ca_o^{2+} homeostasis by virtue of its capacity for regulated absorption of dietary Ca_o^{2+} through the action of $1{,}25(OH)_2D_3$, the most active naturally occurring metabolite of vitamin D (Bringhurst *et al.,* 1998; Brown, 1991). The duodenum is the principal site for 1,25-dihydroxyvitamin D_3 $[1{,}25(OH)_2D_3]$-stimulated intestinal Ca^{2+} absorption through a transcellular pathway of active transport. The latter is thought to involve initial Ca^{2+} entry through the recently identified Ca^{2+}-permeable channel, TRPV6 (previously known as CaT1 or ECaC2; Peng *et al.,* 1999; Hoenderop *et al.,* 1999). As with the transcellular transport of Ca^{2+} in DCT, calcium ions subsequently diffuse across the cell—a process facilitated by the vitamin D − dependent Ca^{2+}-binding protein, calbindin D_{9K}—and are eventually extruded at the basolateral cell surface by the Ca^{2+}-ATPase and, perhaps, the Na^+-Ca^{2+} exchanger (Thebault *et al.,* 2006). Jejunum and ileum absorb less Ca^{2+} than duodenum (particularly when expressed as absorption per unit surface area). They also secrete Ca^{2+}, which may chelate fatty acids and bile acids, thereby producing insoluble "calcium soaps" and mitigating possible damaging effects of free fatty acids and bile salts on colonic epithelial cells. The major function of the colon in fluid and electrolyte metabolism is the absorption of water and Na^+. Nevertheless, it absorbs substantial amounts of Ca^{2+} in humans by both vitamin D-dependent and -independent routes, especially in its proximal segments (Favus, 1992), where it expresses levels of TRPV6 similar to those in the duodenum (Peng *et al.,* 1999).

The CaR is expressed in all segments of the intestine in the rat. The highest levels of expression of the receptor are on the basal surface of the small intestinal absorptive cells, the epithelial cells of the crypts of both the small intestine and colon, and in the enteric nervous system (Chattopadhyay *et al.,* 1998). Does the CaR have any role in systemic Ca_o^{2+} homeostasis in these locations? There are several direct actions of Ca_o^{2+} on intestinal function. Hypercalcemia inhibits dietary Ca^{2+} absorption (Krishnamra *et al.,* 1994). Recent studies have also shown apparently direct actions of dietary and/or extracellular Ca^{2+} on the expression of TRPV6, calbindin D_{9K}, and PMCA2B in mice with knockout of the 1-hydroxylase gene, which are of uncertain physiological significance at present but document the capacity of the GI tract to sense Ca_o^{2+} (Hoenderop *et al.,* 2004). Moreover, direct measurements of Ca_o^{2+} within the interstitial fluid underneath small intestinal absorptive epithelial cells has shown that Ca_o^{2+} increases by nearly twofold if luminal levels of Ca_o^{2+} are elevated to 5 to 10 mM—similar to those achieved after the intake of Ca^{2+}-containing foods (Mupanomunda *et al.,* 1999). This level of Ca_o^{2+} would be more than sufficient to stimulate the CaR expressed on the basolateral surface of small intestinal absorptive cells. The CaR expressed in the enteric nervous system, which regulates the secretomotor functions of the gastrointestinal tract, could potentially mediate known effects of high and low Ca_o^{2+} to reduce and increase GI motility, respectively, in hyper- and hypocalcemic individuals (Bringhurst *et al.,* 1998). Such an effect on gastrointestinal motility, however, even if it were CaR-mediated, would not have any obvious relevance to systemic Ca_o^{2+} homeostasis, other than perhaps indirectly, by modulating the time available for absorption of Ca^{2+} and other nutrients.

Bone and Cartilage

The levels of Ca_o^{2+} achieved within the bony microenvironment probably vary substantially during the regulated turnover of the skeleton via osteoclastic resorption of bone followed by its restoration by bone-forming osteoblasts—a process that totally replaces the human skeleton approximately every 10 years (Bringhurst *et al.,* 1998). In fact, Ca_o^{2+} directly beneath resorbing osteoclasts can be as high as 8 to 40 m*M* (Silver *et al.,* 1988). Moreover, Ca_o^{2+} has a variety of actions on bone cell functions *in vitro* that could serve physiologically useful purposes, although it is not yet known whether these same actions occur *in vivo*. For example, high Ca_o^{2+} stimulates parameters of osteoblastic functions that could enhance their recruitment to sites of future bone formation, including chemotaxis and proliferation, and promote their differentiation to osteoblasts with a more mature phenotype (Quarles, 1997; Yamaguchi *et al.,* 1999; Dvorak *et al.,* 2004). Furthermore, elevated levels of Ca_o^{2+} suppress both the formation (Kanatani *et al.,* 1999) and the activity (Zaidi *et al.,* 1999) of osteoclasts *in vitro*. Therefore, Ca_o^{2+} has effects on cells of both the osteoblastic and osteoclastic lineages and/or their precursors that are homeostatically appropriate. Raising Ca_o^{2+} would, for example, produce net movement of Ca^{2+} into bone by stimulating bone formation and inhibiting its resorption. In addition, locally elevated levels of Ca_o^{2+} produced by osteoclasts at sites of active bone resorption could potentially contribute to "coupling" bone resorption to the ensuing replacement of the missing bone by osteoblasts, by promoting proliferation and recruitment of preosteoblasts within the vicinity to these sites and enhancing their differentiation (Quarles, 1997; Yamaguchi *et al.,* 1999). As detailed later, the molecular identity of the Ca_o^{2+}-sensing mechanism(s) in bone cells remain(s) controversial, although the CaR has been found by several groups of investigators to be present in at least some cells of both osteoblast and osteoclast lineages and could, therefore, potentially participate in this process.

Substantial indirect evidence amassed prior to and around the time that the CaR was cloned suggested that the Ca_o^{2+}-sensing mechanism in osteoblasts and osteoclasts differed in certain pharmacological and other properties from those exhibited the CaR (Quarles, 1997; Zaidi *et al.,* 1999). Moreover, some investigators have been unable to detect expression of the CaR in osteoblast-like (Pi *et al.,* 1999) and osteoclast-like cells (Seuwen *et al.,* 1999). More recent studies, however, have provided strong support for the presence of the CaR in a variety of cells originating from the bone and bone marrow, although its physiological and functional implications in these cells remain uncertain. These CaR-expressing cells include hematopoietic stem cells (Adams *et al.,* 2006), erythroid and platelet progenitors, cells of the monocytic lineage (Yamaguchi *et al.,* 1998d) [but not granulocytes (House *et al.,* 1997)], and cells of both the osteoblast and osteoclast lineages when studied *in situ* in sections of bone (Yamaguchi *et al.,* 1999; Chang *et al.,* 1999b; Dvorak *et al.,* 2004). ST-2 stromal cells (Yamaguchi *et al.,* 1998a), a stromal cell line derived from the same mesenchymal stem cells giving rise to osteoblasts, express CaR mRNA and protein, as do osteoblast-like cell lines (e.g., the Saos-2, MC-3T3-E1, UMR-106, and MG-63 cell lines; Chang *et al.,* 1999b; Yamaguchi *et al.,* 1998b, 1998c). Regarding cells of the osteoclast lineage, more than 80% of human peripheral blood monocytes, which arise from the same hematopoietic lineage that gives rise to osteoclast precursors, express abundant levels of CaR mRNA and protein (Yamaguchi *et al.,* 1998d). Preosteoclast-like cells generated *in vitro* also show expression of the CaR (Kanatani *et al.,* 1999), and osteoclasts isolated from rabbit or mouse bone likewise express the receptor (Kameda *et al.,* 1998; Mentaverri *et al.,* 2006). In murine, rat, and bovine bone sections, in contrast, only a minority of the multinucleated osteoclasts expressed CaR mRNA and protein (Chang *et al.,* 1999b). Additional studies are required to clarify whether primarily osteoclast precursors, rather than mature osteoclasts, express the CaR and whether the CaR actually mediates some or even all of the known actions of Ca_o^{2+} on these cells. One study failed to detect expression of the CaR in transformed osteoblast-like cells derived from either wild-type mice or those with knockout of the CaR (Pi *et al.,* 2000). However, these cells still showed some responses to Ca_o^{2+} and Al^{3+} (e.g., mitogenesis), suggesting the presence of another Ca_o^{2+} sensor, as this group has suggested in earlier studies (Quarles *et al.,* 1997). This group has recently suggested that the basic amino acid-sensing, family C receptor, GPRC6A, represents the key Ca_o^{2+}–sensing mechanism present in osteoblasts (Pi *et al.,* 2005).

Although chondrocytes—the cells that form cartilage—do not participate directly in systemic Ca_o^{2+} homeostasis, they play a key role in skeletal development and growth by providing a cartilaginous model of the future skeleton that is gradually replaced by actual bone. Furthermore, the growth plate represents a site where chondrocytes play a crucial role in the longitudinal growth that persists until the skeleton is fully mature at the end of puberty. The availability of Ca^{2+} is important for ensuring proper growth and differentiation of chondrocytes and resultant skeletal growth *in vivo* (Jacenko and Tuan, 1995; Reginato *et al.,* 1993). Moreover, changing the level of Ca_o^{2+} *in vivo* modulates the differentiation and/or other properties of cells of the cartilage lineage (Bonen and Schmid, 1991; Wong and Tuan, 1995). Chondrocytes arise from the same mesenchymal stem cell lineage that gives rise to osteoblasts, smooth muscle cells, adipocytes, and fibroblasts (Boyan *et al.,* 1999; Dennis *et al.,* 1999). It is interesting, therefore, that the rat cartilage cell line, RCJ3.1C5.18, showed readily detectable levels of CaR mRNA and protein (Chang *et al.,* 1999a). In addition, some cartilage cells in sections of intact bone express CaR mRNA and protein, including the hypertrophic chondrocytes in the growth plate, which are key participants in the growth of long bones (Chang *et al.,* 1999b). Thus the CaR is a candidate for mediating, at least in part, the previously described direct actions of Ca_o^{2+} on chondrocytes and cartilage growth.

In fact, elevating Ca_o^{2+} exerts several direct effects on RCJ3.1C5.18 cells, namely, dose dependently reducing the levels of the mRNAs that encode a major proteoglycan in cartilage, aggrecan, the α_1 chains of type II and X collagens, and alkaline phosphatase (Chang *et al.,* 1999a). In addition, treatment of this cell line with a CaR antisense oligonucleotide for 48 to 72 hours reduced the level of CaR protein expression significantly, in association with enhanced expression of aggrecan mRNA (Chang *et al.,* 1999a), suggesting a mediatory role of the CaR in regulating this gene. This same group has recently shown that chondrocytes isolated from CaR knockout mice retain responsiveness to Ca_o^{2+}. However, this residual activity may result from the fact that these mice are able to splice out exon 5, which was inactivated by insertion of the neomycin gene, to yield an in-frame, modestly truncated receptor that may have residual biological activity (Rodriguez *et al.,* 2005). These results demonstrate, therefore, that (1) Ca_o^{2+} modulates the expression of several important genes in chondrocytic cells, (2) chondrocytic cells express CaR mRNA and protein, and (3) the CaR mediates at least some of these actions of Ca_o^{2+} in this cell type. Thus the CaR could potentially not only modulate bone turnover and/or the coupling of bone resorption to its later replacement by osteoblasts through its actions on bone cells and/or their precursors, but might also participate in the control of skeletal growth through its effects on chondrocytes.

THE CaR AND INTEGRATION OF CALCIUM AND WATER METABOLISM

In addition to its roles in tissues involved directly in Ca_o^{2+} homeostasis, increasing evidence indicates that the CaR is located in other cells and tissues where it contributes to integrating the functions of distinct homeostatic systems, as discussed in this section. An illustrative example is the capacity of the CaR to integrate certain aspects of mineral and water metabolism. Some hypercalcemic patients exhibit reduced urinary concentrating capacity and, occasionally, frank nephrogenic diabetes insipidus (Gill and Bartter, 1961; Suki *et al.,* 1969). In addition, simply the presence of hypercalciuria, without accompanying hypercalcemia, can impair urinary concentrating ability, as illustrated by the enuresis present in some hypercalciuric children, which resolves with dietary calcium restriction and resolution of the hypercalciuria (Valenti *et al.,* 2002). The presence of the CaR in several segments of the nephron that participate in regulating urinary concentration (Riccardi *et al.,* 1998; Sands *et al.,* 1997) has suggested a novel mechanism(s) underlying the long-recognized but poorly understood inhibitory actions of high Ca_o^{2+} on renal-concentrating capacity. Studies have shown that high Ca_o^{2+}, probably by activating CaRs residing on the apical membrane of cells of the IMCD, reversibly inhibits vasopressin-elicited water flow by about 35% to 40% in perfused rat IMCD tubules (Sands *et al.,* 1997). Indeed, the CaR is present in the same apical endosomes that contain the vasopressin-regulated water channel, aquaporin-2 (Sands *et al.,* 1997). This observation indicates that the receptor potentially reduces vasopressin-enhanced water flow in the IMCD by either promoting the endocytosis or, more likely, blocking the exocytosis of these endosomes (Procino *et al.,* 2004). Furthermore, induction of chronic hypercalcemia in rats through treatment with vitamin D reduces the level of expression of the aquaporin-2 protein but not its mRNA (Sands *et al.,* 1998), which would further decrease vasopressin-activated water flow in the terminal-collecting duct. In addition to the mechanisms just described, high Ca_o^{2+}-elicited, CaR-mediated reduction in the reabsorption of NaCl in the MTAL (Wang *et al.,* 1996, 2001), by decreasing the medullary countercurrent gradient, would diminish even further the maximal urinary-concentrating power of hypercalcemic patients (Figure 3). CaR-evoked generation of prostaglandins, especially PGE_2, due to upregulation of Cox-2 in MTAL, may impair NaCl reabsorption in this nephron segment and, in turn, generation of the hypertonic interstitium needed for normal urinary concentrating ability (Wang *et al.,* 2001).

What is the evidence that these effects of high Ca_o^{2+} on the renal concentrating mechanism are mediated by the CaR? Of interest in this regard, persons with inactivating mutations of the CaR are capable of concentrating their urine normally despite their hypercalcemia (Marx *et al.,* 1981), presumably because they are “resistant” to the usual suppressive effects of high Ca_o^{2+} on the urinary-concentrating mechanism. Conversely, individuals with activating CaR mutations can develop symptoms of diminished urinary-concentrating capacity (e.g., polyuria and polydipsia), even at normal levels of Ca_o^{2+}, when treated with vitamin D and calcium supplementation, probably because their renal CaRs are overly sensitive to Ca_o^{2+} (Pearce *et al.,* 1996). These experiments in nature, therefore, support the postulated, CaR-mediated link between Ca_o^{2+} and water homeostasis.

Is the defective renal handling of water in hypercalcemic patients of any physiological relevance? We have suggested previously that it provides a mechanism that integrates the renal handling of divalent cations, especially

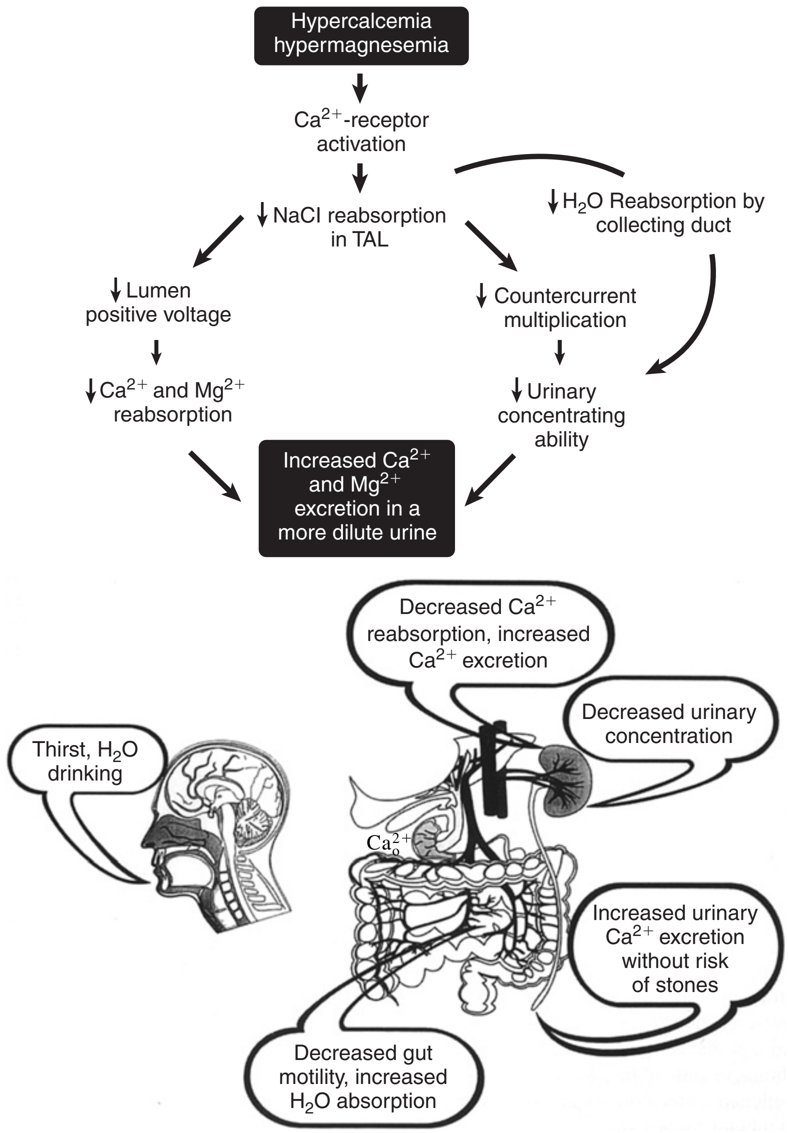
Hypercalcemia hypermagnesemia
Ca^{2+}-receptor activation
↓NaCl reabsorption in TAL
↓H_2O Reabsorption by collecting duct
↓Lumen positive voltage
↓Countercurrent multiplication
↓Ca^{2+} and Mg^{2+} reabsorption
↓Urinary concentrating ability
Increased Ca^{2+} and Mg^{2+} excretion in a more dilute urine
Decreased Ca^{2+} reabsorption, increased Ca^{2+} excretion
Decreased urinary concentration
Thirst, H_2O drinking
Ca_o^{2+}
Increased urinary Ca^{2+} excretion without risk of stones
Decreased gut motility, increased H_2O absorption

FIGURE 3 Mechanisms that may interrelate systemic Ca_o^{2+} and water homeostasis (see text for further details). (*Top*) Mechanisms through which the CaR reduces maximal urinary-concentrating ability. Reproduced with permission from Brown and Hebert (1997b). (*Bottom*) Additional extrarenal mechanisms integrating Ca_o^{2+} and water homeostasis, such as Ca_o^{2+} -evoked activation of the CaR in the SFO, which would enhance water intake and mitigate loss of free water that would otherwise result from a diminished maximal urinary-concentrating capacity. Reproduced with permission from Brown *et al*. (1996).

Ca^{2+}, and water, thereby allowing appropriate "trade-offs" in how the kidney coordinates calcium and water metabolism under specific physiological conditions (Hebert *et al.,* 1997; see Fig. 3). For example, in a situation where a systemic load of Ca^{2+}, must be disposed of, the resultant CaR-mediated inhibition of PTH secretion and direct inhibition of tubular reabsorption of Ca^{2+}, promote calciuria. The consequent elevation in luminal Ca_o^{2+} in the IMCD, particularly in a dehydrated individual, could predispose to the formation of Ca^{2+}-containing renal stones, were not for the concomitant, CaR-mediated inhibition of maximal urinary concentration. Furthermore, there are abundant CaRs in the subfornical organ (SFO; Rogers *et al.,* 1997)—an important hypothalamic thirst center (Simpson and Routenberg, 1975)—that may ensure a physiologically appropriate increase in drinking. This increased intake of free water could prevent dehydration that might otherwise be a consequence of renal loss of free water due to the concomitant, CaR-induced inhibition of the urinary-concentrating mechanism (see Fig. 3).

Finally, available data support the existence of a "calcium appetite" in rats (Tordoff, 1994) that may furnish a mechanism for modulating the intake of calcium-containing foods in a physiologically relevant manner during hypo- and hypercalcemia. Some reduction in the intake of Ca_o^{2+}-containing foods might also occur as a result of the activation of CaRs in the area postrema of the brain—a "nausea center" (Rogers *et al.,* 1997)—due to the resultant anorexia/nausea. Calcium appetite, in contrast, appears to be mediated by the subfornical organ (McCaughey *et al.,* 2003). The role of the CaR in either of these actions, however, is currently conjectural. We have hypothesized, therefore, that the CaR mediates multiple layers of integration and coordination of the homeostatic systems governing water and calcium metabolism. In doing so, it may contribute to the ability of terrestrial organisms to adjust to the only intermittent availability of environmental calcium and water (Hebert *et al.,* 1997).

CaR-mediated modulation of vasopressin-induced water flow in the IMCD represents an example of "local" Ca_o^{2+} homeostasis. That is, Ca_o^{2+} within a specific microenvironment, which is outside of the blood and the various compartments of the ECF in direct contact with the circulation, is only allowed to rise to a certain level (Brown *et al.,* 1999). Interestingly, changes in the level of Ca_o^{2+} resulting from the mechanism governing systemic Ca_o^{2+} homeostasis are traditionally thought to occur through fine adjustments of the movements of Ca^{2+} into or out of the ECF (i.e., by intestine, bone, or kidney; Brown, 1991). In contrast, CaR-mediated regulation of Ca_o^{2+} in the IMCD primarily results from alterations in the movement of water. Perhaps even this formulation is oversimplified. On the one hand, vasopressin is known to increase distal tubular reabsorption of calcium (Hoenderop *et al.,* 2000), which would also reduce the level of Ca_o^{2+} in the collecting duct. On the other, the increased thirst in hypercalcemic patients, in addition to providing more free water so as to mitigate any associated rise in Ca_o^{2+} in the IMCD, would also dilute Ca_o^{2+} in the ECF. Further studies will no doubt illuminate additional subtleties related to how the body integrates divalent mineral and water metabolism.

OTHER CaR AGONISTS AND MODULATORS: THE CaR AS AN INTEGRATOR OF PHYSIOLOGICAL SIGNALS AND AS A "NUTRIENT SENSOR"

A variety of divalent cations (Sr_o^{2+}), trivalent cations (e.g., Gd_o^{3+}), and even organic polycations (i.e., spermine; Quinn *et al.,* 1997) are effective CaR agonists. It is likely that they all interact with one or more binding sites within the ECD of the receptor (Brown *et al.,* 1999). Only a few of these polycationic agonists, however, are thought to be present within biological fluids at levels that would activate the CaR (Quinn *et al.,* 1997). In addition to Ca_o^{2+}, Mg_o^{2+} and spermine are two such putative, physiological CaR agonists. It is probable that in specific microenvironments [e.g., within the gastrointestinal (GI) tract and central nervous system] the concentrations of spermine are high enough to activate the CaR even at levels of Ca_o^{2+} that are insufficient to do so by themselves (Brown *et al.,* 1999; Quinn *et al.,* 1997). In fact, all of the polycationic CaR agonists potentiate one another's stimulatory effects on the receptor. In other words, only small increments in the level of a given agonist (i.e., spermine) may be sufficient to activate the CaR when a threshold level of another agonist is present in the local microenvironment (e.g., Ca_o^{2+}; Brown *et al.,* 1999).

Is the CaR also a Mg_o^{2+} -sensing receptor? Some evidence supporting the role of the CaR in sensing and, therefore, "setting" Mg_o^{2+} comes from the experiments in nature that firmly established the role of the CaR as a central element in Ca_o^{2+} homeostasis. Namely, persons with hypercalcemia due to heterozygous-inactivating mutations of the CaR (e.g., FHH) exhibit serum Mg^{2+} levels that are in the upper part of the normal range or mildly elevated. Moreover, some patients with neonatal severe hyperparathyroidism due to homozygous inactivating CaR mutations can have more pronounced hypermagnesemia (Aida *et al.,* 1995a). Conversely, persons harboring activating mutations of the CaR can manifest mild reductions in Mg_o^{2+} (Brown, 1999). Mg_o^{2+} is about twofold less potent than Ca_o^{2+} on a molar basis in activating the CaR (Brown *et al.,* 1993; Butters *et al.,* 1997). One might justifiably ask, therefore, how Mg_o^{2+} could regulate its own homeostasis by modulating PTH secretion—an important component of CaR-mediated control of Ca_o^{2+}—when circulating levels of Mg_o^{2+} are, if anything, lower than those of Ca_o^{2+} (Bringhurst *et al.,* 1998)? It is possible that even small changes in Mg_o^{2+} can modulate the activity of the CaR in parathyroid cells because the receptor has been sensitized by ambient levels of Ca_o^{2+} that are close to its "set point" (i.e., on the steepest portion of the curve relating PTH to Ca_o^{2+}). A more likely scenario, however, is that acts on the CaR in the CTAL to regulate its own level in the ECF, as follows: The fraction of Mg^{2+} reabsorbed in the proximal tubule is less than for other solutes (e.g., Ca^{2+}, Na^+, Cl^-, and water). As a result, there is a 1.6- to 1.8-fold rise in the level of Mg_o^{2+}, in the tubular fluid of the CTAL (De Rouffignac and Quamme, 1994), which should, therefore, reach a sufficiently high level to activate the CaR in this nephron segment and, therefore, reduce the reabsorption of Mg_o^{2+}. Recall that not only Ca_o^{2+} but also Mg_o^{2+} inhibits the reabsorption of both divalent cations in perfused CTAL (De Rouffignac and Quamme, 1994).

Another factor modulating the actions of Ca_o^{2+} and other polycations on the CaR is ionic strength per se (e.g., alterations in the concentration of NaCl; Quinn *et al.,* 1998). Elevating ionic strength decreases and reducing ionic strength enhances the sensitivity of the CaR to activation by Ca_o^{2+} and Mg_o^{2+}. The impact of changing ionic strength on the responsiveness of the CaR to divalent cations may be especially relevant in particular microenvironments, such as the GI or urinary tracts, where ionic strength can vary greatly, easily encompassing the range over which this parameter modulates the function of the receptor (Quinn *et al.,* 1998).

In addition to the polycationic CaR agonists just noted, novel "calcimimetic," allosteric activators of the receptor have been developed. These are small hydrophobic molecules, which are derivatives of phenylalkylamines and activate the CaR by increasing its apparent affinity for Ca_o^{2+}. They do so by interacting with the transmembrane domains of the receptor (Nemeth *et al.,* 1998b), in contrast to Ca_o^{2+} which binds to the ECD (Hammerland *et al.,* 1999). Calcimimetics are called "modulators" rather than "agonists" because they only activate the CaR in the presence of Ca_o^{2+}. In contrast, the polycationic agonists of the CaR (e.g., Gd_o^{3+}) activate it even in the nominal absence of Ca_o^{2+} (Nemeth *et al.,* 1998b). Calcimimetics have been approved for use in patients with stage 5 chronic kidney disease (i.e., on dialysis) and those with parathyroid cancer, where they provide an effective medical means of lowering PTH secretion through their direct action on the parathyroid CaR. A detailed discussion of the therapeutic utility of these agents is beyond the scope of this review, but detailed descriptions of their mechanism of action and clinical use can be found in recent reviews (Nemeth, 2004). CaR antagonists, so-called "calcilytics," are also entering clinical trials. The principal therapeutic application envisioned for these agents at the moment is in the treatment of osteoporosis (Nemeth, 2004). Because intermittent exogenous administration of PTH can produce sizable increases in bone mineral density, once-daily administration of a short-acting calcilytic would presumably accomplish the same goal by producing a "pulse" of endogenous PTH secretion (Gowen *et al.,* 2000; Nemeth *et al.,* 1998a).

Amino acids represent another class of endogenous CaR modulators (Conigrave *et al.,* 2000; Figure 4). Activation of the CaR by specific amino acids, particularly the aromatic amino acids, phenylalanine, tyrosine, histidine, and tryptophan, only occurs when Ca_o^{2+} is 1 m*M* or higher. Although individual amino acids are of relatively low potency (e.g., they act at concentrations of 0.1 to 1 m*M* or higher), a mixture of amino acids with a composition similar to that present in human serum under fasting conditions substantially enhances the sensitivity of the CaR to Ca_o^{2+}. That is, optimal concentrations of the mixture reduce the EC_{50} for Ca_o^{2+} (the level of Ca_o^{2+} half-maximally activating the receptor) by nearly 2 mM (e.g., by 40% to 50%) in HEK293 cells stably transfected with the CaR (Conigrave *et al.,* 2000). Amino acids also inhibit PTH secretion in vitro from human parathyroid cells, further supporting their biological relevance as CaR activators (Conigrave *et al.,* 2004).

Although the implications of these direct actions of amino acids on the CaR are not yet clear, they could potentially explain several long-standing but poorly understood observations appearing to link protein and Ca_o^{2+} metabolism. Additionally, they suggest future lines of research directed at elucidating the possible role of the receptor in "nutrient-sensing" more generally, rather than just as a sensor of divalent cations. For example, ingestion of a high-protein diet nearly doubles the rate of urinary calcium excretion relative to that observed on a low protein intake (Insogna and Broadus, 1987). This effect of dietary protein has traditionally been ascribed to the buffering of acidic products of protein metabolism by bone as well as a calciuric action of the acid load (Lemann *et al.,* 1966). However, direct activation of CaRs in the CTAL as a result of increases in serum levels of amino acids might contribute as well. Moreover, reducing dietary protein intake has been shown to have marked effects on circulating calciotropic hormones, nearly doubling serum PTH and $1{,}25(OH)_2D_3$ levels in normal women (Kerstetter *et al.,* 1997). Could these latter changes result from the concomitant decrease in the circulating levels of amino acids, despite little, if any, change in Ca_o^{2+}? Is it possible

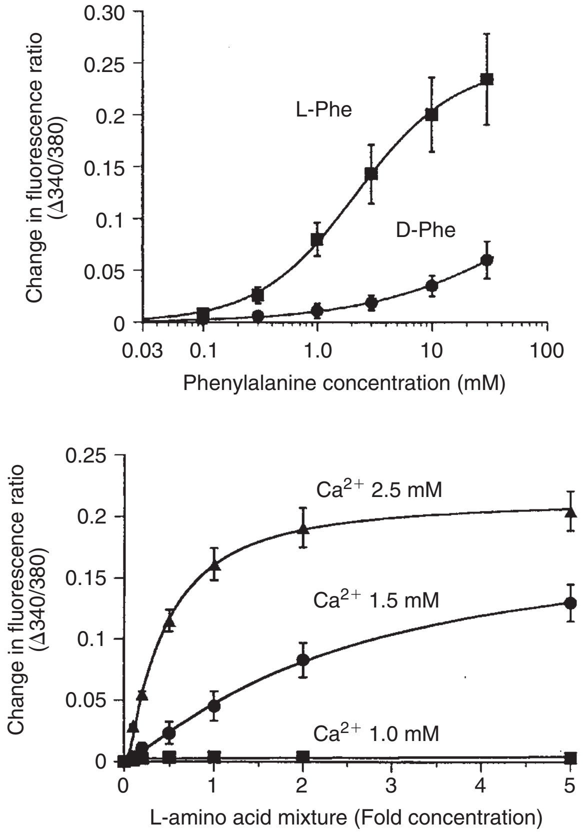

FIGURE 4 Amino acid sensing by the CaR. (*Top*) Activation of the CaR by phenylalanine (L-phenylalanine > D-phenylalanine) at 2.5 mM Ca_o^{2+} in HEK293 cells transfected stably with the CaR as reflected by amino acid-induced increases in the cytosolic Ca_o^{2+} concentration in cells loaded with the Ca_o^{2+}-sensitive intracellular dye fura-2. Increases in the fluorescence ratio (340/380 nm) indicate CaR-mediated increases in Ca_i^{2+}. (*Bottom*) Marked impact on the level of Ca_o^{2+} on the capacity of the CaR to sense the mixture of l-amino acids that emulates that present in the blood under fasting conditions (see text for details). Reproduced with permission from Conigrave *et al.* (2000).

that the reduced intake of dietary protein commonly recommended for patients with chronic renal insufficiency (Bringhurst *et al.,* 1998) actually exacerbates their secondary hyperparathyroidism?

These observations suggest that the CaR is not solely a Ca_o^{2+} (and probably a Mg_o^{2+}) receptor but may also serve as a more general "nutrient" and environmental sensor, which detects changes in Ca_o^{2+} and Mg_o^{2+}, not in isolation, but in the context of the ambient levels of certain amino acids. Further testing of this hypothesis may enhance our understanding of the mechanisms by which complex organisms coordinate homeostatic systems that have traditionally been thought of as functioning largely independently, such as those controlling protein and mineral metabolism. This homeostatic integration may be particularly important within specific parts of the life cycle, such as during somatic growth. Skeletal growth in childhood requires the precisely coordinated deposition of both bone matrix and mineral. Moreover, mineral ions and amino acids must also be assimilated during the growth of soft tissues—all of which contain varying mixtures of mineral ions and protein. For instance, smooth muscle cells contain half as much calcium as bone when expressed on the basis of wet weight (Brown and MacLeod, 2001).

Coordinating mineral ion and protein metabolism might be particularly relevant in the GI tract. Indeed, the presence of an "amino acid receptor" regulating the secretion of gastrin, gastric acid, and cholecystokinin has been postulated (Conigrave *et al.,* 2000). Furthermore, the pharmacological profile of the actions of different amino acids on these parameters is strikingly similar to that for the effects of the same amino acids on the CaR (Mangel *et al.,* 1995; McArthur *et al.,* 1983; Taylor *et al.,* 1982; Conigrave *et al.,* 2000). CaRs in the GI tract system could serve as a particularly suitable target for sensing the availability of dietary protein and mineral ions, which are generally ingested together (i.e., in milk). Further studies are needed, therefore, to investigate whether the CaR represents, in fact, this putative amino acid receptor. Such investigations may reveal whether the sensing of amino acids by the CaR, taken in the context of ambient levels of Ca_o^{2+} and Mg_o^{2+} within the GI tract and elsewhere, provides the molecular basis for a physiologically important link between the systems governing protein and mineral metabolism.

ARE THERE ADDITIONAL Ca_o^{2+} SENSORS?

As noted earlier and described in detail in other reviews (Quarles, 1997; Zaidi *et al.,* 1999; Pi *et al.,* 2005), Ca_o^{2+} sensors in addition to the CaR may exist on osteoblasts and osteoclasts. Moreover, studies have revealed that some of the mGluRs can sense Ca_o^{2+} in addition to recognizing glutamate as their principal physiological agonist, although the physiological importance of this Ca_o^{2+} sensing is not clear at present. Kubo *et al.* (1998) showed that mGluRs 1, 3, and 5 sense levels of Ca_o^{2+} between 0.1 and 10 mM, whereas mGluR2 is substantially less responsive to Ca_o^{2+}. All three of the mGluRs that are capable of sensing Ca_o^{2+} have identical serines and threonines, respectively, at amino acid positions that are equivalent to amino acid residues 165 and 188 in mGluR1a (Brauner-Osborne *et al.,* 1999). These two residues have been shown to play key roles in the binding of glutamate to the respective ECDs of the MGluRs (O'Hara *et al.,* 1993). In contrast, whereas mGluRs 1a, 3, and 5 have serines at positions equivalent to amino acid residue 166 in mGluR1a, mGluR2 has an aspartate rather than a serine at this position (Kubo *et al.,* 1998). Changing this serine to an aspartate in mGluRs 1a, 3, and 5 substantially reduces their capacities to sense Ca_o^{2+}, whereas substituting the aspartate in mGluR2 with a serine enhances its apparent affinity for Ca_o^{2+} to a level comparable to those of mGluRs 1, 3, and 5 (Kubo *et al.,* 1998). Thus the serines in mGluRs 3 and 5 at amino acid positions homologous to residue 166 in mGluR1a appear to play important roles in the capacities of these three receptors to sense Ca_o^{2+}.

Interestingly, another study has shown that changes in Ca_o^{2+} also modulate the function of the activated $GABA_B$ receptors, whereas Ca_o^{2+} has no effect on these receptors in the absence of added GABA (Wise *et al.,* 1999). Ca_o^{2+} potentiates the stimulatory effect of GABA on the binding of GTP to the receptor and increases the coupling of the $GABA_B$ receptor to stimulation of a K^+ channel and inhibition of forskolin-stimulated adenylate cyclase activity. The effects of Ca_o^{2+} on the $GABA_B$ receptor, unlike those on the CaR, were not reproduced by other polyvalent cations. Thus, similar to the CaR, which senses Ca_o^{2+} but is modulated by various amino acids (although not by glutamate; Conigrave *et al.,* 2000), mGluRs and $GABA_B$ receptors sense their primary physiological ligands, glutamate and GABA, respectively, as well as Ca_o^{2+}. These observations further emphasize the structural, functional, and evolutionary relationships among the three types of receptors.

Finally, Ca_o^{2+} could also, of course, modulate the functions of proteins other than GPCRs. For instance, the recently cloned Ca_o^{2+} channels TRPV5 and 6 can be viewed as operating on a macroscopic level as facilitated transporters. That is, they exhibit Michaelis–Menten-like kinetics, their activities (measured as $^{45}Ca^{2+}$ uptake in *X. laevis* oocytes) increase with the level of Ca_o^{2+} until Ca^{2+} uptake saturates above about 1 m*M* Ca_o^{2+}. They could potentially function, therefore, as Ca_o^{2+} sensors. That is, they would tend to "set" the level of Ca_o^{2+} within the local ECF by increasing Ca^{2+} uptake when Ca_o^{2+} is high and reducing it when it is low.

SUMMARY

The discovery of the CaR has provided a molecular mechanism that mediates many of the known actions of Ca_o^{2+} on the cells and tissues that participate directly in systemic Ca_o^{2+} homeostasis, such as parathyroid and certain renal cells. There is still much to be learned, however, about the various functions of the receptor in these tissues, particularly in intestinal and bone cells, as well as in the numerous other CaR-expressing cells that are not directly involved in systemic Ca_o^{2+} homeostasis. In these latter, "nonhomeostatic" cells, the CaR probably serves a variety of roles that enable it to serve as a versatile first messenger, capable of regulating numerous cellular functions. Moreover, the ability of the CaR to integrate and coordinate several different ionic and nutritional signals may permit it to act as a central homeostatic element not only for mineral ion homeostasis, but also for processes relevant to Mg_o^{2+}, water, and protein metabolism.

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Chapter 27

Receptors for Parathyroid Hormone (PTH) and PTH-Related Protein

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INTRODUCTION

The biological actions of parathyroid hormone (PTH) and parathyroid hormone-related protein (PTHrP) have attracted ever wider interest in recent years because of the rapid advances in the study of the developmental biology of bone in which PTHrP and its receptor play a major role, as well as demonstration of the therapeutic potential of PTH in fracture prevention in osteoporosis.

One principal receptor, the type-1 PTH/PTHrP receptor (PTH1R), is the chief mediator of both the homeostatic actions of PTH and the paracrine actions of PTHrP on endochondral bone development. As discussed later, however, additional receptors may interact differentially with PTH versus PTHrP and/or with regions of the two ligands other than their amino-terminal domains. The tools of molecular biology have been central in the efforts to characterize both ligand-binding requirements and signaling properties of these receptors. Work with the principal receptor for PTH and PTHrP, the PTH1R, has proved pivotal in studies aimed at understanding in greater depth the physiological role of PTH in calcium and phosphate homeostasis and the critical paracrine role played by PTHrP in the complex network of different signaling factors that directs endochondral bone development.

At the same time that the reductionist approaches based on analyses of cloned receptors expressed in cell lines have helped clarify initial steps in PTH action and provided a detailed analysis of ligand/receptor/signaling events *in vitro*, the tools of molecular biology have also made possible a new level of integrative physiological analyses of bone biology *in vivo* through the use of mice modified genetically through selective gene knockout and/or transgenic overexpression of the PTH1R and/or its ligands. Much of this latter work, as well as the overall biological actions and physiological role of PTH, is outlined in subsequent chapters on PTH. This chapter focuses on the receptors per se, particularly the cloned and well characterized PTH1R, and those that are still uncloned but of potential biological significance in overall PTH or PTHrP action, especially the receptor for the carboxyl-terminal portion of PTH for which much recent biochemical data have accumulated.

RECEPTORS FOR PTH, PTHrP, AND TIP39: THE PTH1R AND PTH2R

Cloning, Gene Structure, Evolution, and Expression

Because of the pleiotropic actions of PTH, which involve both direct and indirect effects, as well as multiple signal transduction mechanisms, it was initially thought that several different receptors mediated the biological responses of this peptide hormone. Furthermore, the realization that

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some of these actions were PTHrP rather than PTH dependent seemed to increase the probability that more than one receptor would be involved. It was somewhat surprising, therefore, that initial cloning approaches led to the isolation of cDNAs encoding only a single G protein-coupled receptor, PTH1R. The recombinant PTH1R interacts well with PTH and PTHrP and activates several distinct second messenger pathways: adenylate cyclase/protein kinase A (AC/PKA) and phospholipase C/protein kinase C (PLC/PKC; Abou-Samra *et al.*, 1992; Jüppner *et al.*, 1991; Schipani *et al.*, 1993). Based on subsequent findings, such as the similar phenotypes observed in mice that are null for either PTHrP or the PTH1R (Karaplis *et al.*, 1994; Lanske *et al.*, 1996; Vortkamp *et al.*, 1996), it now seems that most of the endocrine actions of PTH and paracrine/autocrine actions of PTHrP on bone development are mediated through the PTH1R. Subsequent to the cloning of the PTH1R, another G protein-coupled receptor that is closely related to the PTH1R was identified in humans and rodents. This receptor, the PTH2R, shows some capacity to interact with PTH ligands but interacts much more efficiently with a peptide isolated from the hypothalamus called TIP39, the presumed native ligand for this receptor (Usdin, 1999; Usdin *et al.*, 1995). Another related receptor, the PTH-3 receptor (PTH3R), was identified in zebrafish and displays functionality more similar to that of the PTH1R (Rubin and Jüppner, 1999; Hoare *et al.*, 2000b). A review of the published sequence of the human genome indicates that there is no gene sequence detected that might be expected to yield the PTH-3 receptor (Venter *et al.*, 2001; Fredriksson and Schioth, 2005).

The PTH1R belongs to a distinct family of G protein-coupled receptors (GPCRs), called class II (or family B) receptors (Cardoso *et al.*, 2006; Fredriksson and Schioth, 2005). The first cDNAs encoding mammalian PTH1Rs were isolated through expression cloning techniques from cell lines that had been widely used in classical PTH/PTH receptor studies: the opossum kidney cell line OK and the rat osteosarcoma cell line ROS 17/2.8 (Abou-Samra *et al.*, 1992; Jüppner *et al.*, 1991). Subsequently, cDNAs encoding human (Eggenberger *et al.*, 1997; Schipani *et al.*, 1993; Schneider *et al.*, 1993), mouse (Karperien *et al.*, 1994), rat (Pausova *et al.*, 1994), chicken (Vortkamp *et al.*, 1996), pig (Smith *et al.*, 1996), dog (Smock *et al.*, 1999), frog (Bergwitz *et al.*, 1998), and fish (Rubin and Jüppner, 1999) PTH1Rs were isolated through hybridization techniques from various tissue and cell sources (i.e., kidney, brain, whole embryos, osteoblast-like cells, and embryonic stem cells). Northern blot and *in situ* studies (Tian *et al.*, 1993; Urena *et al.*, 1993; van de Stolpe *et al.*, 1993), as well as data provided through available public [expressed sequence tag (EST)] databases, confirmed that the PTH1R is expressed in a wide variety of fetal and adult tissues. With the exception of the tetraploid African clawed frog, *Xenopus laevis*, which expresses two nonallelic isoforms of the PTH1R (Bergwitz *et al.*, 1998), all investigated species have only one copy of the PTH1R per haploid genome. Evolutionary studies have identified PTH1Rs in all vertebrate species (Fig. 1), as well as an apparent PTH1R homologue in the urochordate tunicate *Ciona intestinalis* (Cardoso *et al.*, 2006).

The possible existence of other receptors for PTH or PTHrP with unique, organ-specific pharmacological characteristics had been suggested by the distinct ligand binding (Chorev *et al.*, 1990a, 1990b; McKee *et al.*, 1988) and second messenger signaling profiles observed in different clonal cell lines (Cole *et al.*, 1987; Yamaguchi *et al.*, 1987a, 1987b). However, the molecular cloning of identical full-length PTH1R cDNAs from human kidney, brain, and bone-derived cells (Eggenberger *et al.*, 1997; Schipani *et al.*, 1993; Schneider *et al.*, 1993) suggested that the previously observed pharmacological differences arose from species-specific variations in the receptor primary sequence rather than the tissue-specific expression of distinct receptors.

The gene encoding the human PTH1R is located on chromosome 3 (locus 3p22-p21.1). The intron/exon structure of the gene has been analyzed in detail (Bettoun *et al.*, 1997; Manen *et al.*, 1998; Schipani *et al.*, 1995) and was shown to have an organization similar to that of genes encoding the rat and mouse homologues (Kong *et al.*, 1994; McCuaig *et al.*, 1994; Fig. 2). In each of these mammals, the PTH1R gene spans at least 20 kbp of DNA and consists of 14 coding exons and at least three noncoding exons. The size of the coding exons in the human PTH1R gene ranges from 42 bp (exon M7) to more than 400 bp (exon T); the size of the introns varies from 81 bp (between exons M6 and M6/7) to more than 10 kbp (between exons S and E1). Two promoters for the PTH1R have been described in rodents (Joun *et al.*, 1997; Kong *et al.*, 1994; McCuaig *et al.*, 1994, 1995). The P1 promoter (also referred as U3) is active mainly in the adult kidney, whereas the P2 promoter (also referred to as U1) is active in several fetal and adult tissues, including cartilage and bone. In humans, a third promoter, P3 (also referred to as S), also appears to be active in some tissues, including kidney and bone (Bettoun *et al.*, 1998; Giannoukos *et al.*, 1999; Manen *et al.*, 1998). Several frequent polymorphisms were identified within the human PTH1R gene; these include an intronic *Bsm*I polymorphism located between the 5′ noncoding exon U1 and the coding exon S (Hustmyer *et al.*, 1993) and a silent *Bsr*DI polymorphism in exon M7 (nucleotide 1417 of human PTH1R cDNA; Schipani *et al.*, 1994).

At the protein level, all mammalian PTH1Rs have a relatively long amino-terminal extracellular domain (~170 amino acids in the human PTH1R after removal of the signal sequence by signal peptidase cleavage). This domain is encoded by five exons: S (encoding the signal sequence) and E1, E2, E3, and G (encoding the four N-linked glycosylation sites; Zhou *et al.*, 2000). Genes encoding other class II G protein-coupled receptors for which the genomic structure has been explored have a similar organization, except that the equivalent of exon E2

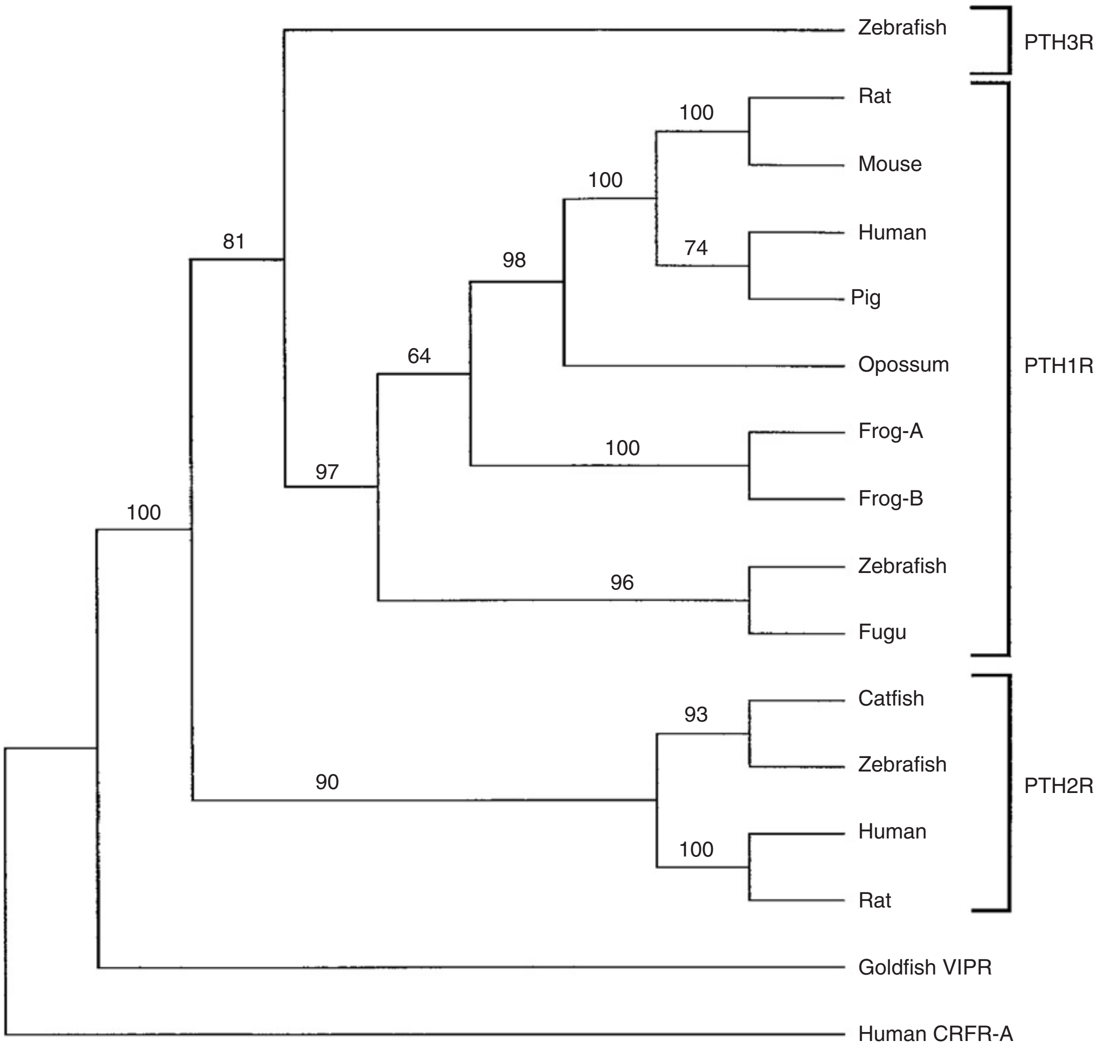
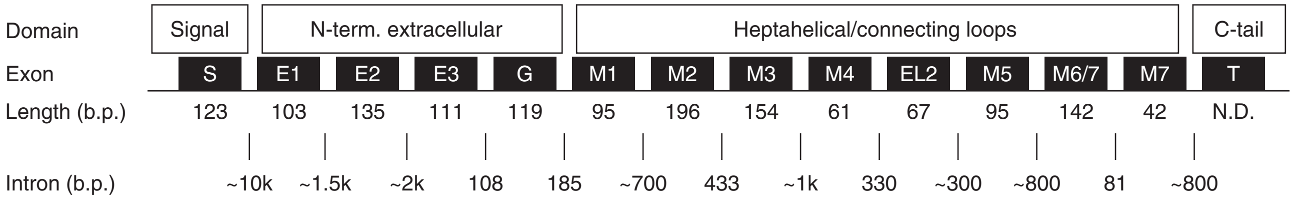

Zebrafish
PTH3R
Rat
Mouse
Human
Pig
Opossum
Frog-A
Frog-B
Zebrafish
Fugu
PTH1R
Catfish
Zebrafish
Human
Rat
PTH2R
Goldfish VIPR
Human CRFR-A
100
100
74
98
64
81
97
100
100
96
93
90
100

FIGURE 1 Evolutionary tree diagram of all known receptors for parathyroid hormone (PTH) and PTH-related peptide (PTHrP) from different vertebrate species. The tree was generated by comparing the amino sequences of the corresponding proteins derived from cDNA cloning or genome analysis. (Reproduced with permission from Rubin, D.A., and Jüppner, H. *J. Biol. Chem.*, 1999).



Domain	Signal	N-term. extracellular				Heptahelical/connecting loops								C-tail
Exon	S	E1	E2	E3	G	M1	M2	M3	M4	EL2	M5	M6/7	M7	T
Length (b.p.)	123	103	135	111	119	95	196	154	61	67	95	142	42	N.D.
Intron (b.p.)	~10k	~1.5k	~2k	108	185	~700	433	~1k	330	~300	~800	81	~800	

FIGURE 2 Intron/exon structure of the human PTH1R gene. The 14 coding exons of the human PTH1R gene are indicated as black boxes, and the corresponding receptor domains are indicated above in open boxes. The lengths in nucleotide base pairs ($k = 1/1000$) of the exons and the intervening introns are also indicated.

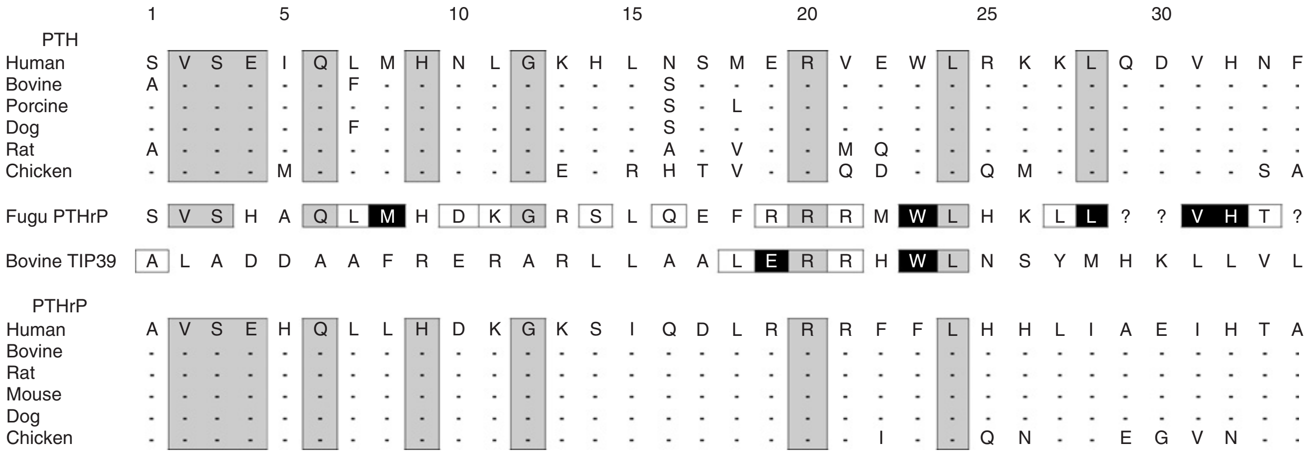
is lacking (Jüppner, 1994; Jüppner and Schipani, 1996). The protein segment encoded by exon E2 is also missing in the PTH2Rs, as well as in the PTH1Rs from *X. laevis* and zebrafish (Bergwitz *et al.*, 1998; Rubin *et al.*, 1999). Earlier *in vitro* mutational studies showed that the E2 segment of the PTH1R can be modified or deleted without a measurable impact on receptor surface expression or function (Jüppner *et al.*, 1994; Lee *et al.*, 1994). Taken together, these findings led to the conclusion that the addition of this exon in the mammalian PTH1Rs was a relatively recent evolutionary modification to the PTH1R gene (Rubin and Jüppner, 1999); the biological role of this receptor region, if any, is unknown.

Class II Receptor Family

The molecular cloning of the PTH1R (Abou-Samra *et al.*, 1992; Jüppner *et al.*, 1991), along with the receptors for secretin (Ishihara *et al.*, 1991) and calcitonin (Lin *et al.*, 1991) that same year, made it clear that these peptide hormone receptors formed a distinct GPCR family. This GPCR family, called the class II or family B GPCRs, includes in humans 15 unique receptors, each of which binds a peptide ligand of moderate size (Cardoso *et al.*, 2006; Fredriksson and Schioth, 2005). Except for the structural similarity provided by the seven membrane-spanning helices, members of the class II (family B) peptide hormone GPCR family share virtually no amino acid sequence homology with most other GPCRs, such as the β2-adrenergic receptor, a class I GPCR. All members of the secretin/calcitonin/PTH receptor family, including an insect and several other invertebrate peptide hormone receptors (Cardoso *et al.*, 2006; Reagan, 1994, 1996; Sulston *et al.*, 1992) share about 45 strictly conserved amino acid residues. Furthermore, all receptors of this family have a relatively long amino-terminal, extracellular domain, and most use at least two different signal transduction pathways, adenylate cyclase and phospholipase C (Jüppner, 1994; Jüppner and Schipani, 1996). The amino-terminal extracellular domain of each of these related receptors typically contains four sites for potential asparagine-linked glycosylation, as well as six conserved cysteine residues that most likely form a disulfide network important for proper domain folding (Gaudin *et al.*, 1995; Grace *et al.*, 2007; Knudsen *et al.*, 1997; Lee *et al.*, 1994; Parthier *et al.*, 2007, Qi *et al.*, 1997; Sun *et al.*, 2007). Within the amino-terminal domain, as well as in the body of the receptor, there are several other "signature" residues conserved among the class II receptors (Cardoso *et al.*, 2006). It is predicted that within the membrane-embedded region there is an overall topological similarity between these class II heptahelical receptors and G protein-coupled receptors of the other families, such as the β2-adrenergic receptor, a class I GPCR (Sheikh *et al.*, 1999) for which an x-ray crystal structure has been solved (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007; Rosenbaum *et al.*, 2007), and those represented by the metabotropic glutamate receptor and the calcium-sensing receptor (class III receptors; reviewed in Chapter 23), although the receptors from each class share no primary sequence homology.

TIP39 AND THE PTH2R

The PTH2R subtype was initially identified through hybridization cloning methods in a human brain cDNA library. At the amino acid level, this receptor is 51% identical to the human PTH1R (Usdin *et al.*, 1995). The cloning of the PTH2R prompted a search for a peptide ligand that is its naturally occurring agonist and would selectively activate the PTH2R. The effort resulted in the discovery of a peptide of 39 amino acids that potently activates both rat and human PTH2R subtypes without activating the PTH1R (Usdin, 1999). The human PTH2R responds to PTH but not to PTHrP, whereas the rat PTH2R responds to neither PTH nor PTHrP (Hoare *et al.*, 1999a). The newly discovered peptide, called TIP39 (tuberoinfundibular peptide of 39 amino acids), was initially purified from bovine hypothalamus extracts and was shown to be only weakly homologous to PTH and PTHrP (Usdin, 1999; Fig. 3).



	1				5					10					15					20					25					30				
PTH																																		
Human	S	V	S	E	I	Q	L	M	H	N	L	G	K	H	L	N	S	M	E	R	V	E	W	L	R	K	K	L	Q	D	V	H	N	F
Bovine	A	-	-	-	-	-	F	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Porcine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dog	-	-	-	-	-	-	F	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rat	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	V	-	-	M	Q	-	-	-	-	-	-	-	-	-	-	-	-
Chicken	-	-	-	-	M	-	-	-	-	-	-	-	E	-	R	H	T	V	-	-	Q	D	-	-	Q	M	-	-	-	-	-	-	S	A
Fugu PTHrP	S	V	S	H	A	Q	L	M	H	D	K	G	R	S	L	Q	E	F	R	R	R	M	W	L	H	K	L	L	?	?	V	H	T	?
Bovine TIP39	A	L	A	D	D	A	A	F	R	E	R	A	R	L	L	A	A	L	E	R	R	H	W	L	N	S	Y	M	H	K	L	L	V	L
PTHrP																																		
Human	A	V	S	E	H	Q	L	L	H	D	K	G	K	S	I	Q	D	L	R	R	R	F	F	L	H	H	L	I	A	E	I	H	T	A
Bovine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mouse	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dog	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chicken	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	-	-	Q	N	-	-	E	G	V	N	-	-

FIGURE 3 Alignment of the PTH(1–34) amino acid sequences of all known vertebrate PTH and PTHrP species, as well as fugu PTHrP and bovine TIP39. Amino acid residues that are conserved in all PTH and PTHrP species are shown in shaded boxes. Amino acid residues found in either fugu PTHrP or bovine TIP39 that are also found in all known PTH species are shown in black boxes with white letters, and residues found either in fugu PTHrP or in bovine TIP39 that are also found in all PTHrP species are boxes; numbers indicate amino acid positions in mammalian PTH or PTHrP. Question marks refer to sequence positions in fugu fish PTHrP not definitely deduced from nucleotide sequence. (Reproduced with permission from Jüppner et al., 2006).

Although TIP39 fails to activate the PTH1R, it binds to it with moderate affinity (~100 n*M*; Hoare *et al.*, 2000a). Interestingly, TIP(7-39) and TIP(9–39) bind with higher affinity to the PTH1R than TIP39 and the truncated peptides function as PTH1R-specific antagonists (Hoare and Usdin, 2000; Jonsson *et al.*, 2001). The physiological role of TIP39 and the PTH2R has not yet been identified, but their abundant expression in the central nervous system suggests a possible neuroendocrine function (Usdin, 2000) that is apparently preserved in evolution, as the PTH2R is found in zebrafish (Rubin and Jüppner, 1999).

MECHANISMS OF LIGAND RECOGNITION AND ACTIVATION BY PTH RECEPTORS

Current data indicate that the PTH receptor interacts with multiple regions of PTH peptide ligands; these contacts establish binding affinity and/or promote receptor activation. Much information on these interactions has been gained from studies that used intact native PTH receptors expressed in various cell systems and synthetic PTH and PTHrP analogues. PTH(1–34) and PTHrP(1–34) bind to and activate the PTH1R with affinities and potencies in the low nanomolar range. The first 13 amino acids of PTH and PTHrP have been highly conserved in evolution with six identities; the (15–34) regions share only moderate homology with three amino acid identities. The N-terminal portions of the two peptides play key roles in receptor activation, whereas the (15–34) portions are required for high-affinity receptor binding (Abou-Samra *et al.*, 1989; Caulfield *et al.*, 1990; Nussbaum *et al.*, 1980).

Ligand Determinants of PTH Receptor Activation

cAMP Signaling Response

Amino-terminally truncated PTH or PTHrP analogues, such as PTH(3-34), PTH(7-34), and PTHrP(7-34), bind to the PTH1R with high affinity and elicit little or no increase in cAMP accumulation. Such fragments yield the most potent PTH1R competitive antagonists (Nutt *et al.*, 1990). Bulky amino acid modifications within the amino-terminal portion of (1–34)-length peptides (e.g., at positions 2, 3, and 6) also confer antagonistic properties to the peptides (Behar *et al.*, 1999; Carter *et al.*, 1999a; Cohen *et al.*, 1991; Gardella *et al.*, 1991). Peptides consisting only of the amino-terminal residues of PTH exhibit severely diminished receptor-binding affinity and hence cAMP-signaling potency. The shortest amino-terminal peptide of the native sequence that retains full PTH1R-binding affinity and cAMP-signaling potency is PTH(1–31; Whitfield and Morley, 1995). PTH(1–14) has been shown to be the shortest native amino-terminal PTH peptide for which at least some cAMP agonist activity can be detected, albeit the EC_{50} of the cAMP response induced by PTH(1–14) in LLC-PK1 porcine kidney cells transfected stably with high levels of the human PTH1R (~100 μM) is markedly higher than that observed for PTH(1–34) (~3 nM; Luck *et al.*, 1999). A series of structure–activity relationship studies on the PTH(1–14) scaffold peptide was undertaken as part of an effort to better understand how residues in the amino-terminal portion of PTH mediate receptor activation (Carter and Gardella, 2001; Luck *et al.*, 1999; Shimizu *et al.*, 2000b, 2001a). An alanine scan analysis of PTH(1–14) demonstrated the functional importance of residues (1–9) and suggested that this sequence represents a minimum-length receptor-activation domain (Luck *et al.*, 1999). Further substitution analyses revealed that the PTH(1–14) sequence could accommodate amino acid changes at a number of positions (Carter and Gardella, 2001; Shimizu *et al.*, 2000b, 2001b) many of which improved signaling potency and binding affinity. The most active analogue was [$Aib^{1,3}$, Gln^{10}, $homoArg^{11}$, Ala^{12}, Trp^{14}]PTH(1–14), which is several thousandfold more potent as a cAMP agonist in stably transfected LLC-PK1 cells than is native PTH(1–14) and approximately as potent as PTH(1–34) (Shimizu *et al.*, 2001b). The relevant substitutions also conferred activity to the otherwise inactive PTH(1–11) fragment; these modified PTH(1–11) analogues are currently the shortest free peptide sequences that can activate the PTH1R (Shimizu *et al.*, 2001b, 2004).

Non-cAMP Signaling Responses

Although it is well established that the amino-terminal residues of PTH mediate AC/PKA signaling, there is still some uncertainty regarding the ligand determinants of PLC/PKC/calcium signaling. Several studies have indicated that residues in the C-terminal portion of PTH(1–34) mediate PKC activation; perhaps most notably, the tetrapeptide PTH(29–32) was shown to be sufficient for activating PKC in ROS 17/2 rat osteosarcoma cells (Jouishomme *et al.*, 1994), as well as in Chinese hamster ovary cells transfected with the rat PTH1R (Azarani *et al.*, 1996). Stimulation of PKC is generally thought to be mediated through PLC signaling; however, other data indicate that determinants of PLC activation reside at the amino terminus of PTH. Thus, even minor N-terminal truncations, as in [$desNH_2$-Gly^1]PTH(1–34), PTH(2–34), or PTH(3–34), severely diminish the capacity of the peptide to stimulate inositol polyphosphate (IP) production via PLC in porcine kidney LLC-PK1 cells transfected with the human PTH1R (Takasu *et al.*, 1999a). In addition, the activity-enhanced PTH(1–14) analogues mentioned above stimulate IP production in transfected COS-7 cells, indicating that residues in this N-terminal portion of the ligand can be sufficient for PLC signaling (Shimizu *et al.*, 2000b).

One possible explanation for the apparent discrepancy in the mapping of PKC and PLC activation determinants is that residues (29–32) of PTH mediate PKC activation via a phospholipase other than PLC. In support of this possibility, Friedman and co-workers (1999) have shown that in the distal tubule cells of the kidney, the PTH1R couples to phospholipase D, whereas in the proximal tubule cells it couples to phospholipase C; moreover, distinct structural components of the ligand were required for the altered signaling responses in the two different cell types. Stern and co-workers have shown that PTH(1–34) can activate PLD in primary and transformed (UMR 106) osteoblastic cells through a Gα12/13-coupled pathway involving activation of the small GTP-binding protein RhoA (Radeff *et al.*, 2004; Singh *et al.*, 2003, 2005). Weaker stimulation of PLD was observed with PTH(3–34) and PTH(1–31) (Singh *et al.*, 1999). The PTH1R may therefore be capable of recognizing different portions of the ligand as activation determinants for various phospholipases, a capacity that may be modulated by the cellular milieu (Whitfield *et al.*, 2001).

Ligand stimulation of the PTH1R can also result in modulation of the mitogen-activated protein kinase (MAPK) or extracellular signal-regulate (ERK)-1/2 signaling cascade via both G protein–dependent and G protein-independent mechanisms. It was shown in distal convoluted tubule cells of the kidney that PTH stimulates activation of ERK2 via a PKC-dependent, PKA-independent mechanism, and this ERK activation likely plays a role in the calcium transport processes mediated by these cells (Sneddon *et al.*, 2000). In transfected HEK-293 cells, PTH stimulates activation of ERK1/2 via both a Gq/PLC-mediated pathway, as well as an arrestin-mediated pathway that is linked to the clathrin-mediated receptor internalization process, does not require activation of the cAMP pathway, and may be induced by the PTH(7-34) fragment (Gesty-Palmer *et al.*, 2006; Rey *et al.*, 2006; Syme *et al.*, 2005).

Ligand Determinants of PTH Receptor Binding

For both PTH and PTHrP, the (15–34) fragment can inhibit the binding of either radiolabeled PTH(1–34) or PTHrP (1–34) to the PTH1R with an IC_{50} in the micromolar range, thus demonstrating that the (15–34) domain contains the principal determinants of receptor-binding affinity (Abou-Samra *et al.*, 1989; Caulfield *et al.*, 1990). The (15–34) domains of both ligands are predicted to form amphiphilic α helices with the hydrophobic face of PTH being formed principally by Trp-23, Leu-24, and Leu-28 (Epand *et al.*, 1985; Neugebauer *et al.*, 1992). Substitution of Leu-24 or Leu-28 in PTH(1–34) by glutamate results in 100-fold reductions in binding affinity, consistent with the view that the hydrophobic face plays a key role in receptor binding (Gardella *et al.*, 1993). It has been suggested for PTH (Epand *et al.*, 1985; Neugebauer *et al.*, 1992; Rölz *et al.*, 1999) that this role involves nonspecific interaction of the hydrophobic surface of the peptide with the phospholipid bilayer of the cell membrane, which then facilitates a two-dimensional diffusion of the hormone to the receptor. Such a model has been suggested for other peptide hormones (Sargent and Schwyzer, 1986). However, more recent structure–activity studies (Dean *et al.*, 2006a) strongly suggest that the hydrophobic surface of the PTH (15–34) domain directly contacts the amino-terminal domain of the receptor. The crosslinking of a PTHrP(1–36) analogue having tryptophan-23 replaced by the photolabile benzophenone-containing amino acid analogue benzoylphenylalanine (Bpa) to a 16-amino-acid segment at the extreme amino terminus of the PTH1R further suggests that at least some residues in the (15–34) domain of the ligand interact with the amino-terminal domain of the receptor (Mannstadt *et al.*, 1998).

MECHANISMS OF PTH1R FUNCTION

Role of the Amino-Terminal Receptor Domain

The large glycosylated amino-terminal extracellular domain of the PTH1R contains six highly conserved cysteine residues that are likely to form an intramolecular network of disulfide bonds. A possible arrangement of these disulfide bonds has been suggested from a biochemical study on a recombinant protein corresponding to the amino-terminal domain of the PTH1R (Tyr^{23}-Ile^{191}) that was overproduced in *Escherichia* coli (Grauschopf *et al.*, 2000). The purified protein was refolded in a glutathione-containing redox buffer system to a homogeneous state and was shown to retain specific, but predictably weak ($K_d \sim 4\,\mu M$), binding affinity for PTH(1–34). The biochemical behavior of this protein suggests that the observed disulfide bond pattern, illustrated in Fig. 4, may faithfully replicate that which occurs in native PTH1Rs expressed in eukaryotic cells. Indeed, this disulfide bonding pattern is confirmed in the three-dimensional structures of isolated amino-terminal domains of several other family B GPCRs solved either by solution-phase NMR (Grace *et al.*, 2007; Sun *et al.*, 2007) or x-ray crystallography (Parthier *et al.*, 2007). These structures, which were obtained as complexes with the C-terminal binding domain of the cognate ligand, show a consistent, beta sheet-rich folding pattern stabilized by three predicted disulfide bonds.

Functional studies on PTH receptor chimeras and mutants generated by site-directed mutagenesis and expressed in COS-7 cells have shown that the amino-terminal domain of the receptor provides important contact sites for at least some residues in the C-terminal-binding

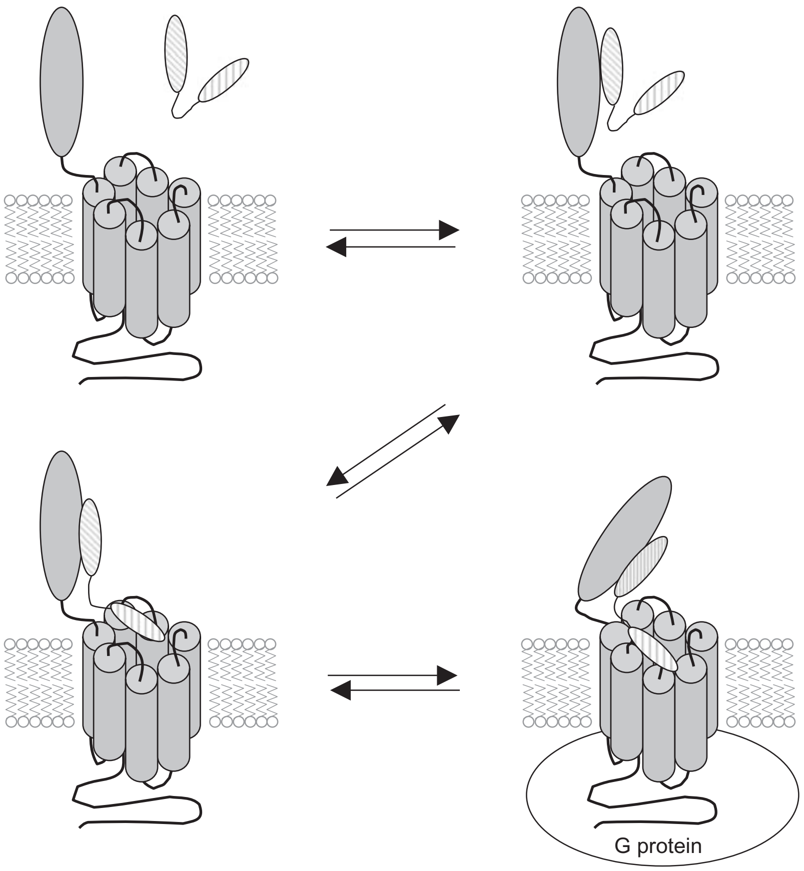


FIGURE 4 Hypothetical model of the PTH–PTH receptor interaction mechanism. The schematic illustrates current hypotheses regarding the mechanism by which PTH binds to the PTH1 receptor and induces G protein coupling. The interaction with PTH(1-34) (hatched ovals) involves two principal components: (1) binding of the C-terminal domain of PTH(1-34) to the amino-terminal extracellular domain of the receptor and (2) the association of the amino-terminal domain of PTH with the juxtamembrane region of the receptor. These two components of the interaction contribute predominantly to affinity and activation, respectively, and may occur in a sequential manner, as depicted. Upon association of the N-terminal portion of the ligand with the juxtamembrane region, a conformational change occurs, which results in the formation of a "closed" high-affinity ligand-receptor complex that is coupled to G protein. (Reproduced with permission from Hoare *et al.*, 2001.)

domains of PTH(1–34) and PTHrP(1–34) (Bergwitz *et al.*, 1996; Jüppner *et al.*, 1994). The crosslinking studies with [Bpa^{23}]PTHrP(1–36) mentioned earlier support this conclusion (Mannstadt *et al.*, 1998; see also Chapter 26). Within the 17-amino-acid interval identified with the Bpa-23 analogue, the specific residues of threonine-33 and glutamine-37 were shown by functional methods to be determinants of PTH(7–34) binding affinity (Mannstadt *et al.*, 1998). A second segment of the amino-terminal extracellular domain involved in ligand interaction maps to the boundary of the amino-terminal domain and the first transmembrane helix. A PTH(1–34) analogue having the benzophenone photophore attached to the ε amino group of lysine 13 crosslinked to this region, most likely at Arg-186 (Adams *et al.*, 1998), and point mutations at the neighboring hydrophobic residues of Phe-184 and Leu-187 and Ile-190 impaired interaction with PTH(3–34) and PTH(1–14) but not PTHrP(15–36) (Carter *et al.*, 1999b). Residues in this segment of the receptor thus appear to be important interaction determinants for residues in the (3–14) region of the ligand.

Residues in the midportion of the amino-terminal domain of the PTH1R are also likely to contribute to ligand interaction, but candidate contact points have not been identified. Use of PTHrP(1–36) analogues modified with Bpa at positions 27 and 28 revealed crosslinking to the E2 region of the receptor's amino-terminal domain, which does not appear to be involved in ligand binding (Gensure *et al.*, 2001). This same study found that Bpa at position 33 resulted in crosslinking to the segment 151–172 of the amino-terminal domain, which may contain determinants of binding (Lee *et al.*, 1995b), although, again, none has been identified. Another study using PTH(1–34) analogues modified with Bpa at positions 11, 15, or 21 found photocrosslinking to the amino-terminal regions: [165–176], [183–189], and [165–176], respectively (Wittelsberger *et al.*, 2006). Overall, the combined crosslinking studies suggest that a rather broad surface of contact between the mid- to C-terminal portion of PTH(1-34) and the receptor's amino-terminal extracellular domain is involved in establishing and maintaining the affinity with which PTH or PTHrP ligands bind to the PTH1R.

Juxtamembrane Region

A number of studies have indicated that interactions between the amino-terminal portion of PTH and the juxtamembrane region of the PTH receptor are important for inducing receptor activation. One such study that utilized PTH1R/calcitonin receptor chimeras and PTH/calcitonin hybrid ligands showed that efficient functional responses were obtained only when a chimeric receptor was paired with a hybrid ligand such that the amino-terminal portion of the ligand was cognate to the juxtamembrane region of the receptor (Bergwitz *et al.*, 1996). Some specific residues in the juxtamembrane region have been identified as candidate interaction sites for the amino-terminal residues of PTH, such as Ser-370 and Leu-427 at the extracellular ends of transmembrane domain(TM)5 and 6, respectively, which determine the agonist/antagonist response profile observed with [Arg^2]PTH(1–34) (Gardella *et al.*, 1994), and Trp-437 and Gln-440 in the third extracellular loop, at which mutations impair the binding of PTH(1–34) but not PTH(3–34) (implying an interaction site for ligand residues 1 and 2; Lee *et al.*, 1995a). Consistent with these mutational data, the receptor crosslinking sites for [Bpa^1]PTH(1–34) (Bisello *et al.*, 1998) and [Bpa^2]PTHrP(1–36) (Behar *et al.*, 1999) were mapped to the extracellular end of TM6 (Behar *et al.*, 1999). Interestingly, both of these ligands utilized Met-425 for covalent attachment, although [Bpa^2]PTHrP(1–36), an antagonist, utilized an additional second site in TM6, whereas [Bpa^1]PTH(1–34), an agonist, utilized only the methionine. These results raise the possibility that the photochemical crosslinking approach can be used to discern differences in the active and inactive states of the PTH1R (Behar *et al.*, 1999).

Other receptor residues involved in interactions with the amino-terminal portion of the ligand have been identified in studies aimed at elucidating the molecular basis by which the human PTH2R discriminates between PTH and PTHrP, an effect that is largely due to the amino acid divergence at position 5 in these ligands (Ile in PTH and His in PTHrP; Behar *et al.*, 1996; Gardella *et al.*, 1996c). Four PTH2R residues involved in this specificity were identified at the extracellular ends of several of the TM helices: Ile-244 in TM3, Tyr-318 in TM5, and Cys-397 and Phe-400 in TM7, corresponding to Leu-289, Ile-363, Tyr-443, and Leu-446, respectively, in the human PTH1R (Bergwitz *et al.*, 1997; Turner *et al.*, 1998). Other residues in the TM domains that have been identified as determinants of PTH(1-34) agonist responsiveness include Ser-229, Arg-233, Ser-236, which may form a hydrophilic surface on TM2 (Turner *et al.*, 1996), and the conserved Gln-451 in TM7 (Gardella *et al.*, 1996a).

A series of studies conducted with a truncated PTH1R (P1R-delNt) that lacks most (residues 24–181) of the amino-terminal extracellular domain has helped discern the role of interactions between the N-terminal residues of PTH and the juxtamembrane region of the receptor in mediating signal transduction. Thus, the modified PTH (1–14) peptides described earlier stimulate cAMP formation with this truncated receptor nearly as effectively as they do with the intact wild-type receptor (Shimizu *et al.*, 2000b, 2001b). The near-full activity of the PTH(1–14) analogues with P1R-delNt stands in dramatic contrast to the markedly (approximately 1000-fold) reduced activity that unmodified PTH(1–34) exhibits with P1R-delNt, as compared to the intact receptor (Shimizu *et al.*, 2000b, 2001). The weak activity of PTH(1–34) on P1R-delNt highlights the importance of interactions between the (15–34) domain of PTH(1–34) and the N-terminal extracellular domain of the receptor in stabilizing the native hormone–receptor complex (see later), whereas the potent activity of modified PTH(1–14) on P1R-delNt indicates that most, if not all, of the key functional residues in the amino-terminal peptide interact primarily, if not exclusively, with the juxtamembrane region of the receptor.

The most potent PTH(1–14) analogues so far, exhibiting low nanomolar potency on both the intact wild-type PTH1R and on P1R-delNt have conformationally constraining substitutions at positions 1 and/or 3 (Shimizu *et al.*, 2004, 2001b). Presumably, the modifications in these peptides stabilize a ligand conformation that has high affinity for the juxtamembrane region of the receptor. Most likely, this conformation is α-helical, as suggested by nuclear magnetic resonance spectroscopy analysis of PTH fragment analogues (Chen *et al.*, 2000; Fiori *et al.*, 2007; Rölz *et al.*, 1999; Tsomaia *et al.*, 2004a, 2004b), as well as X-ray crystallographic analysis of unmodified PTH(1–34) (Jin *et al.*, 2000). At least some of the substitutions in the multisubstituted PTH(1–14) analogues described by Shimizu *et al.* (2000b, 2001b), are likely to provide new and favorable interactions with the receptor that compensate for the loss of binding energy that normally derives from residues in the (15–34) domain of PTH; some, however, may directly facilitate the receptor activation process without affecting binding affinity. One of the goals of the research on these short N-terminal PTH peptides is to discern how individual key residues in the ligand contribute to binding affinity and receptor activation. Data so far on the PTH(1–14) analogues demonstrate that it is possible to achieve full potency and efficacy with a peptide ligand as short as 14 amino acids, and also that a relatively small agonist ligand can fully activate the PTH receptor by interacting solely with the juxtamembrane region of the receptor. As an extension of these studies, a PTH1R mutant was constructed in which residues (1–9) or (1–11) of PTH were tethered directly to the juxtamembrane region of the receptor (at Glu-182). When expressed in COS-7 cells, these constructs resulted in basal cAMP levels that closely approached the cAMP level seen with the wild-type PTH1R fully stimulated with the PTH(1–34) agonist ligand (Shimizu *et al.*, 2000a). A similar result has been reported for corticotropin-releasing factor (CRF) and its class II GPCR (Nielsen *et al.*, 2000). A radiolabeled modified PTH(1-14) peptide was also used in drug screening research to identify small molecules that bind to the PTHR, and this approach indeed revealed a compound that inhibited the binding of the radioligand with micromolar affinity and behaved as a competitive antagonist, suggesting an orthosteric binding mechanism (Carter *et al.*, 2007). An unrelated, activity-based PTHR screening effort identified another small molecule ligand for the PTHR which acts as a weak, micromolar agonist, possibly via an allosteric, rather than an orthosteric, mechanism (Rickard *et al.*, 2006). These studies together suggest promise in the search for potent non-peptidic compounds for the PTHR that can be used to treat diseases such as osteoporosis.

Two-Site Model of PTH/PTH Receptor Interaction

Combined functional and crosslinking data are consistent with a mechanism for the PTH–PTH receptor interaction that involves two principal components: (1) an interaction between the C-terminal domain of the ligand and the amino-terminal domain of the receptor, which contributes predominantly to binding affinity, and (2) an interaction between the amino-terminal portion of the ligand and the juxtamembrane region of the receptor, which contributes to signaling (Fig. 4). This general interaction model is likely to apply to at least some of the other class II receptors, including those for CRF, calcitonin, secretin, and glucagon (Bergwitz *et al.*, 1996; Nielsen *et al.*, 2000; Stroop *et al.*, 1995; Turner *et al.*, 1998). It has been proposed that the interactions at the two receptor domains occur in a sequential manner (Hoare *et al.*, 2001; Ji *et al.*, 1998; see Fig. 4). There is also the possibility that a higher order of folding is involved. In support of this possibility is the crosslinking study showing that a PTH(1–34) agonist analogue having a benzophenone group attached to lysine-27 contacts the first extracellular loop of the PTH1R (Greenberg *et al.*, 2000); thus the amino-terminal extracellular domain of the receptor [and (15–34) portion of the ligand] must be close to the juxtamembrane region of the receptor, at least in the agonist bound state (Piserchio *et al.*, 2000). The model proposed in Fig. 4, based on binding of modified short amino-terminal PTH(1–14) sequences versus the amino-terminally truncated PTH(3–34) antagonist peptide, suggests that the binding steps may even be independent such that the binding of PTH(1–14) analogues to the juxtamembrane regions cannot be blocked by binding of the carboxyl portion of PTH(3–34) to the extracellular domain of the receptor (Hoare *et al.*, 2001). This finding, demonstrated in membrane preparations of receptors, is not seen in cell-based assays that have higher receptor number and differ in other respects (internalization, phosphorylation, etc.; Shimizu *et al.*, 2000b).

Data seen and the models proposed regarding PTH/PTH1R interaction mechanisms have clear implications for drug discovery efforts aimed at finding new PTH receptor agonists. Clinical trial data showing that PTH (given as daily subcutaneous injections) can effectively treat osteoporosis (Neer *et al.*, 2000) are likely to heighten interest in developing orally available nonpeptide mimetics for this receptor. So far, however, no such compounds have been reported. Although it is possible that the agonist-dependent activation of the PTH1R requires multiple ligand contacts to a large and diffuse surface of the receptor, including the amino-terminal extracellular domain, the finding that short peptide sequences [modified PTH(1–11) and (1–14), as well as PTH(1–9) in a tethered construct] can activate the receptor (Shimizu *et al.*, 2000a) suggests, as the model in Figure 27.5 predicts, that it should be possible for a small nonpeptide molecule that interacts only with the juxtamembrane region of the receptor to function as a potent PTH1R agonist.

Conformational Changes in the PTH1R

As for all GPCRs, the binding of an agonist peptide to the PTH1R is thought to induce conformational changes in the receptor, including movements of the TM domains that render the cytoplasmic loops more accessible to G proteins (Gether, 2000). The model shown in Fig. 4 deduced from kinetic data (Hoare *et al.*, 2001) that G protein association by a ligand-bound receptor results in a "tightening" of the receptor–ligand complex reflects this proposed conformational change. An agonist-induced movement of TM3 away from TM6 has been demonstrated for the PTH1R by Sheikh *et al.* (1999), who showed that the chelation of zinc between histidine residues (native and introduced) at the intracellular ends of TM3 and TM6 blocked receptor-mediated G protein activation. An analogous movement was also shown in the β_2-adrenergic receptor, suggesting that the mechanisms of activation for the class I and class II GPCRs are fundamentally similar (Sheikh *et al.*, 1999). Several residues on the cytoplasmic surface of the PTH1R have been identified within regions that are candidate G protein interaction sites. In intracellular loop 3, these include Val-384 and Leu-385 (PLC coupling), Thr-387 (AC coupling), and Lys-388 (AC and PLC coupling; Huang *et al.*, 1996). In intracellular loop 2, Lys-319 has been implicated in PLC signaling (Iida-Klein *et al.*, 1997). Mutation of this Lys to Glu, together with the adjacent mutations of Glu-317→Asp, Lys-318→Ser and Tyr-320→Leu, results in a mutant PTHR that is strongly defective in PLC signaling but maintains near-normal cAMP signaling (Iida-Klein *et al.*, 1997). Guo and colleagues generated “knock in” mice having this “DSEL” mutant PTHR allele in place of the normal PTHR allele, and thus were able to show that the PLC signaling pathway does not play a critical role in PTHR-mediated control of the endochondral bone formation process (Guo *et al.*, 2002).

Three different activating mutations in the PTH1R have been identified in patients with Jansen’s metaphyseal chondrodysplasia (see later discussion). These mutations occur at the cytoplasmic termini of TM2 (Arg-233→His), TM6 (Thr-410→Pro), and TM7 (Ile-458→Arg) and each results in agonist-independent cAMP signaling. Whether the conformational changes induced by the activating mutations are the same as those that occur in the agonist occupied wild-type PTH receptor is unknown, but the study of these mutant PTH1Rs, along with certain peptide ligand analogues, such as [Leu^{11}, D-Trp^{12}]PTHrP(7–34), that behave as inverse agonists with the mutant receptors and depress their basal signaling (Carter *et al.*, 2001; Gardella *et al.*, 1996b) is likely to provide insights into the conformational states that are possible for active and inactive PTH1Rs.

CONFORMATIONAL SELECTIVITY OF LIGAND BINDING

Pharmacological binding studies performed in membranes prepared from cells expressing transfected or endogenous PTHRs have suggested that the PTHR can adopt different conformational states, and that these states can be differentially stabilized by structurally distinct ligands to produce quantitatively different biological response profiles (Dean *et al.*, 2007; Hoare *et al.*, 2001). The studies thus suggest two distinct high-affinity PTH1R conformations: a G protein-coupled conformation, RG, and a novel, G protein-uncoupled conformation, R^0. The findings further suggest a shift in the mechanistic view of how a PTH ligand engages the receptor, and the role of G proteins. Previous views, based largely on the classical ternary complex models of GPRC action (Kenakin, 2003), hold that the initial binding of the ligand to the receptor produces a low-affinity state complex, denoted LR in Figure 27.5, which converts to a high-affinity state only upon engaging a heterotrimeric G protein to form the classical ternary complex, denoted LRG. This model predicts that uncoupling the G protein from the LRG complex—for example, by adding the nonhydrolyzable GTP analogue, GTPγS—would produce the low-affinity LR state and result in the rapid release of bound ligand.

In testing this prediction for the cloned PTHR in membranes from transfected LLC-PK-1 cells, however, it was found that the major fraction of radiolabeled PTH(1–34) which initially bound to the receptor remained stably bound upon GTPγS addition (Dean *et al.*, 2006b). On the other hand, parallel studies showed that a PTH(1–15) radioligand analogue mostly dissociated from the receptor upon addition of GTPγS. PTHrP(1–36) dissociated rapidly from the receptor after GTPγS addition, although more slowly than PTH(1–15). Moreover, in membranes prepared from mouse embryonic fibroblast cells genetically lacking Gα_s, ^{125}I-PTH(1–34) bound well whereas ^{125}I-PTH(1–15) did not (Dean *et al.*, 2006b). The studies thus suggested that certain PTH1R ligands—e.g., PTH(1–34)—could stabilize a novel high-affinity PTHR conformation, R^0, whereas other ligands—e.g., PTH(1–15) or PTHrP(1–36)—bound more selectively to the RG conformation. Because PTH(1–34) interacts with both the N and J domains of the receptor, and PTH(1–15) interacts solely with the J domain, the findings suggested that stable binding to R^0 involves interactions to both the N and J domains of the receptor, a binding weaker for PTHrP(1–36) than for PTH(1–34).

The biological implications for the apparent selectivity that different ligands can exhibit for different PTHR conformations is still uncertain but has become a topic of considerable focus for our group. PTH(1–34) bound more stably to R^0 and produced a more prolonged cAMP response in intact cells than did PTHrP(1–36) (Dean *et al.*, 2007). Thus, stable binding to R^0 was found to correlate with the capacity to produce prolonged cAMP signaling responses in intact cells. The mechanism of this prolonged cAMP signaling response is unknown. It may simply reflect the isomerization, over time, of stable LR^0 complexes to the active-state LRG conformation. Alternatively, the mechanism of the persistent signaling may be more complex involving binding of other proteins to the ligand–receptor complex or even persistent signaling after receptor internalization. Although most earlier studies suggest largely similar binding and activation mechanisms for PTH and PTHrP, these newer kinetic studies suggest differences in the conformational selectivity exhibited by the two ligands.

Biophysical studies of the process using the technique of fluorescence resonance energy transfer (FRET) illustrates a rapid drop in FRET signal with activation. PTH(1–34) remains bound to the receptor with persistence of the FRET signal associated with activation, despite a washing step to remove ligand. However, such maneuvers with PTH(1–15) or PTHrP(1–36), though illustrating the same change in FRET signal with initial binding, reveal immediate or rapid reversal of the FRET signal, respectively, with the washing step. These results thus mirror the persistent ligand binding after use of GTPγS and prolonged cAMP signaling following the buffer washing step with PTH(1–34), in contrast to results with PTH(1–15) or PTHrP(1–36).

These differences for PTH and PTHrP may well have biological and pharmacological relevance *in vivo*. For example, they may underlie the difference reported in the endogenous actions of the two ligands—i.e., endocrine versus paracrine—as well some of the differences seen in clinical studies reported by Stewart and co-workers for PTH(1–34) and PTHrP(1–36), in that the former appears to have a greater tendency to promote bone-resorption and stimulate synthesis of 1,25-vitamin D_3 than does the latter (Horwitz *et al.*, 2003, 2005, 2006). Recently, our group has designed analogues that further accentuate the R^0 binding affinity noted with PTH(1–34). On such an analogue, Ala_1,Aib_3[M]PTH(1–28) exhibits remarkably persistent activity in *in vitro* test systems with renal cell models and *in vivo* with prolonged duration of hypercalcemia and hypophosphatemia when compared to results even with PTH(1–34) (Nagai *et al.*, 2007; Okazaki *et al.*, 2007).

PTH1R REGULATION

The agonist-dependent response capacity of the PTH1R is diminished markedly within minutes following an initial exposure to agonist (Bergwitz *et al.*, 1994; Fukayama *et al.*, 1992). This desensitization is accompanied by rapid internalization of the PTH–PTH1R complex (Ferrari *et al.*, 1999; Huang *et al.*, 1999; Malecz *et al.*, 1998). Phosphorylation of other G protein-coupled receptors on cytoplasmic domains is known to play an important role in the internalization/desensitization process (Lefkowitz, 1998). The PTH1R is phosphorylated on its cytoplasmic tail immediately following agonist activation (Blind *et al.*, 1996), specifically on as many as seven serine residues that cluster to within the midregion of the cytoplasmic tail (Malecz *et al.*, 1998; Qian *et al.*, 1998). The second messenger–activated kinases PKA and PKC both appear to contribute to PTH1R phosphorylation, because both forskolin and phorbol 12-myristate 13-acetate increase PTH1R phosphorylation (Blind *et al.*, 1995). The inhibitory effect of staurosporine on PTH1R phosphorylation (Qian *et al.*, 1998) and internalization (Ferrari *et al.*, 1999) supports a role for PKC, and potentially other kinases, in these processes. Cotransfection experiments have indicated that the G protein receptor kinase-2 (GRK-2) also contributes to PTH1R phosphorylation (Dicker *et al.*, 1999; Malecz *et al.*, 1998).

With other G protein-coupled receptors, receptor phosphorylation enables the binding of β-arrestin2 (ββ-Arr2), which then interferes sterically with G protein coupling and, in an adapter role, binds the receptor directly to clathrin (Lefkowitz, 1998). PTH1R endocytosis occurs largely via a clathrin-coated vesicle-mediated process (Huang *et al.*, 1995). By expressing moderate levels of a phosphorylation-deficient PTH1R mutant having the clustered serines of the C-tail replaced by alanine, Qian *et al.* (1999) found that agonist-induced internalization in LLC-PK1 cells was reduced markedly in comparison to the wild-type receptor. Moreover, these workers genetically engineered a model mouse strain in which the wild-type PTH receptor gene was replaced at both alleles by the phosphorylation-deficient PTHR mutant; the mutant "PD" mice, although viable and fertile, exhibited a markedly exaggerated, and ultimately fatal, calcemic response to infused PTH(1–34) peptide administered by an Alzet mini pump (Bounoutas *et al.*, 2006). There does not appear to be a simple relationship between phosphorylation of the C-terminal tail of the PTH1R and receptor internalization/desensitization, however. Thus, Malecz *et al.* (1998) found that a similar alanine-substituted phosphorylation-deficient PTH1R mutant expressed at high levels in HEK-293 cells was internalized upon agonist binding just as efficiently as the wild-type receptor; in cotransfection experiments, Dicker *et al.* (1999) found that GRK-2 efficiently crosslinked to, coimmunoprecipitated with, and inhibited agonist-induced PLC signaling by a PTH1R mutant deleted for the C-terminal tail; and finally, using fluorescent confocal microscopy methods, Ferrari and Bisello (2001) found that a PTH1R deleted for the C-tail was internalized upon agonist binding just as efficiently as the intact receptor. This last study also showed that, like the intact receptor, the agonist-occupied C-terminally truncated receptor recruited β-Arr2 tagged with green fluorescent protein (GFP) from the cytosol to the membrane, but where the GFP-β-Arr2 remained associated with the internalized intact receptor, it dissociated rapidly from the internalized truncated receptor. Thus, phosphorylation of the C-terminal tail of the PTH1R appears to play a role in stabilizing the complex formed with the intracellular trafficking and regulatory proteins, but these proteins must also utilize other cytoplasmic components of the receptor for additional docking interactions.

Studies with the constitutively active mutant PTH receptors of Jansen's disease have begun to shed light on how conformational changes in the receptor might play a role in the internalization process. Both the H223R and the T410P mutant receptors spontaneously recruit β-Arr2 from the cytosol to the membrane (Ferrari and Bisello, 2001), but where the H223R mutant, as well as the wild-type receptor, exhibited little or no internalization of antagonist ligands, the T410P mutant internalized antagonist ligands to levels comparable to those seen with the agonist-occupied wild-type receptor (Carter *et al.*, 2001; Ferrari and Bisello, 2001). Thus, cAMP signaling and β-Arr2 binding by the PTH1R are not sufficient to induce internalization. In addition, it appears that a specific receptor conformation, which is presumably induced by agonist binding (or in some way mimicked by the T410P mutation), must be accessed for this process to occur. Further investigation with these PTH1R mutants, new ligand analogues, and fluorescently labeled regulatory proteins should help to elucidate further the molecular mechanisms involved in regulating the PTH1R-mediated signaling response, a possibly important feature of overall physiological regulation of PTH action and its pharmacological use. Paradoxically, the PTH(7–34) fragment can induce internalization of the PTHR in renal distal tubule as well as in rat osteoblastic cells via a mechanism that appears to differ from that used by PTH(1–34), in that the former is inhibited by the presence of the PTH1R-scaffolding protein Na/H exchanger regulatory factor 1 (NHERF1; Mahon *et al.*, 2002, 2003; Mahon and Segre, 2004), whereas the latter is not (Sneddon *et al.*, 2003, 2004).

PTH1R MUTATIONS IN HUMAN DISEASE

Several diseases of bone and mineral metabolism have been linked to mutations in the PTH1R. As mentioned earlier, Jansen's metaphyseal chondrodysplasia is a rare disease associated with skeletal abnormalities, dwarfism, and hypercalcemia and is caused by heterozygous-dominant activating mutations in the PTHR (Calvi and Schipani, 2000). Mutations at the three sites have been found, each located at or near the cytoplasmic termini of one of the transmembrane domain helices: TM2 (Arg-233→His), TM6 (Thr-410→Pro), and TM7 (Ile-458→Arg) (see Fig. 4). The mutations cause constitutive, ligand-independent signaling of the receptor via the Gαs pathway, such that when transfected into COS-7 cells the mutant receptors produce basal cAMP levels that are severalfold higher than those observed for the wild-type PTH1R. Consistent with the skeletal phenotype of the affected individuals and the complex role that the PTHR plays in bone remodeling, transgenic mice having targeted expression of the PTHR1-H223R allele to osteoblasts exhibit increased bone in trabecular compartments but decreased bone in cortical areas (Calvi *et al.*, 2001). A different heterozygous activating mutation was identified in a patient with enchondromatosis (Ollier and Maffucci diseases), a rare disease characterized by cartilage tumors of the bone. The mutation, Arg-150→Cys, is located in the receptor's amino-terminal extracellular domain. When the corresponding receptor mutant was transfected into COS-7 cells and the lower expression levels were corrected for, a moderate elevation of ligand-independent basal cAMP, relative to wild-type, was observed (Hopyan *et al.*, 2002). The mechanism by which this mutation, located on the extracellular surface of the receptor, results in an apparent increase in the efficiency of coupling of the receptor to Gαs is unclear. Another mutation in the receptor's amino-terminal domain, Pro-132→Leu has been found in cases of Blomstrand's chondrodysplasia, a neonatal lethal disorder characterized by dramatically advanced endochondral bone maturation. Investigations into the underlying molecular defects of this disease revealed that Pro-132→Leu was present on both alleles, and when the corresponding mutant was analyzed in COS-7 cells, a loss-of-function phenotype was observed (Karaplis *et al.*, 1998; Zhang *et al.*, 1998). Whether this proline, situated in the core region of the amino-terminal domain, is directly involved in ligand interaction or provides a scaffolding function, as might be inferred from its preservation in all class II receptors, has not yet been determined. Finally, a nonsense mutation, Arg-485→Stop, was found in the C-terminal tail of the PTHR in a patient with Eiken syndrome, a rare homozygous recessive skeletal dysplasia (Duchatelet *et al.*, 2005). The phenotype associated with this mutation is opposite to that of Blomstrand's disease, in that skeletal ossification is extremely delayed. The Eiken skeletal phenotype appears consistent with an activating effect of the mutation, but the underlying mechanism is not yet clear. In any case, the absence of a phenotype for the Arg-485→Stop allele in the heterozygous carrier state, as well as the location of the mutation in the C-terminal tail of the receptor, point to a mechanism that is distinct from that which underlies the effects of the heterozygous activating mutations found in Jansen's chondrodysplasia.

OTHER RECEPTORS FOR PTH AND/OR PTHrP

Receptors for Mid- and Carboxyl-Terminal Portions of PTH and PTHrP

There is a substantial amount of pharmacological evidence in the literature for additional nonclassical receptors for PTH and PTHrP (Jüppner *et al.*, 2006). Competition binding studies and other functional assays have suggested that distinct receptors exist for midregional and carboxyl-terminal fragments of PTH or PTHrP, although the biological importance of such receptors remains to be established. For example, intact PTH and/or larger carboxyl-terminal PTH fragments have been shown to interact with a novel cell surface receptor (see later discussion). Other receptors appear to mediate the effects of midregional PTHrP on placental calcium transport (Kovacs *et al.*, 1996; Wu *et al.*, 1996) and in the skin (Orloff *et al.*, 1996). Carboxyl-terminal portions of PTHrP also have effects on osteoblasts (Cornish *et al.*, 1997, 1999; Fenton *et al.*, 1991a, 1991b), as well as the central nervous system (Fukayama *et al.*, 1995). A novel receptor that interacts with the amino-terminal portion of PTHrP, but not the amino-terminal portion of PTH, has been characterized in the rat supraoptic nucleus, and this PTHrP-selective receptor can regulate the synthesis and release of arginine vasopressin (Yamamoto *et al.*, 1998). Still other receptors that interact with amino-terminal portions of PTH and PTHrP have been identified pharmacologically that signal through changes in intracellular free calcium but not through increases in cAMP (Gaich *et al.*, 1993; Orloff *et al.*, 1995; Soifer *et al.*, 1992). Clearly there is strong interest in isolating complementary cDNAs, which encode such nonclassical receptors for PTH and PTHrP, but so far none have been identified. Such interest is highlighted by studies on the carboxyl-terminal portion of PTH.

Receptors Specific for Carboxyl-Terminal PTH in Bone (CPTHR)

The traditional view of PTH biology has been that the major biologic actions of PTH on bone, cartilage, and kidney result from activation of PTH1R, which is fully achieved by the N-terminal sequence PTH(1–34) (or the homologous N-terminal portion of PTHrP). This concept has derived largely from a heavy focus in the years following the isolation and structural identification of PTH on the use of cAMP production and of biologic responses now known to be largely cAMP dependent. These include calcemia and phosphaturia, as indices of PTH action *in vivo* and *in vitro*, although, as reviewed earlier, it is now known that PTH1R can also trigger cAMP-independent signaling events, including activation of PLC, PLD, PLA2, PKC, and increased cytosolic calcium, and that the structural determinants within both the PTH(1–34) ligand and the PTH1R itself that are required for these activities are not fully congruent.

Until very recently, the possibility that the C-terminal portion of the intact PTH(1–84) molecule might be physiologically important has received inadequate attention in part because (a) peptides such as PTH(39–84) or PTH(53–84) cannot be shown to bind or activate PTH1Rs; (b) the expressed PTH1R interacts equivalently with PTH(1–34) and PTH(1–84); (c) even minimal truncation at the N terminus of PTH(1–34) ablates PTH1R activation; and (d) differences in the bioactivity of PTH(1–34) and PTH(1–84) have not been demonstrated consistently in the usual "PTH bioassays" *in vitro* or *in vivo* (Potts and Jüppner, 1998). Moreover, the rapid production of C-terminal PTH fragments via endopeptidic cleavage of intact PTH *in vivo* (mainly in liver and kidney) has been regarded as the major route of metabolic clearance of active PTH, whereby the active N terminus of the molecule is destroyed *in situ* and long-lived C fragments are released back into the circulation (Potts and Jüppner, 1998). However, large portions of the C-terminal sequence of PTH(1–84) have been tightly conserved during evolution, active N-terminal fragments of PTH have not been demonstrated convincingly in blood of normal subjects, and C fragments of PTH (with N termini between residues 24 and 43) are cosecreted with intact hormone by the parathyroid glands in a manner whereby the ratio of intact fragments to C fragments is subject to regulation by blood calcium (Potts and Jüppner, 1998). Uncertainty regarding the potential importance of these observations, vis-à-vis a possible physiologic role for circulating C-terminal PTH peptides (CPTH), resulted mainly from a lack of evidence for specific receptors, distinct from the PTH1R (upon which all major PTH bioassays have been based), at which these peptides might act.

Initial indications that receptors for CPTH (CPTHRs) might exist actually appeared in the 1980s, when techniques were first developed to radiolabel intact PTH(1–84) in a biologically active form. At that time, careful analysis of ^{125}I-bPTH(1–84) binding to renal membranes and intact osteoblastic cells provided clear evidence of a second binding site that had a 10-fold lower affinity than that now known to pertain to the PTH1R and was specifically displaced by CPTH peptides (Demay *et al.*, 1985; Rao and Murray, 1985; Rao *et al.*, 1983). More recently, Inomata *et al.* reported the first direct measurements of CPTHR binding in ROS 17/2.8 rat osteosarcoma cells and rat parathyroid-derived cells, using radioiodinated recombinant peptides ^{125}I-[Tyr^{34}]hPTHrP(19–84) and ^{125}I-[Leu^{8,18}, Tyr^{34}]hPTHrP(1–84) as tracers (Inomata *et al.*, 1995). These two radioligands, which bind minimally, if at all, to the PTH1R, exhibited binding affinity comparable to that of hPTH(1–84) itself. These results suggested that all of the binding determinants of intact PTH(1–84) for the CPTHR reside within the PTH(19–84) sequence. Specific CPTHR binding was observed for hPTH(53–84), hPTH(39–94), and hPTH(1–84) but not hPTH(44–68) or hPTH(1–34); chemical crosslinking demonstrated a predominant 90-kDa receptor band (Inomata *et al.*, 1995). Unequivocal evidence that these CPTHR sites are distinct from the PTH1R subsequently was obtained via the demonstration of specific ^{125}I-[Tyr^{34}]hPTHrP(19–84) binding to clonal osteoblasts and osteocytes in which both PTH1R alleles had been ablated by gene targeting (Divieti *et al.*, 2001). The apparent binding affinity of these CPTHR sites for intact PTH and longer CPTH fragments (K_d = 0–20 nM) was 10-fold lower than that of the PTH1R for PTH(1–84) or PTH(1–34) (1–2 nM). Interestingly, this difference in affinity of PTH1Rs and CPTHRs for intact PTH and CPTH fragments, respectively, mirrors their relative levels in blood, where evidence suggests a 5- to 10-fold higher concentration of CPTH peptides than intact hormone.

Early work in several laboratories documented increased alkaline phosphatase expression following exposure of rat of human osteosarcoma cells to CPTH peptides, and subsequent research has identified a variety of biologic effects of CPTH fragments, including regulation of collagen and IGFBP-5 mRNA in UMR-106 rat osteosarcoma cells, stimulation of ^{45}Ca uptake in SaOS-2 cells, promotion of osteoclast formation in primary murine bone and marrow cell cultures, regulation of collagen II and X mRNA in primary fetal bovine hypertrophic chondrocytes, induction of cytosolic calcium transients in human primary fetal hypertrophic chondrocytes, and control of dentin and enamel formation in organ-cultured embryonic mouse tooth germ (Erdmann *et al.*, 1996, 1998; Fukayama *et al.*, 1994; Kaji *et al.*, 1994; Murray *et al.*, 1991; Nakamoto *et al.*, 1993; Nasu *et al.*, 1998; Sutherland *et al.*, 1994; Takasu *et al.*, 1996; Tsuboi and Togari, 1998). Most often, these effects of CPTH peptides were different from, if not opposite to, those of PTH(1-34), although mediation by PTH1Rs could not be definitely excluded because all of the cells studied were known, or could be assumed, to express PTH1Rs. More recent work, however, using cells genetically devoid of functional PTH1Rs, has clearly documented biologic responses to CPTHRs that must be distinct from the PTH1R (Divieti *et al.*, 2001). The structural features of the PTH ligand required for CPTHR activation have yet to be fully defined, although the importance of an intact C terminus for some, but not all, responses has been emphasized (Takasu *et al.*, 1996).

It is of interest that CPTHRs have been identified so far primarily in cells of skeletal origin, i.e., marrow stromal cells, osteoblasts, osteocytes, and chondrocytes. However, CPTHRs were also described for cells derived from rat parathyroid cells (rPTs), which have characteristics of fibroblasts (Potts and Jüppner, 1998). The highest levels of CPTHR expression reported to date ($2-3\times10^6$/cell) were observed in clonal cell lines, conditionally transformed by a temperature-sensitive SV40 transgene and isolated from embryonic PTH1R-null mice, with phenotypic features of osteocytes (i.e., a dendritic morphology and abundant expression of osteocalcin and connexin-43 but not of alkaline phosphatase or cbfa-1; Divieti *et al.*, 2001). In such cells, genetically devoid of PTH1Rs, PTH(1–84) could not elicit cAMP generation and, as expected, no binding of ^{125}I-[Tyr^{34}]hPTHrP(1-36) could be detected (Divieti *et al.*, 2001). Analysis of the structural requirements for CPTHR ligand binding in these cells, using the ^{125}I-[Tyr^{34}]hPTH(19–84) radioligand, demonstrated equivalent affinity of hPTH(1–84), [Tyr^{34}]hPTH(19–84), and hPTH(24–84) ($IC_{50} = 20-50\,nM$) that declined substantially with further truncation to hPTH(39–84) ($IC_{50} = 20-50\,nM$), indicating the presence of important binding determinants within the sequence hPTH(24–38). Interestingly, hPTH(1–34), which contains most of this region, also weakly displaced the CPTHR radioligand ($IC_{50} > 10{,}000\,nM$). These key features of ligand recognition were shared in common with PTH1R-null chondrocytes, marrow stromal cells, osteoblasts, and osteocytes (P. Divieti, and F. R. Bringhurst, unpublished data), suggesting that these various cell types may express structurally identical CPTHRs.

A possible physiologic role for CPTHRs expressed by cells of the osteoblast lineage was suggested by observations that, in PTH1R-null clonal osteocytes, intact PTH, as well as CPTH fragments, such as hPTH(39–84) and hPTH(53–84), promote apoptosis. This contrasts with the antiapoptotic effect of PTH1R activation in such cells (Jilka *et al.*, 1998) and suggests that the PTH1R and the still-uncloned CPTHR may exert functionally antagonistic actions upon osteoblastic cells *in vivo*. CPTHR activation in clonal osteocytes also modified expression of connexin-43, suggesting a possible role in the regulation of cell-to-cell communication via gap junctions. The signal transduction mechanisms that may underlie these CPTHR effects remain unknown, although, as noted earlier, coupling to G_s is unlikely.

In summary, evidence that receptors with specificity for the carboxyl-terminal portion of intact PTH(1-84) (i.e., CPTHRs) exist in bone is now unequivocal (Divieti *et al.*, 2001). These receptors, most clearly defined *in vitro* using cell systems genetically devoid of PTH/PTHrP receptors (PTH1Rs), can bind and be activated by intact PTH(1–84) and various synthetic CPTH fragments, such as hPTH(19–84), hPTH(39–84), and hPTH(53–84) (Divieti *et al.*, 2001; Murray *et al.*, 1991). Numerous biologic responses to CPTHR activation have been identified, including regulation of calcium transients, alkaline phosphatase, collagen gene expression, osteoclast formation, connexin-43 expression, and apoptosis (Demay *et al.*, 1985; Divieti *et al.*, 2001; Erdmann *et al.*, 1996, 1998; Fukayama *et al.*, 1994; Kaji *et al.*, 1994; Murray *et al.*, 1991; Nakamoto *et al.*, 1993; Nasu *et al.*, 1998; Rao and Murray, 1985; Rao *et al.*, 1983; Sutherland *et al.*, 1994; Takasu *et al.*, 1996; Tsuboi and Togari, 1998). Observations *in vivo* indicate that the fragment hPTH(7–84), which may exist normally *in vivo* but which clearly can bind CPTHRs with high affinity, antagonizes the PTH1R-mediated calcemic effect of PTH *in vivo* (Nguyen-Yamamoto *et al.*, 2000; Slatopolsky *et al.*, 2000). This *in vivo* action is mirrored in *in vitro* studies showing antagonism by hPTH(7–84) but not by hPTHrP(7–34) of calvarial bone resorption induced by PTH(1–34), suggesting a possible role for CPTHR activation in blocking the bone resorption (P. Divieti and F. R. Bringhurst, unpublished data). Finally, evidence that CPTHR activation promotes apoptosis in cells of the osteoblast lineage, an effect opposite that of PTH(1–34) in such cells, points to a potentially important interaction of PTH1Rs and CPTHRs in control of osteoblast and osteocyte number (Divieti *et al.*, 2001).

Carboxyl fragments of intact PTH are secreted in a calcium-regulated manner by the parathyroid glands, are generated rapidly from secreted or injected PTH(1–84) via peripheral metabolism in liver and kidney, circulate normally at molar levels 5-to 10-fold higher than those of intact PTH(1–84), and accumulate to much higher concentrations in renal failure (Potts and Jüppner, 1998). The possibility that CPTHR activation may play a role in modulating osseous responses to full-length (versus N-terminal) PTH administered as a therapeutic for osteoporosis or in the pathogenesis of currently unexplained features of renal osteodystrophy clearly is worthy of further investigation in light of all the accumulating evidence about high concentrations of CPTH fragments, the distinct CPTHR, and the variety of biological effects seen *in vitro* following the interactions of C fragments with the CPTHR.

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Chapter 28

Parathyroid Hormone

Molecular Biology

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THE PARATHYROID HORMONE GENE

The PTH Gene

The human parathyroid hormone (PTH) gene is localized on the short arm of chromosome 11 at 11p15 (Antonarakis *et al.*, 1983; Zabel *et al.*, 1985). The human and bovine genes have two functional TATA transcription start sites, and the rat has only one. The two homologous TATA sequences flanking the human PTH gene direct the synthesis of two human PTH gene transcripts both in normal parathyroid glands and in parathyroid adenomas (Igarashi *et al.*, 1986). The PTH genes in all species that have been cloned have two introns or intervening sequences and three exons (Kronenberg *et al.*, 1986). Strikingly, even though fish do not have discrete parathyroid glands, they do synthesize PTH by using two distinct genes that share the same exon–intron pattern found in tetrapod PTH genes (Danks *et al.*, 2003; Gensure *et al.*, 2004). The locations of the introns are identical in each case (Bell *et al.*, 2005b). Intron A splits the 5′-untranslated sequence of the mRNA five nucleotides before the initiator methionine codon. Intron B splits the fourth codon of the region that codes for the pro sequence of preproPTH. The three exons that result thus are roughly divided into three functional domains. Exon I contains the 5′-untranslated region. Exon II codes for the pre sequence or signal peptide and exon III codes for PTH and the 3′-untranslated region. The structure of the PTH gene is thus consistent with the proposal that exons represent functional domains of the mRNA (Kemper, 1986; Bell *et al.*, 2005b). Although the introns are at the same location, the size of the large intron A in humans is about twice as large as those in the rat and bovine (3500,1714, and 1600 nt, respectively). It is interesting that the human gene is considerably longer in both intron A and the 3′-untranslated region of the cDNA than the bovine, rat, and mouse. Knowledge of the structures of other PTH genes from other species will be necessary in order to determine whether extra sequences were inserted or are less susceptible to deletion in the human gene. Both introns have the characteristic splice site elements. The second exon, containing 106 and 121 nt in the human and bovine pre-mRNA is much smaller and more homologous in size among the genes than intron A. The genes for PTH and PTHrP (PTH-related protein) are located in similar positions on sibling chromosomes 11 and 12. It is therefore likely that they arose from a common precursor by chromosomal duplication.

The PTH mRNA

Complementary DNA encoding for human (Hendy *et al.*, 1981; Vasicek *et al.*, 1983), bovine (Kronenberg *et al.*, 1979; Weaver *et al.*, 1982), rat (Schmelzer *et al.*, 1987), mouse (He *et al.*, 2002), pig (Schmelzer *et al.*, 1987), chicken (Khosla *et al.*, 1988; Russell and Sherwood, 1989), dog (Rosol *et al.*, 1995), cat (Toribio *et al.*, 2002), horse (Caetano *et al.*, 1999), macaca (Malaivijitnond *et al.*, 2002), fugu fish (Danks *et al.*, 2003), and zebrafish (Gensure *et al.*, 2004) PTH have all been cloned. The PTH gene is a typical eukaryotic gene with consensus sequences for initiation of RNA synthesis, RNA splicing, and polyadenylation. The primary RNA transcript consists of RNA transcribed from both introns and exons, and then RNA sequences derived from the introns are spliced out. The product of this RNA processing, which represents the exons, is the mature PTH mRNA, which will then be translated into preproPTH. There is considerable identity among mammalian PTH genes, which is reflected in an 85% identity between human and bovine proteins

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and 75% identity between human and rat proteins. There is less identity in the 3′-noncoding region. The actual initiator ATG codons for the human and bovine have been identified by sequencing *in vitro* translation products of the mRNAs (Habener *et al.*, 1975; Kemper *et al.*, 1976). In the bovine sequence, the first ATG codon is the initiator codon, in accord with many other eukaryotic mRNAs (Kozak, 1991a, 1991b). The human and rat sequences have ATG triplets prior to the probable initiator ATG, which are present ten nucleotides before the initiation codon and are immediately followed by a termination codon. The termination codon immediately following the codon for glutamine at position 84 of PTH indicates that there are no additional precursors of PTH with peptide extensions at the carboxyl position. A more extensive review of the structure and sequences of the PTH gene has been published elsewhere in the book, *Molecular Biology of the Parathyroid* (Bell *et al.*, 2005b).

DEVELOPMENT OF THE PARATHYROID AND TISSUE-SPECIFIC EXPRESSION OF THE PTH GENE

The thymus, thyroid, and parathyroid glands in vertebrates develop from the pharyngeal region, with contributions both from pharyngeal endoderm and from neural crest cells in the pharyngeal arches. Studies of gene knockout mice have shown that the hoxa3, pax 1, pax 9, and Eya1 transcription factors are needed to form parathyroid glands as well as many other pharyngeal pouch derivatives, such as the thymus. Hoxa3 mutant homozygotes have defects in the development of all three organs and are completely missing parathyroid glands (Manley and Capecchi, 1998). Pax 1 and Pax 9 mouse mutants are similarly missing parathyroid glands and other pharyngeal pouch structures (Su *et al.*, 2001; Peters *et al.*, 1998). Eyes absent (Eya) genes regulate organogenesis in both vertebrates and invertebrates. Mutations in human EYA1 cause congenital Branchio-Oto-Renal (BOR) syndrome, whereas targeted inactivation of murine Eya1 impairs early developmental processes in multiple organs, including the ears, kidneys, and skeletal system. Eya1 is also important to the morphogenesis of organs derived from the pharyngeal region, including thymus, parathyroid, and thyroid (Xu *et al.*, 2002). The thymus and parathyroid are derived from 3rd pharyngeal pouches and their development is initiated via inductive interactions between neural crest-derived arch mesenchyme, pouch endoderm, and possibly the surface ectoderm of 3rd pharyngeal clefts. Eya1 is expressed in all three cell types during thymus and parathyroid development from E9.5, and the organ primordia for both of these structures failed to form in Eya1−/− embryos. These results indicate that Eya1 is required for the initiation of thymus and parathyroid gland formation. Six1 expression is markedly reduced in the arch mesenchyme, pouch endoderm, and surface ectoderm in the pharyngeal region of Eya1−/− embryos, indicating that Six1 expression in those structures is Eya1 dependent (Xu *et al.*, 2002). In addition, in Eya1−/− embryos, the expression of Gcm2 (see later) in the 3rd pouch endoderm is undetectable at E10.5; however, the expression of Hox and Pax genes in the pouch endoderm is preserved at E9.5–10.5. Therefore, Eya1 controls critical early inductive events involved in the morphogenesis of thymus, parathyroid, and thyroid and is independent of Hox and Pax genes and upstream of Gcm2 in the development of the parathyroid.

The DiGeorge syndrome is a human genetic condition resulting from deletion of contiguous genes. The specific gene causing the hypoparathroidism found in DiGeorge syndrome and the closely related CATCH-22 syndrome (cardiac defects, abnormal facies, thymic hypoplasia, cleft palate, hypocalcemia, associated with chromosome 22 microdeletion) is likely to be Tbx1 (Baldini, 2005). In the DiGeorge syndrome, malformations include absence of the parathyroid glands and thymus as well as the heart outflow tract, and most DiGeorge syndrome patients are hemizygous for a 1.5–3.0 Mb region of 22q11. In mice deletion of one copy of Tbx1 (in the equivalent genetic region in the mouse) affects the development of the parathyroids, the thymus, and the fourth pharyngeal arch arteries, whereas homozygous mutation severely disrupts cardiac outflow as well (Merscher *et al.*, 2001; Lindsay *et al.*, 2001). These data in mice suggest that Tbx1 has a major role in the molecular etiology of the DiGeorge syndrome phenotype.

In addition to the genes just discussed, which function in early differentiation of parathyroid cells in the context of effects more broadly during development of pharyngeal pouches, other genes have more specific effects on parathyroid gland development. Gunther *et al.* (2000) studied Glial cells missing2 (Gcm2), a mouse homologue of *Drosophila* Gcm, a transcription factor whose expression is restricted to the parathyroid glands. They showed that Gcm2-deficient mice lacked parathyroid glands and exhibited hypoparathyroidism, identifying Gcm2 as a master regulatory gene of parathyroid gland development. Thymus development was not affected by Gcm2 deletion. Gcm2-deficient mice were viable and fertile and had only a mildly abnormal bone phenotype. Despite their lack of parathyroid glands, Gcm2-deficient mice had PTH serum levels similar to those of wild-type mice (in the presence of hypocalcemia), as did parathyroidectomized wild-type mice. Expression and ablation studies identified the thymus, where Gcm1, another Gcm homologue, is expressed as the additional, down-regulatable source of PTH. Thus, Gcm2 deletion uncovered an auxiliary mechanism for the regulation of calcium homeostasis in the absence of parathyroid glands. A human patient with a defective Gcm B gene, the human equivalent of Gcm-2, exhibited hypoparathyroidism and complete absence of PTH from the bloodstream (Ding *et al.*, 2000).

Two other genes needed for the formation of parathyroid glands are GATA3 and SOX3. Humans with one mutated copy of the GATA3 transcription factor exhibit hypoparathyroidism, sensorineural deafness, and renal anomalies (Van Esch *et al.*, 2000). Humans with X-linked hypoparathyroidism manifest a deletion–insertion near the end of the SOX3 gene, a finding that suggests an important role for Sox3 in parathyroid development (Bowl *et al.*, 2005).

Okabe and Graham (2004) performed elegant studies that demonstrate a role for Gcm-2 even in fish, which do not have discrete parathyroid glands. They showed that the parathyroid gland of tetrapods and the gills of fish both express Gcm-2 and require this gene for their formation. They also showed that the gill region expresses mRNA encoding the two PTH genes found in fish, as well as mRNA encoding the calcium-sensing receptor. They speculate that gcm-2, PTH, and the calcium-sensing receptor might, therefore be found in the fish equivalent of parathyroid cells. This hypothesis about the origin of parathyroid cells, however, is not supported by other studies that show that fish parathyroid hormones are not synthesized in the gill but instead in lateral line neuromasts, mechanosensory cells that are distributed along the fish's body surface. The relationship between these cells and parathyroid cells of tetrapods is unknown. Thus, the conserved role of Gcm-2 in forming pharyngeal structures is established, but the relationship between Gcm-2 and parathyroid hormone-producing cells in fish requires further experimentation.

PROMOTER SEQUENCES

Regions upstream of the transcribed structural gene often determine tissue specificity and contain many of the regulatory sequences for the gene. For PTH, analysis of this region has been hampered by the lack of a parathyroid cell line. Arnold's group has demonstrated, however, that the 5 kb of DNA upstream of the start site of the human PTH gene was able to direct parathyroid gland-specific expression in transgenic mice (Hosokawa *et al.*, 1997). Analysis of the human PTH promoter region identified a number of consensus sequences by computer analysis (Kel *et al.*, 2005). These included a sequence resembling the canonical cAMP-responsive element 5′-TGACGTCA-3′ at position −81 with a single-residue deviation. This element was fused to a reporter gene (CAT) and then transfected into different cell lines. Pharmacological agents that increase cAMP led to an increased expression of the CAT gene, suggesting a functional role for the cAMP-responsive element (CRE). The role of this putative CRE in the PTH gene in the parathyroid remains to be established. Specificity protein (Sp) and the nuclear factor-Y (NF-Y) complex are thought to be ubiquitously expressed transcription factors associated with basal expression of a host of gene products. Sp family members and NF-Y can cooperatively enhance transcription of a target gene. Koszewski and colleagues (Alimov *et al.*, 2005) identified and characterized a highly conserved Sp1 DNA element present in mammalian PTH promoters. They showed that coexpression of Sp proteins and NF-Y complex leads to synergistic transactivation of the hPTH promoter, with alignment of the Sp1 DNA element essential for full activation (Alimov *et al.*, 2005). They also identified a similar arrangement of DNA response elements and synergism in the bovine PTH (bPTH) promoter, suggesting that this may be a conserved mechanism to enhance transcription of the PTH gene. They demonstrated the presence of a proximal NF-Y-binding site in the hPTH promoter and highlight the potential for synergism between distal and proximal NF-Y DNA elements to strongly enhance transcription (Koszewski *et al.*, 2004). Moreover, their data raised the possibility that the repressive effects of vitamin D on hPTH gene transcription may involve displacement of NF-Y binding to the proximal site by the 1,25$(OH)_2D_3$ receptor (VDR) heterodimer, which subsequently attenuates synergistic transactivation (Koszewski *et al.*, 2004).

Several groups have identified DNA sequences that might mediate the negative regulation of PTH gene transcription by 1,25-dihydroxyvitamin D [1,25$(OH)_2D_3$]. Demay *et al.* (1992) identified DNA sequences in the human PTH gene that bind the 1,25$(OH)_2D_3$ receptor. Nuclear extracts containing the 1,25$(OH)_2D_3$ receptor were examined for binding to sequences in the 5′-flanking region of the hPTH gene. A 25-bp oligonucleotide containing sequences from −125 to −101 from the start of exon 1 bound nuclear proteins that were recognized by monoclonal antibodies against the 1,25$(OH)_2D_3$ receptor. The sequences in this region contained a single copy of a motif (AGGTTCA) that is homologous to the motifs repeated in the upregulatory 1,25$(OH)_2D_3$ response element of the osteocalcin gene. When placed upstream to a heterologous viral promoter, the sequences contained in this 25-bp oligonucleotide mediated transcriptional repression in response to 1,25$(OH)_2D_3$ in GH4C1 cells but not in ROS 17/2.8 cells. Therefore, this downregulatory element differs from upregulatory elements both in sequence composition and in the requirement for particular cellular factors other than the 1,25$(OH)_2D_3$ receptor (VDR) for repressing PTH transcription (Demay *et al.*, 1992). Russell *et al.* (1999) have shown that there are two negative VDREs in the rat PTH gene. One is situated at −793 to −779 and bound a VDR/RXR heterodimer with high affinity and the other at −760 to −746 bound the heterodimer with a lower affinity. Transfection studies with VDRE-CAT constructs showed that they had an additive effect. Liu *et al.* (1996) identified such sequences in the chicken PTH gene and demonstrated their functionality after transfection into the opossum kidney (OK) cell line. They converted the negative activity imparted by the PTH VDRE to a positive transcriptional response through selective mutations introduced into the element. They showed that there was a p160 protein that specifically interacted with a heterodimer complex bound to the wild-type VDRE, but was absent from complexes bound to response elements associated with positive transcriptional activity. Thus, the sequence of the individual VDRE appears to play an active role in dictating transcriptional responses that may be mediated by altering the ability of a vitamin D receptor heterodimer to interact with accessory factor proteins. Further work is needed to demonstrate that any of these differing negative VDREs function in this fashion in parathyroid cells.

The transrepression by 1,25-$(OH)_2D_3$ has also been shown to depend on another promoter element. Kato's laboratory have identified an E-box (CANNTG)-like motif as another class of nVDRE in the human 1α(OH)ase promoter (Murayama *et al.*, 2004; Fujiki *et al.*, 2005). In sharp contrast to the previously reported DR3-like motif in the hPTH gene promoter, a basic helix-loop-helix factor, designated VDR interacting repressor (VDIR), transactivates through direct binding to this E-box-type element (1αnVDRE). However, the VDIR transactivation function is transrepressed through ligand-induced protein–protein interaction of VDIR with VDR/RXR. In the absence of 1,25-$(OH)_2D_3$, VDIR appears to bind to 1αnVDRE for transactivation through the histone acetylase (HAT) coactivator, p300/CBP. Binding of 1α,25$(OH)_2D_3$ to VDR induces interaction with VDIR and dissociation of the HAT coactivator, resulting in recruitment of histone deacetylase (HDAC) corepressor for ligand-induced transrepression (Murayama *et al.*, 2004). They have also characterized the functions of VDIR and E-box motifs in the human (h) PTH and hPTHrP gene promoters (Kim *et al.*, 2007). They identified E-box-type elements acting as nVDREs in both the hPTH promoter (hPTHnVDRE; −87 to −60 bp) and in the hPTHrP promoter (hPTHrPnVDRE; −850 to −600 bp; −463 to −104 bp) in a mouse renal tubule cell line. The hPTHnVDRE alone was enough to direct ligand-induced transrepression mediated through VDR/retinoid X receptor and VDIR. Direct DNA binding of hPTHnVDRE to VDIR, but not VDR/retinoid X receptor, was observed and ligand-induced transrepression was coupled with recruitment of VDR and histone deacetylase 2 (HDAC2) to the hPTH promoter. They concluded that negative regulation of the hPTH gene by liganded VDR is mediated by VDIR directly binding to the E-box-type nVDRE at the promoter, together with recruitment of an HDAC corepressor for ligand-induced transrepression (Kim *et al.*, 2007). These studies were specific to a mouse proximal tubule cell line and await the development of a parathyroid cell line to confirm them in a homologous cell system.

MUTATIONS IN THE PTH GENE

Rare patients have been found with abnormal parathyroid hormone genes that result in hypoparathyroidism (Gafni and Levine, 2005). The PTH gene of a patient with familial isolated hypoparathyroidism (Ahn *et al.*, 1986) was studied by Arnold *et al.* (1990), and a point mutation in the hydrophobic core of the signal peptide-encoding region of preproPTH was identified. This T-to-C point mutation changed the codon for position 18 of the 31-amino-acid prepro sequence from cysteine to arginine, and in functional studies the mutant protein was processed inefficiently. The mutation impaired interaction of the nascent protein with signal recognition particles and the translocation machinery, and cleavage of the mutant signal sequence by solubilized signal peptidase was slow (Karaplis *et al.*, 1995). Sunthornthepvarakul *et al.* (1999) reported a novel mutation of the signal peptide of the prepro-PTH gene associated with autosomal recessive familial isolated hypoparathyroidism. The affected members in this family presented with neonatal hypocalcemic seizures. Their intact PTH levels were undetectable during severe hypocalcemia. A replacement of thymine with a cytosine was found in the first nucleotide of position 23 in the 25-amino-acid signal peptide. This results in the replacement of the normal Ser (TCG) with a Pro (CCG). Only affected family members were homozygous for the mutant allele, whereas the parents were heterozygous, supporting autosomal recessive inheritance. Because this mutation is at the −3 position in the signal peptide of the prepro-PTH gene, the authors hypothesized that the prepro-PTH mutant might not be cleaved by signal peptidase at the normal position and might be degraded in the rough endoplasmic reticulum. Parkinson and Thakker (1992) studied one kindred with autosomal recessive isolated hypoparathyroidism and identified a G-to-C substitution in the first nucleotide of intron 2 of the parathyroid hormone gene. Restriction enzyme cleavage revealed that the patients were homozygous for mutant alleles, unaffected relatives were heterozygous, and unrelated normals were homozygous for the wild-type alleles. Defects in messenger RNA splicing were investigated by the detection of illegitimate transcription of the PTH gene in lymphoblastoid cells. The mutation resulted in exon skipping with a loss of exon 2, which encodes the initiation codon and the signal peptide, thereby causing parathyroid hormone deficiency. Somatic mutations identified in the PTH gene in some parathyroid adenomas are discussed elsewhere in this book. Goswami *et al.* (2004) studied 51 patients with sporadic idiopathic hypoparathyroidism. Neither the clinical manifestations nor the biochemical indexes of the disease were related to the occurrence of mutations or SNPs in the PTH gene. Because neither patient nor control samples exhibited any variations in the sequence of their 3′-UTR regions, they concluded that it is unlikely that mRNA instability is a factor in the pathogenesis of the disease (Goswami *et al.*, 2004).

REGULATION OF PTH GENE EXPRESSION

1,25-Dihydroxyvitamin D

PTH regulates serum concentrations of calcium and phosphate, which, in turn, regulate the synthesis and secretion of PTH. 1,25-Dihydroxyvitamin D (calcitriol) has independent effects on calcium and phosphate levels and also participates in a well-defined feedback loop between calcitriol and PTH.

PTH increases the renal synthesis of calcitriol. Calcitriol then increases blood calcium largely by increasing the efficiency of intestinal calcium absorption. Calcitriol also potently decreases transcription of the PTH gene. This action was first demonstrated *in vitro* in bovine parathyroid cells in primary culture, where calcitriol led to a marked decrease in PTH mRNA levels (Silver *et al.*, 1985; Russell *et al.*, 1984) and a consequent decrease in PTH secretion (Cantley *et al.*, 1985a, 1985b; Karmali *et al.*, 1989; Chan *et al.*, 1986). The physiological relevance of these findings was established by *in vivo* studies in rats (Silver *et al.*, 1986). The localization of $1,25(OH)_2D_3$ receptor mRNA (VDR mRNA) to parathyroids was demonstrated by *in situ* hybridization studies of the thyroparathyroid and duodenum. VDR mRNA was localized to the parathyroids in the same concentration as in the duodenum, the classic target organ of calcitriol (Naveh-Many *et al.*, 1990). Rats injected with amounts of calcitriol that did not increase serum calcium had marked decreases in PTH mRNA levels, reaching $<4\%$ of control at 48 hours. This effect was shown to be transcriptional both in *in vivo* studies in rats (Silver *et al.*, 1986) and in *in vitro* studies with primary cultures of bovine parathyroid cells (Russell *et al.*, 1986). When 684 bp of the 5′-flanking region of the human PTH gene was linked to a reporter gene and transfected into a rat pituitary cell line (GH4C1), gene expression was lowered by $1,25(OH)_2D_3$ (Okazaki *et al.*, 1988). These studies suggest that $1,25(OH)_2D_3$ decreases PTH transcription by acting on the 5′-flanking region of the PTH gene, probably at least partly through interactions with the vitamin D receptor-binding sequences and/or the E-box that binds VDIR noted earlier. The effect of $1,25(OH)_2D_3$ may involve heterodimerization with the retinoid acid receptor. This is because 9 *cis*-retinoic acid, which binds to the retinoic acid receptor, when added to bovine parathyroid cells in primary culture, led to a decrease in PTH mRNA levels (MacDonald *et al.*, 1994). Moreover, combined treatment with 1×10^{-6} M retinoic acid and 1×10^{-8} M $1,25(OH)_2D_3$ decreased PTH secretion and preproPTH mRNA more effectively than either compound alone (MacDonald *et al.*, 1994). Alternatively, retinoic acid receptors might synergize with VDRs through actions on distinct sequences.

A further level at which $1,25(OH)_2D_3$ might regulate the PTH gene would be at the level of the $1,25(OH)_2D_3$ receptor. $1,25(OH)_2D_3$ acts on its target tissues by binding to the $1,25(OH)_2D_3$ receptor, which regulates the transcription of genes with the appropriate recognition sequences. Concentration of the $1,25(OH)_2D_3$ receptor in $1,25(OH)_2D_3$ target sites could allow a modulation of the $1,25(OH)_2D_3$ effect, with an increase in receptor concentration leading to an amplification of its effect and a decrease in receptor concentration dampening the $1,25(OH)_2D_3$ effect. Naveh-Many *et al.* (1990) injected $1,25(OH)_2D_3$ into rats and measured the levels of $1,25(OH)_2D_3$ receptor mRNA and PTH mRNA in the parathyroid tissue. They showed that $1,25(OH)_2D_3$ in physiologically relevant doses led to an increase in VDR mRNA levels in the parathyroid glands in contrast to the decrease in PTH mRNA levels. This increase in VDR mRNA occurred after a time lag of 6 hours, and a dose response showed a peak at 25 pmol. Weanling rats fed a diet deficient in calcium were markedly hypocalcemic at 3 weeks and had very high serum $1,25(OH)_2D_3$ levels. Despite the chronically high serum $1,25(OH)_2D_3$ levels, there was no increase in VDR mRNA levels; furthermore, PTH mRNA levels did not fall and were increased markedly. The low calcium in the bloodstream may have prevented the increase in parathyroid VDR levels, which may partially explain PTH mRNA suppression. Whatever the mechanism, the lack of suppression of PTH synthesis in the setting of hypocalcemia and increased serum $1,25(OH)_2D_3$ is crucial physiologically because it allows an increase in both PTH and $1,25(OH)_2D_3$ at a time of chronic hypocalcemic stress. Russell *et al.* (1993) studied the parathyroids of chicks with vitamin D deficiency and confirmed that $1,25(OH)_2D_3$ regulates PTH and VDR gene expression in the avian parathyroid gland. Brown *et al.* (1995) studied vitamin D-deficient rats and confirmed that calcitriol upregulated parathyroid VDR mRNA. Rodriguez *et al.* (2007) showed that administration of the calcimimetic R-568 resulted in increased VDR expression in parathyroid tissue. *In vitro* studies of the effect of R-568 on VDR mRNA and protein were conducted in cultures of whole rat parathyroid glands. Incubation of rat parathyroid glands *in vitro* with R-568 resulted in a dose-dependent decrease in PTH secretion and an increase in VDR expression. Together with previous work on the effect of extracellular calcium to increase parathyroid VDR mRNA *in vitro* (Garfia *et al.*, 2002), they concluded that activation of the CaR upregulates the parathyroid VDR mRNA.

All these studies show that $1,25(OH)_2D_3$, and calcium in certain circumstances, increases the expression of the VDR gene in the parathyroid gland, which would result in increased VDR protein synthesis and increased binding of $1,25(OH)_2D_3$. This ligand-dependent receptor upregulation would lead to an amplified effect of $1,25(OH)_2D_3$ on the PTH gene and might help explain the dramatic effect of $1,25(OH)_2D_3$ on the PTH gene.

Vitamin D may also amplify its effect on the parathyroid by increasing the activity of the calcium receptor (CaR). Canaff and Hendy (2002) showed that, in fact, there are VDREs in the human CaR promoter. The calcium-sensing receptor (CaR), expressed in parathyroid chief cells, thyroid C-cells, and cells of the kidney tubule, is essential for maintenance of calcium homeostasis. They showed that parathyroid, thyroid, and kidney CaR mRNA levels increased 2-fold at 15 hours after intraperitoneal injection of $1,25(OH)_2D_3$ in rats. Human thyroid C-cell (TT) and kidney proximal tubule cell (HKC) CaR gene transcription increased approximately 2-fold at 8 and 12 hours after $1,25(OH)_2D_3$ treatment. The human CaR gene has two promoters yielding alternative transcripts containing either exon 1A or exon 1B 5′-untranslated region sequences that splice to exon 2 some 242 bp before the ATG translation start site. Transcriptional start sites were identified in parathyroid gland and TT cells; the transcriptional start site for promoter P1 lies 27 bp downstream of a TATA box, whereas that for promoter P2, which lacks a TATA box, lies in a GC-rich region. In HKC cells, transcriptional activity of a P1 reporter gene construct was 11-fold and of P2 was 33-fold above basal levels. $1,25(OH)_2D_3$ (10^{-8}M) stimulated P1 activity 2-fold and P2 activity 2.5-fold. Vitamin D response elements (VDREs), in which half-sites (6 bp) are separated by three nucleotides, were identified in both promoters and shown to confer $1,25(OH)_2D_3$ responsiveness to a heterologous promoter. This responsiveness was lost when the VDREs were mutated. In electrophoretic mobility shift assays with either *in vitro* transcribed/translated vitamin D receptor and retinoid X receptor-alpha, or HKC nuclear extract, specific protein–DNA complexes were formed in the presence of $1,25(OH)_2D_3$ on oligonucleotides representing the P1 and P2 VDREs. In summary, functional VDREs have been identified in the CaR gene and probably provide the mechanism whereby $1,25(OH)_2D_3$ upregulates parathyroid, thyroid C-cell, and kidney CaSR expression.

The use of calcitriol is limited by its hypercalcemic effect, and therefore a number of calcitriol analogs have been synthesized that are biologically active but are less hypercalcemic than calcitriol (Brown, 2005). These analogs usually involve modifications of the calcitriol side chain, such as 22-oxa-$1,25(OH)_2D_3$, which is the chemical modification in oxacacitriol (Nishii *et al.*, 1991), or a cyclopropyl group at the end of the side chain in calcipotriol (Kissmeyer and Binderup, 1991; Evans *et al.*, 1991). Brown *et al.* (1989) showed that oxacalcitriol *in vitro* decreased PTH secretion from primary cultures of bovine parathyroid cells with a similar dose response to that of calcitriol. *In vivo* the injection of both vitamin D compounds led to a decrease in rat parathyroid PTH mRNA levels (Brown *et al.*, 1989). However, detailed *in vivo* dose–response studies showed that *in vivo* calcitriol is the most effective analog for decreasing PTH mRNA levels, even at doses that do not cause hypercalcemia (Naveh-Many and Silver, 1993). Oxacalcitriol and calcipotriol are less effective for decreasing PTH RNA levels but have a wider dose range at which they do not cause hypercalcemia; this property might be useful clinically. The marked activity of calcitriol analogs *in vitro* compared with their modest hypercalcemic actions *in vivo* probably reflects their rapid clearance from the circulation (Bouillon *et al.*, 1991). There is much interest in the development and marketing of new calcitriol analogs to decrease PTH gene expression and serum PTH levels without causing hypercalcemia, but there have been few rigorous comparisons of their biological effects compared with those of calcitriol itself (Brown, 1998; Verstuyf *et al.*, 1998). There has been a lot of debate about the relative advantages of the so-called "nonhypercalcemic" vitamin D analogs over $1,25(OH)_2$ vitamin D_3. Drueke (2005) has analyzed the clinical trials that have been performed and concluded that all clinical studies were retrospective in nature and suffered from the limitations of retrospective data analysis. The question is still open.

However, the ability of calcitriol to decrease PTH gene transcription is used therapeutically in the management of patients with chronic renal failure. They are treated with calcitriol, or its prodrug 1α(OH)-vitamin D_3 in order to prevent the secondary hyperparathyroidism of chronic renal failure. The poor response in some patients may well result from poor control of serum phosphate, decreased vitamin D receptor concentration in the patients' parathyroids (Fukuda *et al.*, 1993), an inhibitory effect of a uremic toxin(s) on VDR-VDRE binding (Patel and Rosenthal, 1985), or tertiary hyperparathyroidism with monoclonal parathyroid tumors (Arnold *et al.*, 1995).

Calreticulin and the Action of $1,25(OH)_2D_3$ on the PTH Gene

Another possible level at which $1,25(OH)_2$ vitamin D_3 might regulate PTH gene expression involves calreticulin. Calreticulin is a calcium-binding protein present in the endoplasmic reticulum of the cell and may also have a nuclear function. It regulates gene transcription via its ability to bind a protein motif in the DNA-binding domain of nuclear hormone receptors of sterol hormones. It has been shown to prevent vitamin D's binding and action on the osteocalcin gene *in vitro* (Wheeler *et al.*, 1995). Sela-Brown *et al.* (1998) showed that calreticulin might inhibit the action of vitamin D on the PTH gene. Both rat and chicken VDRE sequences of the PTH gene were incubated with recombinant VDR and retinoic acid receptor (RXR) proteins in a gel retardation assay and showed a clear retarded band. Purified calreticulin inhibited binding of the VDR-RXR complex to the VDREs in gel retardation assays. This inhibition was caused by direct protein–protein interactions between VDR and calreticulin. OK cells were transiently cotransfected with calreticulin expression vectors and either rat or chicken PTH gene promoter-CAT constructs. The cells were then assayed for $1,25(OH)_2D_3$-induced CAT gene expression. $1,25(OH)_2D_3$ decreased PTH promoter-CAT transcription. Cotransfection with sense calreticulin, which increases calreticulin protein levels, completely inhibited the effect of $1,25(OH)_2D_3$ on the PTH promoters of both rat and chicken. Cotransfection with the antisense calreticulin construct did not interfere with the effect of vitamin D on PTH gene transcription. Calreticulin expression had no effect on basal CAT mRNA levels. In order to determine a physiological role for calreticulin in regulation of the PTH gene, levels of calreticulin protein were determined in the nuclear fraction of rat parathyroids. The rats were fed either a control diet or a low calcium diet, which leads to increased PTH mRNA levels, despite high serum $1,25(OH)_2D_3$ levels that would be expected to inhibit PTH gene transcription (Sela-Brown *et al.*, 1998). It was postulated that high calreticulin levels in the nuclear fraction would prevent the effect of $1,25(OH)_2D_3$ on the PTH gene. In fact, hypocalcemic rats had increased levels of calreticulin protein, as measured by Western blots, in their parathyroid nuclear faction. This may help explain why hypocalcemia leads to increased PTH gene expression, despite high serum $1,25(OH)_2D_3$ levels, and may also be relevant to the refractoriness of the secondary hyperparathyroidism of many chronic renal failure patients to $1,25(OH)_2D_3$ treatment. These studies, therefore, indicate a role for calreticulin in regulating the effect of vitamin D on the PTH gene and suggest a physiological relevance to these studies (Sela-Brown *et al.*, 1998).

Calcium

In Vitro Studies

A remarkable characteristic of the parathyroid is its sensitivity to small changes in serum calcium, which leads to large changes in PTH secretion. This remarkable sensitivity of the parathyroid to increase hormone secretion after small decreases in serum calcium levels is unique to the parathyroid. All other endocrine glands increase hormone secretion after exposure to a high extracellular calcium. This calcium sensing is also expressed at the levels of PTH gene expression and parathyroid cell proliferation. *In vitro* and *in vivo* data agree that calcium regulates PTH mRNA levels, but data differ in important ways. *In vitro* studies with bovine parathyroid cells in primary culture showed that calcium regulated PTH mRNA levels (Russell *et al.*, 1983; Brookman *et al.*, 1986), with an effect mainly of high calcium to decrease PTH mRNA. These effects were most pronounced after more prolonged incubations, such as 72 hours. The physiological correlates of these studies in tissue culture are hard to ascertain, because the parathyroid calcium sensor might well have decreased over the time period of the experiment (Mithal *et al.*, 1995). This may explain why the dose response differs from *in vivo* data, but the dramatic difference in time course suggests that *in vivo* data reflect something not seen in cultured cells.

In Vivo Studies

Calcium and phosphate both have marked effects on the levels of PTH mRNA *in vivo*. The major effect is for low calcium to increase PTH mRNA levels and low phosphate to decrease PTH mRNA levels (Fig. 1). Naveh-Many *et al.* (1989) studied rats *in vivo*. They showed that a small decrease in serum calcium from 2.6 to 2.1 mmol/L led to large increases in PTH mRNA levels, reaching threefold that of controls at 1 and 6 hours. A high serum calcium had no effect on PTH mRNA levels even at concentrations as high as 6.0 mmol/L. Interestingly, in these same thyroparathyroid tissue RNA extracts, calcium had no effect on the expression of the calcitonin gene (Naveh-Many, 1989; Naveh-Many *et al.*, 1992b). Thus, whereas a high calcium is a secretagogue for calcitonin, it does not regulate calcitonin gene expression. Yamamoto *et al.* (1989) also studied the *in vivo* effect of calcium on PTH mRNA levels in rats. They showed that hypocalcemia induced by a calcitonin infusion for 48 hours led to a sevenfold increase in PTH mRNA levels. Rats made hypercalcemic (2.9–3.4 mM) for 48 hours had the same PTH mRNA levels as controls that had received no infusion (2.5 mM); these levels were modestly lower than those found in rats that had received a calcium-free infusion. In further studies, Naveh-Many *et al.* (1992b) transplanted Walker carcinosarcoma 256

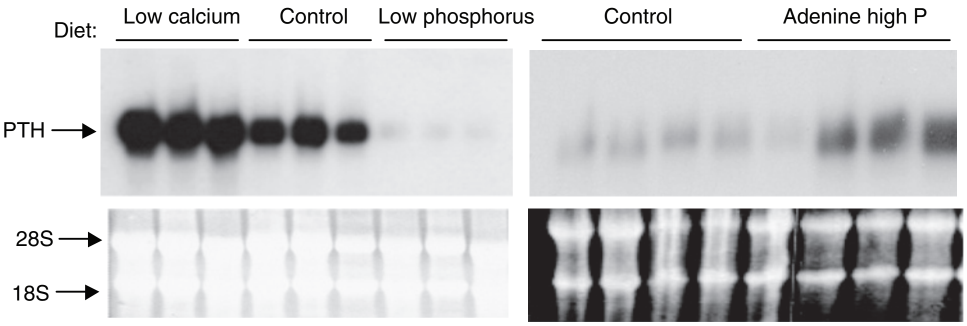

FIGURE 1 The effect of dietary phosphorus and calcium depletion and adenine high phosphorus-induced renal failure on PTH mRNA levels. PTH mRNA levels are shown for individual weanling rats fed diets containing low calcium, control, or low phosphorus, and mature rats fed control or adenine-high phosphorus.

cells into rats. Serum calciums increased to 18 mg/dL at day 10 after transplantation. There was no change in PTH mRNA levels in the rats with marked chronic hypercalcemia (Naveh-Many *et al.*, 1992b). Differences between *in vivo* and *in vitro* results probably reflect the instability of the *in vitro* system, but it is also impossible to eliminate the possibility that *in vivo* effects are influenced by indirect effects of a high or low serum calcium or by other variables changed in the *in vivo* protocols. Nevertheless, the physiological conclusion is that common causes of hypercalcemia *in vivo* do not importantly decrease PTH mRNA levels; these results emphasize that the gland is geared to respond to hypocalcemia and not hypercalcemia.

Mechanisms of Regulation of PTH mRNA by Calcium

The mechanism whereby calcium regulates PTH gene expression is particularly interesting. Changes in extracellular calcium are sensed by a calcium sensor that then regulates PTH secretion (Brown *et al.*, 1993; Yano and Brown, 2005). Signal transduction from the CaSR involves activation of phospholipase C, D, and A_2 enzymes (Kifor *et al.*, 1997). It is not known what mechanism transduces the message of changes in extracellular calcium leading to changes in PTH mRNA. However, it has been shown that the response to changes in serum calcium involve the protein phosphatase type 2B, calcineurin (Bell *et al.*, 2005a). *In vivo* and *in vitro* studies demonstrated that inhibition of calcineurin by genetic manipulation or pharmacological agents affected the response of PTH mRNA levels to changes in extracellular calcium (Bell *et al.*, 2005a).

Okazaki *et al.* (1992) identified a negative calcium regulatory element (nCaRE) in the atrial natiuretic peptide gene, with a homologous sequence in the PTH gene. They identified a redox factor protein (ref1), which bound a putative nCaRE, and the level of ref1 mRNA and protein were elevated by an increase in extracellular calcium concentration (Okazaki *et al.*, 1992). They suggested that ref1 had transcription repressor activity in addition to its function as a transcriptional auxiliary protein (Okazaki *et al.*, 1992). Because no parathyroid cell line is available, these studies were performed in nonparathyroid cell lines, so their relevance to physiological PTH gene regulation remains to be established.

Moallem *et al.* (1998) have performed *in vivo* studies on the effect of hypocalcemia on PTH gene expression. The effect is post-transcriptional *in vivo* and involves protein–RNA interactions at the 3′-untranslated region of the PTH mRNA (Moallem *et al.*, 1998). A similar mechanism is involved in the effect of phosphate on PTH gene expression so the mechanisms involved will be discussed after the independent effect of phosphate on the PT is considered.

Phosphate

Phosphate Regulates the Parathyroid Independently of Calcium and 1,25(OH)$_2$D$_3$

The demonstration of a direct effect of high phosphate on the parathyroid *in vivo* has been difficult. One of the reasons is that the various maneuvers used to increase or decrease serum phosphate invariably leads to a change in the ionized calcium concentration. In moderate renal failure, phosphate clearance decreases and serum phosphate increases; this increase becomes an important problem in severe renal failure. Hyperphosphatemia has always been considered central to the pathogenesis of secondary hyperparathyroidism, but it has been difficult to separate the effects of hyperphosphatemia from those of the attendant hypocalcemia and decrease in serum $1,25(OH)_2D_3$ levels. In the 1970s, Slatopolsky and Bricker (1973) showed in dogs with experimental chronic renal failure that dietary phosphate restriction prevented secondary hyperparathyroidism. Clinical studies (Portale *et al.*, 1984) demonstrated that phosphate restriction in patients with chronic renal insufficiency is effective in preventing the increase in serum PTH levels (Lucas *et al.*, 1986; Portale *et al.*, 1984; Lafage *et al.*, 1992; Aparicio *et al.*, 1994; Combe and Aparicio, 1994). The mechanism of this effect was not clear, although at least part of it was considered to be caused by changes in serum $1,25(OH)_2D_3$ concentrations. *In vitro* (Tanaka and DeLuca, 1973; Condamine *et al.*, 1994) and *in vivo* (Portale *et al.*, 1984, 1989) phosphate directly regulated the production of $1,25(OH)_2D_3$. A raised serum phosphate decreases serum $1,25(OH)_2D_3$ levels, which then leads to decreased calcium absorption from the diet and eventually a low serum calcium. The raised phosphate complexes calcium, which is then deposited in bone and soft tissues and decreases serum calcium. However, a number of careful clinical and experimental studies suggested that the effect of phosphate on serum PTH levels was independent of changes in both serum calcium and $1,25(OH)_2D_3$ levels. In dogs with experimental chronic renal failure, Lopez-Hilker *et al.* (1990) showed that phosphate restriction corrected their secondary hyperparathyroidism independent of changes in serum calcium and $1,25(OH)_2D_3$ levels. They did this by placing the uremic dogs on diets deficient in both calcium and phosphate. This led to lower levels of serum phosphate and calcium, with no increase in the low levels of serum $1,25(OH)_2D_3$. Despite this, there was a 70% decrease in PTH levels. This study suggested that, at least in chronic renal failure, phosphate affected the parathyroid cell by a mechanism independent of its effect on serum $1,25(OH)_2D_3$ and calcium levels (Lopez-Hilker *et al.*, 1990). Therefore, phosphate plays a central role in the pathogenesis of secondary hyperparathyroidism, both by its effect on serum $1,25(OH)_2D_3$ and calcium levels and possibly independently. A raised serum phosphate also stimulates the secretion of FGF23, which in turn decreases PTH gene expression and serum PTH levels (T.N.-M. and J.S., work in progress). This effect would act as a counterbalance to the stimulatory effect of phosphate on the parathyroid and is discussed separately in this chapter.

Kilav *et al.* (1995) were the first to establish *in vivo* that the effects of serum phosphate on PTH gene expression and serum PTH levels were independent of any changes in serum calcium or $1,25(OH)_2D_3$. In a particularly informative experiment, they bred second-generation vitamin D-deficient rats and then placed the weanling vitamin D-deficient rats on a diet with no vitamin D, low calcium, and low phosphate. After one night of this diet, serum phosphate had decreased markedly with no changes in serum calcium or $1,25(OH)_2D_3$. These rats with isolated hypophosphatemia had marked decreases in PTH mRNA levels and serum PTH. However, the very low serum phosphates in these *in vivo* studies may have no direct relevance to possible direct effects of high phosphate in renal failure. It is necessary to separate nonspecific effects of very low phosphate from true physiological regulation. To establish that the effect of serum phosphate on the parathyroid was indeed a direct effect, *in vitro* confirmation was needed, which was provided by three groups. Rodriguez was the first to show that increased phosphate levels increased PTH secretion from isolated parathyroid glands *in vitro*; the effect required maintenance of tissue architecture (Almaden *et al.*, 1996). The effect was found in whole glands or tissue slices but not in isolated cells. This result was confirmed by Slatopolsky *et al.* (1996). Olgaard's laboratory provided elegant further evidence of the importance of cell–cell communication in mediating the effect of phosphate on PTH secretion (Nielsen *et al.*, 1996). The requirement for intact tissue suggests either that the sensing mechanism for phosphate is damaged during the preparation of isolated cells or that the intact gland structure is important to the phosphate response.

Parathyroid responds to changes in serum phosphate at the level of secretion, gene expression, and cell proliferation, although the mechanism of these effects is unknown. The effect of high phosphate to increase PTH secretion may be mediated by phospholipase A_2-activated signal transduction. Bourdeau *et al.* (1992, 1994) showed that arachidonic acid and its metabolites inhibit PTH secretion. Almaden *et al.* (2000) showed *in vitro* that a high phosphate medium increased PTH secretion, which was prevented by the addition of arachidonic acid. When dog parathyroid tissue was cultured in a high calcium and normal phosphate medium, there was an increase in arachidonic acid production at 30 and 45 min, returning to baseline at 60 min. A high phosphate medium prevented the increase in arachidonic acid production at 30 and 45 min, and there was a modest increase in PTH secretion only after 2 and 3 hours of incubation. These results suggest that phosphate decreases the production of arachidonic acid in the parathyroid and that arachidonic acid decreases PTH secretion, but it is less clear to what extent the effect of phosphate on PTH secretion depends on this pathway. The use of inhibitors of the phospholipase A_2 pathway may help clarify this question. We can now provide some of the answers for the effect of phosphate and calcium on PTH gene expression.

Protein–PTH mRNA Interactions Determine the Regulation of PTH Gene Expression by Serum Calcium and Phosphate

The clearest rat *in vivo* models for effects of calcium and phosphate on PTH gene expression are diet-induced hypocalcemia with a large increase in PTH mRNA levels and diet-induced hypophosphatemia with a large decrease in PTH mRNA levels (Fig. 1). In both instances the effect was post-transcriptional, as shown by nuclear transcript run-on experiments (Kilav *et al.*, 2005). Parathyroid cytosolic proteins were found to bind *in vitro*-transcribed PTH mRNA, with three bands at about 50, 60, and 110 kDa (Moallem *et al.*, 1998). Interestingly, this binding was increased with parathyroid proteins from hypocalcemic rats (with increased PTH mRNA levels) and decreased with parathyroid proteins from hypophosphatemic rats (with decreased PTH mRNA levels). Proteins from other tissues bound to PTH mRNA, but this binding is regulated by calcium and phosphate only with parathyroid proteins. Intriguingly, binding requires the presence of the terminal 60 nucleotides of the PTH transcript.

PTH mRNA is Degraded *in vitro* by Parathyroid Cytosolic Proteins, which are Regulated by Calcium and Phosphate

Naveh-Many and colleagues utilized an *in vitro* degradation assay to study the effects of hypocalcemic and hypophosphatemic parathyroid proteins on PTH mRNA stability (Moallem *et al.*, 1998). In this assay, control rats' parathyroid cytosolic proteins led to the degradation of a radiolabeled PTH transcript in about 40–60 min (Fig. 2). Hypocalcemic parathyroid proteins degraded the transcript only in 180 min, whereas hypophosphatemic parathyroid proteins degraded the transcript within 5 min. Moreover, the rapid degradation of PTH mRNA by hypophosphatemic proteins was totally dependent on an intact 3′-untranslated region (UTR) and, in particular, on the terminal 60 nucleotides. Proteins from other tissues in these rats were not regulated by calcium or phosphate. Therefore, calcium and phosphate change the properties of parathyroid cytosolic proteins, which bind specifically to the PTH mRNA 3′-UTR and determine its stability. What are these proteins?

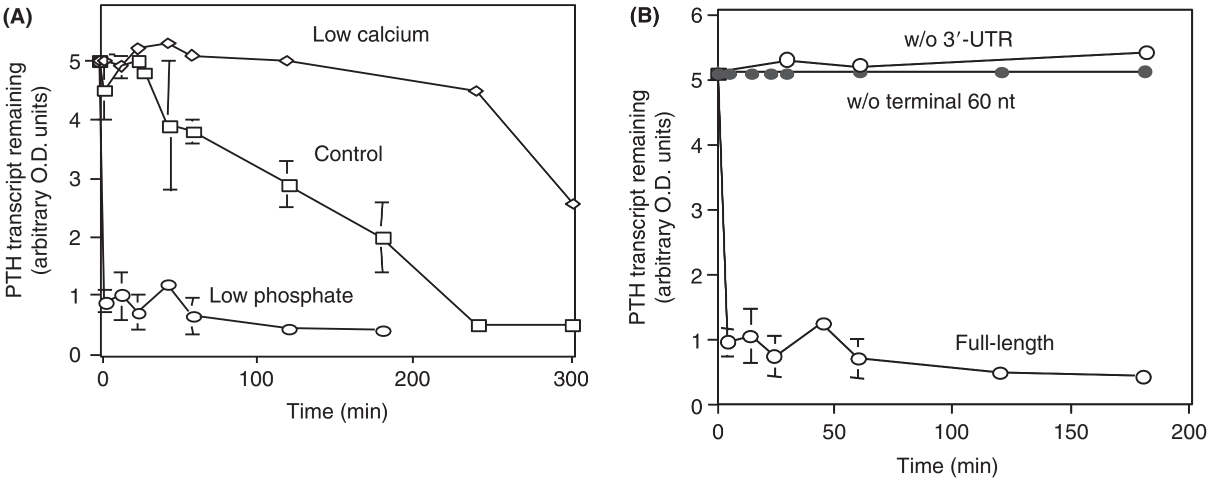

FIGURE 2 *In vitro* degradation of PTH mRNA by parathyroid cytosolic proteins. **(A)** Time–response curves of intact full-length PTH mRNA after incubation with parathyroid cytosolic proteins. Each point represents the mean±SE of three to four different experiments. At some points the SE is less than the size of the graphic symbols. The PTH transcript was degraded very rapidly by proteins from −P rats and remained intact for a longer time period with proteins from −Ca rats. **(B)** Mapping a region in the PTH 3′-UTR that mediates degradation by proteins from −P rats. PTH mRNA probes used were intact PTH mRNA (probe A), without the 3′-UTR (probe C), and without the 3′-terminal 60 nucleotides of the 3′-UTR (probe B). Reproduced with permission from Moallem *et al.* (1998).

Identification of the PTH mRNA 3′-UTR-Binding Proteins that Determines PTH mRNA Stability

AU-rich Binding Factor (AUF1)

Sela-Brown *et al.* (2000) and Dinur *et al.* (2006) utilized affinity chromatography to isolate these RNA-binding proteins.. The proteins, which bind the PTH mRNA, are also present in other tissues, such as brain, but only in the parathyroid is their binding regulated by calcium and phosphate. The PTH RNA 3′-UTR-binding proteins were purified by PTH 3′-region RNA affinity chromatography of rat brain S-100 extracts. The eluate from the column was enriched in PTH RNA 3′-UTR-binding activity. Addition of eluate to the *in vitro* degradation assay with parathyroid protein extracts stabilized the PTH transcript. A major band from the eluate at 50 kDa was sequenced and was identical to AU-rich binding factor (AUF1) (Sela-Brown *et al.*, 2000). Recombinant AUF1 bound the full-length PTH mRNA and the 3′-UTR. Added recombinant AUF1 also stabilized the PTH transcript in the *in vitro* degradation assay. These results showed that AUF1 is a protein that binds to the PTH mRNA 3′-UTR and stabilizes the PTH transcript.

The regulation of protein–PTH mRNA binding involves post-translational modification of AUF1. AUF1 levels are not regulated in PT extracts from rats fed calcium- and phosphorus-depleted diets. However, two-dimensional gels showed post-translational modification of AUF1 that included phosphorylation (Bell *et al.*, 2005a). There is no parathyroid cell line, but a PTH mRNA *cis*-acting 63-nt element (Kilav *et al.*, 2001) is recognized in HEK 293 cells as an instability element (Fig. 3). RNA interference for AUF1 decreased human PTH secretion in cotransfection experiments (Bell *et al.*, 2005a). Stably transfected cells with a chimeric GH gene containing the PTH 63-nt *cis*-acting element were used to study the signal transduction pathway that regulates AUF1 modification and chimeric gene mRNA stability. Cyclosporine A, the calcineurin inhibitor, regulated AUF1 post-translationally, and this correlated with an increase in the stability of GH-PTH 63-nt mRNA but not of the control GH mRNA. Mice with genetic deletion of the calcineurin Aβ gene had markedly increased PTH mRNA levels that were still regulated by low-calcium and -phosphorus diets. Therefore, calcineurin regulates AUF1 post-translationally *in vitro* and PTH gene expression *in vivo* but still allows its physiological regulation by calcium and phosphate (Bell *et al.*, 2005a).

Most patients with chronic kidney disease develop secondary hyperparathyroidism with disabling systemic complications. Calcimimetic agents are effective tools in the management of secondary hyperparathyroidism, acting through allosteric modification of the calcium-sensing receptor (CaR) on the parathyroid gland to decrease PTH secretion and parathyroid cell proliferation. R-568 decreased both PTH mRNA and serum PTH levels in adenine high-P-induced uremia (Levi *et al.*, 2006). The effect of the calcimimetic on PTH gene expression was post-transcriptional and correlated with differences in protein–RNA binding and post-translational modifications of the *trans*-acting factor AUF1 in the parathyroid. The AUF1 modifications as a result of uremia were reversed to those of normal rats by treatment with R-568. Therefore, uremia and activation of the CaR mediated by calcimimetics modify AUF1 post-translationally. These modifications in AUF1 correlate with changes in protein–PTH mRNA binding and PTH mRNA levels (Levi *et al.*, 2006).

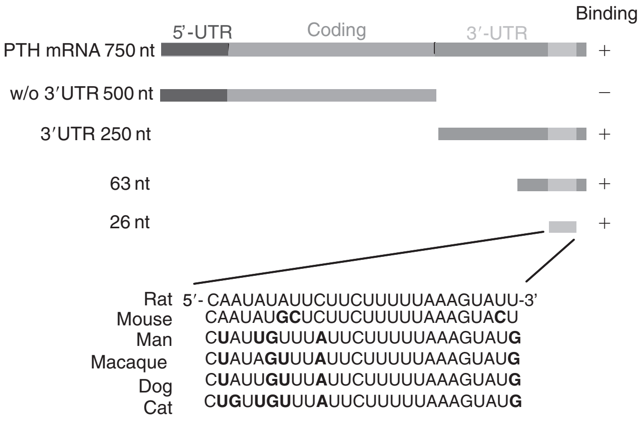
Binding
5′-UTR
Coding
3′-UTR
PTH mRNA 750 nt
+
w/o 3′UTR 500 nt
–
3′UTR 250 nt
+
63 nt
+
26 nt
+
Rat 5′- CAAUAUAUUCUUCUUUUUAAAGUAUU-3’
Mouse CAAUAUGCUCUUCUUUUUAAAGUACU
Man CUAUUGUUUAUUCUUUUUAAAGUAUG
Macaque CUAUAGUUUAUUCUUUUUAAAGUAUG
Dog CUAUUGUUUAUUCUUUUUAAAGUAUG
Cat CUGUUGUUUAUUCUUUUUAAAGUAUG

FIGURE 3 Schematic representation of the rat PTH mRNA and the sequence of the PTH mRNA protein-binding *cis*-acting element in the PTH mRNA 3′-UTR in different species. The full-length PTH mRNA and transcripts for different regions of PTH mRNA were analyzed for binding with parathyroid cytosolic proteins by UV and REMSA and their binding summarized by +/ − . The 26-nt minimal proximal *cis*-acting element of the PTH mRNA 3′-UTR is indicated and its sequence in different species is shown. The nucleotides that are different from the rat sequence are shown in bold.

Unr (Upstream of N-Ras)

A second parathyroid cytosolic protein that is part of the stabilizing PTH mRNA 3′-UTR-binding complex was shown to be Unr by affinity chromatography (Dinur *et al.*, 2006). Recombinant Unr bound the PTH 3′-UTR transcript and supershift experiments with antibodies to Unr showed that Unr is part of the parathyroid RNA-binding complex. Depletion of Unr by small interfering RNA decreased PTH mRNA levels in HEK293 cells transiently cotransfected with the human PTH gene. Overexpression of Unr increased the rat full-length PTH mRNA levels but not a PTH mRNA lacking the terminal 60-nucleotide *cis*-acting protein-binding region. Therefore, Unr binds to the PTH *cis*-element and increases PTH mRNA levels, as does AUF1. Unr, together with the other proteins in the RNA-binding complex, determines PTH mRNA stability (Dinur *et al.*, 2006). Recent findings have identified an additional decay-promoting protein that differentially interacts with PTH mRNA to recruit the degradatory machinery (T.N.-M., manuscript in preparation). The balance between the stabilizing and destabilizing proteins determines PTH mRNA levels in response to physiological stimuli (Fig. 4).

A Conserved Sequence in PTH mRNA 3′-UTR Binds Parathyroid Cytosolic Proteins and Determines RNA Stability in Response to Changes in Calcium and Phosphate

Kilav *et al.* (2001) have identified the minimal sequence for protein binding in the PTH mRNA 3′-UTR and determined its functionality. A minimum sequence of 26 nucleotides was sufficient for PTH RNA–protein binding and competition (see Figure 3). Significantly, this sequence was preserved among species (Bell *et al.*, 2005b). To study the functionality of the sequence in the context of another RNA, a 63-bp cDNA PTH sequence consisting of the 26 nucleotide and flanking regions was fused to growth hormone (GH) cDNA. The conserved PTH RNA protein-binding region was necessary and sufficient for responsiveness to calcium and phosphate and determines PTH mRNA stability and levels (Kilav *et al.*, 2001) (see Fig. 2).

The PTH mRNA 3′-UTR binding element is AU rich and is a type III AU-rich element (ARE). Sequence analysis of the PTH mRNA 3′-UTR of different species revealed a preservation of the 26-nt protein-binding element in rat, murine, human, macaque, feline, and canine 3′-UTRs (Bell *et al.*, 2005b). In contrast to protein-coding sequences that are highly conserved, UTRs are less conserved. The conservation of the protein-binding element in the PTH mRNA 3′-UTR suggests that this element represents a functional unit that has been evolutionarily conserved. The *cis*-acting element is at the 3′ distal end in all species where it is expressed. This element is not present in the 3′-UTRs of bovine, porcine, and gallus PTH mRNA. Protein–RNA-binding experiments using bovine PT gland extracts and RNA probes for different regions of the bovine PTH mRNA 3′-UTR identified a protein-binding element in bovine PTH mRNA at the proximal end of the 3′-UTR that is different from the distal protein-binding element. The human 3′-UTR contains both elements, but only the distal element binds proteins (Bell *et al.*, 2005b). Functional studies with HEK293 cells transiently transfected with reporter genes containing the different elements showed that the human distal, but not the proximal element, destabilized a reporter mRNA similarly to the effect of this element in the rat. A reporter mRNA containing the single bovine PTH mRNA protein-binding element was also destabilized in transfected cells (Bell *et al.*, 2005b). Therefore, different species have alternative protein-binding elements involved in the regulation of PTH mRNA stability.

Dynein Light Chain (M_r 8000) Binds the PTH mRNA 3′-Untranslated Region and Mediates its Association with Microtubules

To isolate other proteins binding to the 3′-UTR of parathyroid hormone, mRNA expression cloning was used. Epstein *et al.* (2000) screened an expression library for proteins that bound the PTH mRNA 3′-UTR, and the sequence of one clone was identical to dynein light chain (M_r 8000) (LC8). LC8 is part of the cytoplasmic dynein complexes that function as molecular motors that translocate along microtubules. Recombinant LC8 bound PTH

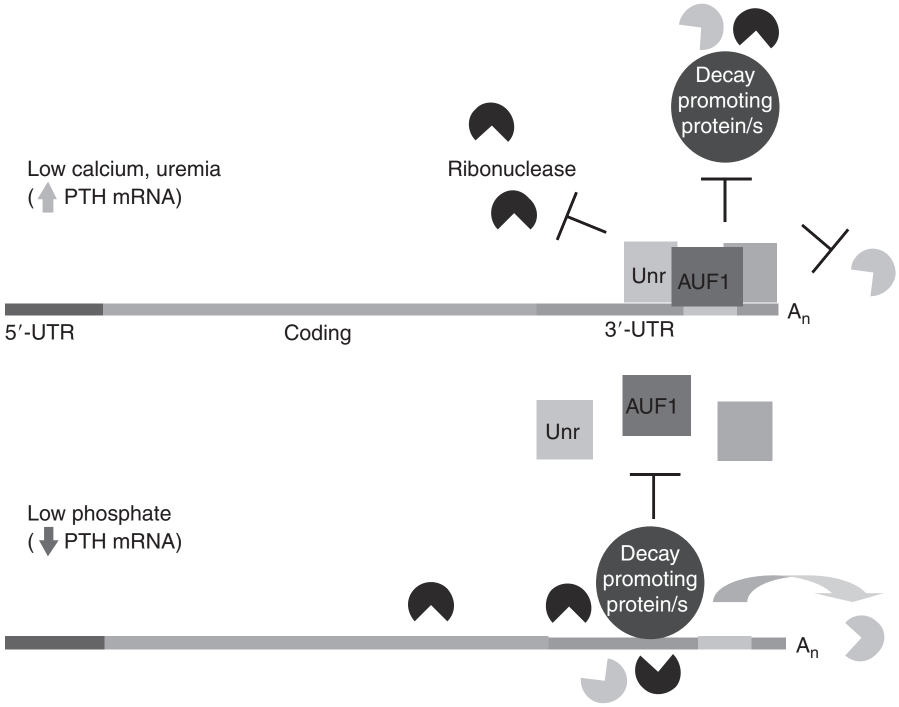

FIGURE 4 Model for the regulation of PTH mRNA stability by calcium and phosphorus depletion. Low P decreases and low Ca increases PTH mRNA stability and levels *in vivo*. Low Ca^{2+} induces the binding of a stabilizing protein complex consisting of AUF1 and Unr, and possibly other unidentified proteins to a defined cis element in the PTH mRNA 3′-UTR and this involves post-translational modifications of AUF1. This complex competes for the binding of decay-promoting proteins and thereby inhibits PTH mRNA degradation leading to increased PTH mRNA stability and levels. Low P increases the association of PTH mRNA with decay-promoting proteins. These proteins then recruit the degradation machinery, consisting of ribonucleases to decrease PTH mRNA stability and levels. The decay-promoting protein KSRP, has recently been identified as a regulator of PTH mRNA decay (Nechama et al., 2008). The cleavage mechanism of PTH mRNA remains to be added.

mRNA 3′-UTR by the RNA mobility shift assay. PTH mRNA colocalized with polymerized microtubules in the parathyroid gland, as well as with a purified microtubule preparation from calf brain, and this was mediated by LC8. This was the first report of a dynein complex protein binding a mRNA. Therefore, the dynein complex may be the motor for the transport and localization of mRNAs in the cytoplasm and the subsequent asymmetric distribution of translated proteins in the cell.

Sex Steroids

PTH is anabolic to bone and is an effective means of treating postmenopausal osteoporosis (Dempster *et al.*, 1993; Finkelstein *et al.*, 1994). In postmenopausal women with osteoporosis, time series analysis has shown that there is a loss in the periodicity of PTH secretion (Prank *et al.*, 1995; Fraser *et al.*, 1998). This suggests that estrogens may have an effect on the parathyroid. Estradiol and progesterone both increased the secretion of PTH from bovine parathyroid cells in primary culture (Greenberg *et al.*, 1987). However, transdermal estrogen did not increase serum PTH levels in postmenopausal patients (Prince *et al.*, 1990). Estrogen receptors were not detected in parathyroid tissue by a hormone-binding method (Prince *et al.*, 1991), but were detected by immunohistochemistry and PCR for the estrogen receptor mRNA (Naveh-Many *et al.*, 1992a). In *in vivo* ovariectomized rats, both estrogen and progestins regulated PTH gene expression. Estrogen receptor mRNA and protein were shown to be present in the parathyroid suggesting that these are target organs for estrogen.

Further studies were performed on the effect of progestins on PTH gene expression (Epstein *et al.*, 1995). The 19-nor progestin R5020 given to weanling rats or mature ovariectomized rats led to a twofold increase in thyroparathyroid PTH mRNA levels. In addition, *in vitro*, in primary cultures of bovine parathyroid cells, progesterone increased PTH mRNA levels. The progesterone receptor (PR) mRNA was demonstrated in rat parathyroid tissue by *in situ* hybridization and in human parathyroid adenoma by immunohistochemistry. Changes in PTH mRNA levels during the rat estrous cycle were also studied. At proestrus and estrus, PTH mRNA levels were increased significantly by three- and fourfold compared with diestrus (Epstein *et al.*, 1995). These results confirm that the parathyroid gland is a target organ for the ovarian sex steroids estrogen and progesterone and are of physiological relevance, as shown by the changes during estrus.

FIBROBLAST GROWTH FACTOR 23 AND THE PARATHYROID

Phosphate homeostasis is maintained by a counterbalance between efflux from the kidney and influx from intestine and bone. Fibroblast growth factor-23 (FGF23) is a bone-derived phosphaturic hormone that acts on the kidney to increase phosphate excretion and suppress biosynthesis of 1,25$(OH)_2$ vitamin D. FGF23 signals through fibroblast growth factor receptors (FGFRs) bound by the transmembrane protein Klotho (Kurosu *et al.*, 2006). Because most tissues express FGFRs, expression of Klotho virtually determines FGF23 target organs. Takeshita *et al.* (2004) were the first to show that Klotho protein is expressed not only in the kidney but also in the parathyroid, pituitary, and sino-atrial node. In addition, Urakawa *et al.* (2006) injected rats with FGF23 and demonstrated increased Egr-1 (early growth response gene-1) mRNA levels in the parathyroid, suggesting that the parathyroid may be a further FGF23 target organ. Imura *et al.* (2007) extended these observations with elegant studies showing that after challenge with a low extracellular Ca^{2+}, Klotho is necessary for the association of Na^+,K^+-ATPase with the plasma membrane. In addition, they showed that Klotho may have a role in the secretion of PTH. In ongoing studies on the functionality of the effect of FGF23 on the parathyroid, we have preliminary results showing that the administration of recombinant FGF23 led to an increase in parathyroid Klotho levels (T.N.-M. and J.S., unpublished work). Using *in vivo* and *in vitro* models, we show that FGF23 suppresses parathyroid hormone (PTH) gene expression and secretion. These data indicate that FGF23 acts directly on the parathyroid to decrease serum PTH. This novel bone–parathyroid endocrine axis adds a new dimension to the understanding of mineral homeostasis.

SUMMARY

The PTH gene is regulated by a number of factors. Calcitriol acts on the PTH gene to decrease its transcription, and this action is used in the management of patients with chronic kidney disease. The major effect of calcium on PTH gene expression *in vivo* is for hypocalcemia to increase PTH mRNA levels, and this is mainly post-transcriptional. Phosphate also regulates PTH gene expression *in vivo*, and this effect appears to be independent of the effect of phosphate on serum calcium and 1,25$(OH)_2D_3$. The effect of phosphate is also post-transcriptional. *Trans*-acting parathyroid cytosolic proteins bind to a defined *cis*-element in the PTH mRNA 3′-UTR. This binding determines the degradation of PTH mRNA by degrading enzymes and thereby PTH mRNA half-life. The post-transcriptional effects of calcium and phosphate are the result of changes in the balance of these stabilizing and degrading factors on PTH mRNA. These interactions also regulate PTH mRNA levels in experimental uremia. In diseases such as chronic renal failure, secondary hyperparathyroidism involves abnormalities in PTH secretion and synthesis. FGF23 also acts on its receptors, the Klotho-FGFR1, 2c, and 3c to decrease PTH mRNA levels and secretion. An understanding of how the parathyroid is regulated at each level will help devise rational therapy for the management of such conditions, as well as treatment for diseases, such as osteoporosis, in which alterations in PTH may have a role.

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Chapter 29

Parathyroid Hormone–Receptor Interactions

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INTRODUCTION

One impetus for elucidating the nature of parathyroid hormone (PTH) receptor − ligand interactions in atomic detail is the desire to design therapeutic agents for osteoporosis with characteristics superior to currently available PTH and related analogs. Such agents should be anabolic for bone and conveniently administered. To date, the only truly anabolic drug approved for treatment of osteoporosis is PTH-(1–34) (Neer *et al.*, 2001). Its mode of administration (subcutaneous injection) and side effects make it far from optimal as therapy. Therefore, the drive to understand structure − function − conformation relations of PTH-(1–34) and its cognate G protein-coupled receptor (GPCR), PTHR1, remains strong as a prerequisite to the design of improved analogs.

The first two parts of this chapter summarize our knowledge on the structure and function of the ligand, PTH-(1–34) (and sometimes PTHrP-(1–36)), and the receptor, PTHR1, respectively. The third part reviews newest approaches to integrated studies of the bimolecular ligand—PTHR1 complex. Significant advances in our understanding of GPCR activation combined with increasing knowledge on the diversity of signaling pathways for PTHR1 make for a fast pace in the flow of new data and new experimental approaches to study of the PTH − PTHR1 system.

THE LIGAND: STRUCTURE AND FUNCTION

Early work on the structure–activity relations of PTH and PTHrP has been covered in extensive reviews (Chorev and Rosenblatt, 1994, 1996; Potts *et al.*, 1997). It established that the N-terminal 1–34 amino acid sequence of both calciotropic hormones is sufficient to induce the entire spectrum of *in vitro* and *in vivo* PTHR1-mediated activities (Dempster *et al.*, 1993; Whitfield and Morley, 1995). Both PTH-(1–34) and PTHrP-(1–36) have similar affinities to PTHR1 and are equipotent in stimulating adenylyl cyclase and intracellular Ca^{2+} transients in cells expressing PTHR1. Significant sequence homology between PTH and PTHrP is limited to the first 13 residues, of which 8 are identical (Fig. 1). The molecular architecture consists of two functional domains. The "activation domain" is assigned to the homologous N-terminal sequences, and the principal "binding domain," located at the structurally divergent mid- and C-terminal sequences, is assigned to residues 14–34 (Abou-Samra *et al.*, 1989; Caulfield *et al.*, 1990; Gardella *et al.*, 1993; Nussbaum *et al.*, 1980). Truncation of 2–6 residues from the N terminus converts the 1–34/36 agonists into potent antagonists (Rosenblatt, 1986; Rosenblatt *et al.*, 1993). The functional similarity of two domains in two hormones that also share a common receptor has promoted the hypothesis that they have similar conformations, despite their limited sequence homology (Caulfield *et al.*, 1990; Mierke *et al.*, 1997).

The new challenges facing structural studies of PTH- and PTHrP-derived ligands originate from advances along several avenues of PTH-related biology. Subtype receptor multiplicity raises the need for receptor subtype- or target-specific ligands. Exploring the multiplicity of signaling pathways and their relevance to physiological and pathophysiological processes requires the development of signaling selective analogs. The concept that a series of receptor conformational states are induced by specific categories of ligand (agonist, antagonist, partial agonist, etc.) represents a new impetus to investigate the structure/activity relations and the pharmacology of PTH and PTHrP analogs. It implies that specific signaling and biological response can be obtained with the right ligand stabilizing the right receptor conformation (Kenakin, 2003). The design of a PTH-like drug with a better therapeutic window than PTH, i.e., a more favorable ratio of anabolic to catabolic activities, remains a goal of great interest.

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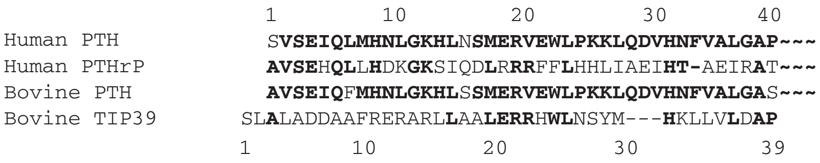

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                    1        10        20        30       40
Human PTH           SVSEIQLMHNLGKHLNSMERVEWLPKKLQDVHNFVALGAP~~~
Human PTHrP         AVSEHQLLHDKGKSIQDLRRRFFLHHLIAEIHT-AEIRAT~~~
Bovine PTH          AVSEIQFMHNLGKHLSSMERVEWLPKKLQDVHNFVALGAS~~~
Bovine TIP39      SLALADDAAFRERARLLAALERRHWLNSYM---HKLLVLDAP
                  1        10        20        30      39
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FIGURE 1 Alignment of human PTH-(1–40) with human PTHrP-(1–39), bovine PTH-(1–40), and bovine TIP39 sequences. Amino acid residues in bold indicate direct homology. Gaps in the sequences of PTHrP and TIP are introduced to maximize homology. Numbering at the top and bottom refers to mature PTH and mature TIP39, respectively. The wavy line indicates C-terminal truncated sequences. It can be appreciated that the highest homology observed is between human and bovine PTH. There is much less homology between human PTH and human PTHrP. The homology among TIP39 and PTH or PTHrP is very limited.

PROBING THE PRIMARY STRUCTURE

A wealth of data has been generated by exhaustively scanning either the entire sequence of PTH-(1−36) or portions thereof. These studies employ both synthetic and biosynthetic methodologies to generate new insights into the tolerance and significance of certain portions and residues with regard to bioactivity (Gardella *et al.*, 1991; Gombert *et al.*, 1995; Oldenburg *et al.*, 1996). Both Gardella and Oldenburg and their coworkers used recombinant DNA methodologies to generate analogs, either by randomly mutating codons coding for positions 1–4 in hPTH (Gardella *et al.*, 1991) or by replacing individual codons with [(A/G)(A/G)G] (coding for Lys, Arg, Glu, or Gly) (Oldenburg *et al.*, 1996). Conversely, Gombert *et al.* (1995) used a parallel multisynthesis approach to generate D-Ala, L-Ala, and D-Xxx scans of hPTH-(1–36), where D-Xxx is the enantiomeric form of the native amino acid residue in the particular position.

The D-Ala scan reveals segments 2–8 and 20–28 to be the least tolerant. Substitutions with D-Ala within these segments result in large decreases in binding affinity and are accompanied by a large loss in ligand-stimulated adenylyl cyclase activity. The latter is most pronounced for substitutions of Arg^{20}, Trp^{23}, Leu^{24}, and Leu^{28} in the sequence 20−28, which overlaps with the principal binding domain (Gombert *et al.*, 1995). A high correlation between binding and adenylyl cyclase activation was observed in the L-Ala scan. The largest loss in adenylyl cyclase activity occurs when the substitutions are within the activation domain (residues 2–8) (Gombert *et al.*, 1995). Only substituting L-Ala for Lys^{13}, Asn^{16}, or Glu^{19} yielded analogs that are slightly more active. The D-Xxx scan resulted in an overall loss of affinity and efficacy. The most affected region was the putative amphiphilic helical domain (residues 23–29) and, to a lesser extent, the C-terminal segment 32–36 (Gombert *et al.*, 1995).

Gardella *et al.* (1991) focused on the evolutionarily conserved, first four N-terminal residues in PTH. Residues Glu^{4} and Val^{2} are less tolerant to substitution than the other two positions, suggesting an important role in receptor binding and activation. The most intriguing finding of this study was the divergent activity displayed by [Arg^{2},Tyr^{34}]PTH-(1−34)NH$_2$ in two different cell lines expressing the wild-type PTHR1: ROS 17/2.8, a rat osteosarcoma cell line, and OK, an opossum kidney cell line (Gardella *et al.*, 1991). This analog is a weak partial agonist for stimulation of adenylyl cyclase in ROS 17/2.8 cells, whereas it is a full agonist for cAMP increases in the OK cell system (Gardella *et al.*, 1991). Gardella and coworkers (1994) further analyzed the activities of [Arg^{2},Tyr^{34}]PTH-(1–34)NH$_2$ in COS-7 cells transfected with rat or opossum PTHR1 or rat/opossum PTHR1 chimeras. They demonstrated that the differences in activity in ROS and OK cells are owing to differences in the interaction of the receptors (rat vs. opossum) with the amino terminus of the ligand and not to tissue-specific (bone vs. kidney) effects.

Cohen and coworkers (1991) reported that the highly conserved PTH and PTHrP residues, Ser^{3} and Gln^{6}, make important contributions to the binding and activation of PTHR1. Substituting of Phe or Tyr for Ser^{3}, and Phe or Ser for Gln^{6}, generates partial agonists. Both [Phe^{3}]hPTH-(1−34) and [Phe^{6}]hPTH-(1–34) competitively inhibit bPTH-(1−34)- and PTHrP-(1–34)-stimulated adenylyl cyclase activity. Specific substitutions within the “activation domain” may therefore convert full agonists into partial antagonists. The study also suggests that structural perturbation of ligand−receptor bimolecular interactions at the N-terminus of PTH-(1–34) can interfere preferentially with the induction of conformational changes, and therefore inhibit intracellular signaling. Such conformational changes are required for the effective coupling of the ligand-occupied receptor to G proteins, but are not important for ligand recognition and binding.

Oldenburg *et al.* (1996) conducted an extensive study in which they introduced single, nonconservative point mutations in PTH-(1–34) and characterized them in UMR106, rat osteosarcoma cells. These mutations, which span the mid- and C-terminal regions, namely, the 11–34 sequence, generated several analogs more potent than the parent peptide. One of the more interesting analogs is [Arg^{19,22,30},Lys^{29},Hse^{34}]hPTH-(1–34), which is equipotent to bPTH (EC$_{50}$ ~0.9 and K_d ~1.5 nM). The high potency of this analog is attributed to the presence and disposition of the seven positive charges in the C-terminal helix and not to its amphiphilic nature. The positive charges may play a critical role in intramolecular, ligand−receptor or ligand − lipid interactions.

Similarly, Barbier *et al.* (2005) and Dean *et al.* (2006a) investigated the role of amino acid side-chains in region 17−31 of PTH-(1–31) in binding to PTHR1. In the first study, backbone methylation was found to disrupt the predicted amphiphilic C-terminal α-helix as reflected in a loss of activity. In particular, introduction of the respective N-methyl amino acids in positions Val^{21}, Leu^{24}, Arg^{25}, and Leu^{28} resulted in a pronounced loss of activity, suggesting that these residues play a critical role in interaction with the N-ECD. When testing the analogs with PTHR-delNt, a PTHR1 mutant lacking the N-ECD, a different set of positions (Ser^{17}, Trp^{23}, and Lys^{26}), located on the other side of the amphiphilic helix, was found to be particularly sensitive to methylation. The authors conclude that the C-terminal helix of PTH-(1–31) must bind to both the N-ECD as well as the extracellular loop regions of PTHR1 (Barbier *et al.*, 2005).

The second study by Dean *et al.* (2006a) reports that substitutions at residues from the hydrophobic face of the predicted amphiphilic C-terminal α-helix (Arg^{20}, Trp^{23}, Leu^{24}, and Leu^{28}) resulted in an approximate 200-fold reduced binding affinity to full-length PTHR1, compared to a 4-fold reduction in binding affinity to PTHR-delNt. In contrast, substitutions at the hydrophilic face of the helix (Arg^{25}, Lys^{26}, Lys^{27}) yielded 4 – 10-fold reduced activity for both receptors. The results are supportive of a role for the hydrophobic face of the C-terminal helix of PTH in contributing strongly to the binding interaction with the N-ECD of PTHR1.

In addition to C-terminal and N-terminal helices, flexible hinge regions around positions 12 and 19 of PTH-(1–34) were proposed to be involved in the bioactive conformation. Therefore, structure-activity relations of residues 11−13 of PTH-(1–34) were studied by introducing either β-amino acids (Peggion *et al.*, 2002) or α-amino isobutyric acid (Aib) (Peggion *et al.*, 2003). Analysis by CD spectroscopy, 2D-NMR and computer simulations stressed the importance of the conformational stability of the helical activation domain as well as the hydrophobic side-chain of Leu^{11} in PTH-(1–34). Introduction of β-amino acids around position 18 resulted in partial or complete loss of functionality in combination with minimal conformational differences in all analogs tested (Schievano *et al.*, 2003)

RECEPTOR SUBTYPE SPECIFICITY SWITCH

The finding that the N-truncated sequence, PTHrP-(7–34), can bind and weakly activate the PTHR2, which selectively binds PTH but not PTHrP (Behar *et al.*, 1996b; Usdin *et al.*, 1995), suggested to Behar *et al.* (1996a) that the N-terminal sequence 1–6 of PTHrP must contain a structural element that disrupts the PTHrP-(1–34) − PTHR2 interaction. The single-point hybrid ligands, [His^5,$Nle^{8,18}$,Tyr^{34}]bPTH-(1–34)NH_2 and [Ile^5]PTHrP-(1–34)NH_2, were generated by swapping the nonconserved residues in position 5 between PTH-(1–34), which binds and activates both PTHR1 and PTHR2, and PTHrP-(1–34) (Behar *et al.*, 1996a). Indeed, in HEK293 cells stably transfected with either hPTHR1 or hPTHR2, the receptor specificity of these point-hybrid ligands is reversed when compared with their parent compounds. Therefore, His^5, a single residue within the activation domain of the ligands, acts as a specificity "switch" between these two highly homologous receptor subtypes.

In parallel, a study conducted by Gardella and colleagues (1996a) in COS-7 cells transiently expressing PTHR1 and PTHR2 also identified position 5 in addition to position 23 to account for the different potencies of PTH and PTHrP for PTHR2. Residue 5 (Ile in PTH and His in PTHrP) determines signaling capability, whereas residue 23 (Trp in PTH and Phe in PTHrP) is a major determinant for binding affinity. [Ile^5,Trp^{23},Tyr^{36}]PTHrP-(1–36) is a full agonist and binds avidly to PTHR2, whereas swapping only position 23 yields [Trp^{23},Tyr^{36}]PTHrP-(1–36), an antagonist on PTHR2 but full agonist on PTHR1.

TIP39, A PUTATIVE ENDOGENOUS LIGAND OF PTHR2

Usdin *et al.* (1999b) have purified an endogenous ligand selective for PTHR2, a tuberoinfundibular peptide of 39 amino acids (bTIP39) from bovine hypothalamic extracts. A homology search reveals that 9 out of the 39 residues of bTIP39 are identical to bPTH (Fig. 1). TIP39 does not appreciably activate adenylyl cyclase in COS-7 cells transfected with either human or rat PTHR1, although it binds to the receptor with moderate affinity [$IC_{50} = 59\,nM$, displacing ^{125}I-bPTH-(3–34)] (Hoare *et al.*, 2000). Jonsson *et al.* (2001) reported that TIP39 binds with weak affinity ($\sim 200\,nM$) to hPTHR1 stably expressed in $LLCPK_1$ cells (HKrk-B7 cells) and fails to stimulate adenylyl cyclase activity. The truncated peptides, TIP-(3–39) and TIP-(9–39), which display 3- and 5.5-fold higher affinity than the intact peptide, are similarly devoid of any adenylyl cyclase activity. Moreover, while TIP39 is a weak antagonist, both of the TIP39-derived N-terminally truncated peptides inhibit PTH-(1–34)-and PTHrP-(1–36)-stimulated adenylyl cyclase with efficiencies similar to a highly potent PTHR1 antagonist, [Leu^{11},D-Trp^{12},Trp^{23},Tyr^{36}]PTHrP-(7–36)NH_2.

Additional work with a hybrid PTHrP/TIP39 peptide and PTHR1/PTHR2 chimeras provides some insight into TIP39 − receptor interactions (Hoare *et al.*, 2000; Jonsson *et al.*, 2001). Jonsson and colleagues reported that the hybrid peptide PTHrP-(1− 20)-TIP39-(23 −39) binds effectively ($IC_{50} = 8$–$11\,nM$) and stimulates cAMP efficaciously in

HKrk-B7 and SaOS-2 cells (EC_{50} = 1.4 and 0.38 n*M*, respectively). Hoare *et al.* (2000) reported that the juxtamembrane domains of the PTHR2, which include ECL's 1–3, determine binding and signaling selectivity of TIP39 for PTHR2 over PTHR1. This was established by studying TIP39 interactions with the reciprocal chimeric PTHR1/PTHR2, in which the N-ECD domains were exchanged. TIP39 fully activated [N-ECD]PTHR1/[JMD]PTHR2 (EC_{50} = 2 n*M*) and bound to it with an affinity equal to wild-type PTHR2 (IC_{50} = 2.3 and 2 n*M*, respectively). However, the reciprocal chimeric receptor, ([N-ECD]PTHR2/[JMD]PTHR1), is not activated by TIP39 and the binding affinity to TIP39 is similar to that of PTHR1. Truncation of the first six amino acid residues from the N-terminus of TIP39 results in a tenfold increase in binding affinity for PTHR1 (IC_{50} = 6 n*M*), making it a potent, selective antagonist of this receptor (Hoare *et al.*, 2000). At the same time, TIP39-(7–39) does not activate PTHR2 and has a seventy-fold lower affinity to it than the intact hormone (IC_{50} =370 and 5.2 n*M*, respectively). Jonsson *et al.* (2001) concluded from their studies with amino-terminal truncated TIP39 and PTHrP/TIP39 hybrid peptides that the carboxyl-terminal portion of TIP39 interacts with PTHR1 very similarly to the analogous domains in PTH-(1–34) and PTHrP-(1−36). At the same time, the amino-terminal portion has destabilizing interactions with this receptor that result in poor affinity and are therefore unproductive.

Taken together, these studies suggest that the dominant molecular determinants of the binding selectivity of TIP39 to PTHR2 are different from those identified for PTH and PTHrP binding to PTHR1. In the TIP39/PTHR2 system, binding specificity is assigned to the juxtamembrane domain of the receptor and the N-terminal domain of the ligand (Hoare *et al.*, 2000), the binding selectivity in the PTH/PTHrP − PTHR1 system is specified by the N-ECD in the receptor and the C-terminal portion of the ligands (Bergwitz *et al.*, 1996; Gardella *et al.*, 1994, 1996a; Rosenblatt *et al.*, 1978).

Although these findings are of major significance for understanding bimolecular ligand−receptor interactions, the physiological role of the TIP39/PTHR2 system still remains to be established (Usdin *et al.*, 2000).

SIGNALING-SELECTIVE LIGANDS

The activation of PTHR1 triggers at least two G protein-dependent signaling pathways, increasing both adenylyl cyclase/PKA via $G_s\alpha$ and PLC/IP_3-DAG/cytosolic transients of $[Ca^{2+}]$/PKC via G_q in homologous as well as heterologous receptor/cell systems (Abou-Samra *et al.*, 1992; Bringhurst *et al.*, 1993; Juppner *et al.*, 1991; Lee *et al.*, 1995b; McCuaig *et al.*, 1994; Pines *et al.*, 1994; Schneider *et al.*, 1993; Smith *et al.*, 1996). In addition, as is the case for several other GPCRs, G protein-independent activation of PTHR1 mediated through β-arrestins was recently demonstrated (Gesty-Palmer *et al.*, 2006). Currently, the relationship between these signaling pathways and the cellular and *in vivo* expression of PTH bioactivity is not fully established. Over the past years, much was learned about PTHR1 internalization and trafficking (Bisello *et al.*, 2002; Syme *et al.*, 2005; Rey *et al.*, 2006; Wheeler *et al.*, 2007, 2008; Wang *et al.*, 2007; Weinman *et al.*, 2007). However, many cellular processes in bone metabolism remain to be elucidated. One of the open questions in the development of PTH-based, anabolic anti-osteoporotic therapies focuses on understanding the mechanism responsible for catabolic versus anabolic actions of PTH induced by continuous versus intermittent administration of hormone, respectively. The direct linkage of one or several specific signaling pathways to Wnt signaling and the anabolic activity of PTH remains to be established with precision.

The design of signaling-selective PTH/PTHrP-derived agonists is of great significance for understanding the role of the different pathways in cellular metabolic processes. Studies carried out using osteoblastic cells and organ cultures suggest that PTH residues 1–7 form the cAMP/PKA activation domain (Fujimori *et al.*, 1991), whereas PTH residues 28–34 comprise the PKC activation domain (Jouishomme *et al.*, 1992, 1994). The latter encompasses the region also associated with PTH mitogenic activity in cultured osteoblast-like cells (residues 30–34) (Schluter *et al.*, 1989; Somjen *et al.*, 1990). Cyclic AMP appears to be involved in the bone formation (Rixon *et al.*, 1994) and resorption activities of PTH (Tregear *et al.*, 1973). Although PTH stimulation of bone resorption *in vitro* is mediated primarily through the cAMP-dependent activation of PKA (Kaji *et al.*, 1992), it may not be the sole second messenger pathway involved (Herrmann-Erlee *et al.*, 1988; Lerner *et al.*, 1991). At the same time, stimulation of TE-85 human osteosarcoma cell proliferation by PTH-(1−34) is not associated with an increase in intracellular cAMP (Finkelman *et al.*, 1992). Taken together, PTH analogs that stimulate increases in cAMP levels have been shown to either inhibit (Kano *et al.*, 1991; Reid *et al.*, 1988; Sabatini *et al.*, 1996) or stimulate (McDonald *et al.*, 1986; Sabatini *et al.*, 1996; Van der Plas *et al.*, 1985) osteoblastic cell proliferation, depending on species, cell models used, and experimental conditions.

The failure to demonstrate bone-forming activity *in vitro* and bone anabolic effects *in vivo* with PTH analogs in which the capacity to activate adenylyl cyclase is severely compromised or completely eliminated turned attention to adenylyl cyclase-selective analogs. The adenylyl cyclase-selective analog hPTH-(1–31)NH_2 (Ostabolin) is equipotent to PTH-(1–34) in stimulating cAMP production in ROS 17/2 (Jouishomme *et al.*, 1994; Neugebauer *et al.*, 1995) and a potent stimulator of cortical and trabecular bone growth in OVX rats (Armamento-Villareal *et al.*, 1997; Hilliker *et al.*, 1996; Rixon *et al.*, 1994; Whitfield *et al.*, 1996, 2000). By truncating the C terminus up to residue 31, Jouishomme *et al.* (1994) were able to compromise the putative PKC-signaling motif, Gln^{28}-His^{32}, and generate hPTH-(1–31)NH_2, a PKA-selective analog. Whitfield *et al.* (1997) developed *c*[Glu^{22},Lys^{26},Leu^{27}]hPTH-(1−31)NH_2, a second-generation PKA signaling-selective analog, in which the helical nature of the C-terminus is enhanced by formation of a side-chain to side-chain lactam ring and the introduction of a hydrophobic residue at position 27. The replacement of Lys^{27} with Leu improves the amphiphilicity of the C-terminal helical domain; the side-chain of Lys^{27} was shown to interact with L^{261} in ECL3 of PTHR1 (Greenberg *et al.*, 2000; Piserchio *et al.*, 2000a). This analog is only a 1.4- to 2-fold stronger stimulator of femoral trabecular bone formation than the linear parent analog. Both hPTH-(1–31)NH_2 and *c*[Glu^{22},Lys^{26},Leu^{27}]hPTH-(1–31)NH_2 have been reported to prevent loss of vertebral and trabecular bone and to raise vertebral and trabecular bone volume and thickness over those of control, vehicle-injected, sham-operated rats (Whitfield *et al.*, 2000). The action of these analogs on vertebral bone was as effective as that of hPTH-(1–34)NH_2. However, unlike hPTH-(1−34)NH_2, their effect on pelvic BMD was equivocal.

Takasu *et al.* (1999a) offered an alternative view regarding the structural determinants associated with signaling pathway activation. Replacement of Glu^{19}→Arg, a receptor-binding and affinity-enhancing modification, generates [Arg^{19}]PTH-(1–28), which is a potent and full stimulator of adenylyl cyclase and PKC. Interestingly, substitution of Ala^1 for Gly generates [Gly^1,Arg^{19}]hPTH-(1–28), a PKA-selective agonist. This study concludes that the extreme N-terminus of hPTH constitutes a critical activation domain for coupling to PLC. The C-terminal region, especially hPTH-(28–31), contributes to PLC activation through receptor binding, but this domain is not required for full PLC activation. Therefore, they suggest that the N-terminal determinants for adenylyl cyclase and PLC activation in hPTH-(1–34) overlap but are not identical and that subtle modifications in this region may dissociate activation of these two effectors.

In the course of designing photoreactive PTHrP analogs for mapping the bimolecular ligand-receptor interface, Behar *et al.* (2000) generated [Bpa^1,Ile^5,$Arg^{11,13}$,Tyr^{36}] PTHrP-(1–36)NH_2. This analog binds and stimulates adenylyl cyclase equipotently to the parent analog [Ile^5, $Arg^{11,13}$,Tyr^{36}]PTHrP-(1–36)NH_2 in HEK293/C-21 cells overexpressing the recombinant human PTHR1 (~400,000 receptors/cell), but does not elicit intracellular calcium transients. Moreover, it does not stimulate the translocation of β-arrestin2-GFP fusion protein, an effect that is PKC dependent (Ferrari *et al.*, 1999). Subsequently, the G_s/PKA signaling selectivity of [Bpa^1,Ile^5,$Arg^{11,13}$,Tyr^{36}]PTHrP-(1–36) and of PTHrP-(2–36) (position 1 modified or deleted, respectively) was correlated with the stabilization of an active, G protein-coupled receptor state, resulting in lack of β-arrestin2 recruitment to the cell membrane, sustained cAMP signaling, and absence of ligand−receptor complex internalization (Bisello *et al.*, 2002). Utilizing molecular modeling, the authors generate a mechanistic explanation for the differences in conformational changes induced by agonist versus the two position 1-modified analogs that stresses a difference in the movement of ICL3.

Gesty-Palmer *et al.* (2006) recently presented another position 1-modified agonist, [Trp^1]PTHrP-(1−36), and likewise demonstrated its G_s/PKA pathway signaling selectivity and lack of β-arrestin recruitment. In this study, they dissociate activation of the mitogen-activated protein kinases (MAPK) ERK1/2 by PTH-(1−34) into two distinct signaling mechanisms: an early G protein-dependent pathway involving PKA and PKC, and a late G protein-independent pathway mediated through β-arrestins. [D-Trp^{12}, Tyr^{34}]PTH-(7−34), previously presented as an inverse agonist for cAMP in cells expressing the constitutively active PTHR1 mutant H223R (Gardella *et al.*, 1996c), selectively inhibits G_s/PKA activation while having no apparent activity in G_q/PKC stimulation. Despite its inability to activate G_s/PKA or G_q/PKC signaling pathways, the inverse agonist is able to activate MAP kinase. The study identifies that this happens through β-arrestin recruitment, and that this pathway corresponds to the late G protein-independent activation phase (Gesty-Palmer *et al.*, 2006).

Such signaling-selective analogs are likely to help in the understanding of the molecular signaling mechanisms of PTH, as well as in the characterization of different active receptor conformations and ultimately in the development of novel therapeutics for the treatment of various bone pathologies.

TARGET-SPECIFIC LIGANDS

The wide distribution of PTHR1 in tissues other than bone and kidney (Urena *et al.*, 1993), the classical target tissues for PTH, and its physiological activation by locally secreted PTHrP (Philbrick *et al.*, 1996) raise concerns about potential side effects following parenteral administration of therapeutic doses of exogenous PTH. Bone-selective PTH/PTHrP-derived analogs may reduce the activation of PTHR1 in kidney and nonosseous tissues and provide better bone anabolic drugs.

To this end, Cohen and coworkers reported that [His^3]- and [Leu^3]hPTH-(1–34) are partial agonists of adenylyl cyclase in a kidney cell line (50% and 20%, respectively), but full agonists in UMR-106 rat osteosarcoma cells (Cohen *et al.*, 1991; Lane *et al.*, 1996). *In vivo,* however, both analogs were less potent than PTH in the induction of bone formation.

In summary, the development of an effective and safe therapeutic modality that would stimulate the formation of new, mechanically competent bone and possibly reconstitute trabecular architecture in osteoporotic patients continues to be a worthy goal. This goal may be approached by analogs that interact with the PTHR1 in a signaling-selective manner or are targeted specifically to bone to achieve a more favorable therapeutic window.

PUTATIVE BIOACTIVE CONFORMATION

In the absence of a crystal structure of the PTH − PTHR1 complex, alternative approaches have been utilized to obtain insight into the three-dimensional structure of ligand while bound to PTHR1. Because GPCRs are embedded in the cell membrane, Schwyzer (1991, 1992, 1995) hypothesized that the initial conformations adapted by a ligand are induced by nonspecific interactions with the membrane. Only some of these membrane-induced conformations are recognized by the membrane-embedded GPCR. Therefore, the study of conformations in a membrane mimetic milieu, like the micellar environment, is probably the best available approximation of the natural state.

Secondary structure prediction methods suggest that the N-terminal 1–34 sequences of both PTH and PTHrP assume helical structures at their N- and C-terminal domains (Chorev *et al.,* 1990; Cohen *et al.,* 1991; Epand *et al.,* 1985). These helical sequences span residues 1–9 and 17–31 in PTH and 1–11 and 21–34 in PTHrP (Cohen *et al.,* 1991). A good correlation between receptor-binding affinity and the extent of helicity was established by circular dichroism (CD), a spectroscopic method that can assess the average conformation of a peptide (Neugebauer *et al.,* 1995). Assessment of helical content by CD estimated PTH-(1−34) to have on the average fewer than eight residues in helical conformation, which is lower than that estimated for PTHrP-(1−34) (Cohen *et al.,* 1991; Epand *et al.,* 1985; Neugebauer *et al.,* 1992; Willis and Szabo, 1992; Zull *et al.,* 1990). In the presence of 45% trifluoroethanol (TFE), a solvent that promotes secondary structure, the total helical content of both bPTH-(1−34) and hPTHrP-(1−34) is about 73% (Cohen *et al.,* 1991). Nevertheless, the relevance of the conformation in TFE to the bioactive conformation is still debated.

According to ^{1}H-NMR studies in water, the structure of PTH-(1−34) is mostly random, except for a short-ordered region encompassing residues 20–24 (Bundi *et al.,* 1976, 1978; Lee and Russell, 1989). Pellegrini *et al.* (1998b) reported that hPTH-(1−34) in water is highly flexible, with some evidence of transient helical loops spanning the sequences 21–26 and 7–8. This CD and NMR study was carried out in aqueous solution, with variable pHs and salt concentrations, and in the presence of dodecylphosphocholine (DPC) micelles. Distance geometry calculations generated conformations that were refined by molecular dynamic simulations explicitly incorporating solvent (H_2O). This study generated high-resolution conformational preferences of hPTH-(1−34), which were later used in the construction of an experimentally based model of the hormone−receptor complex. Both in aqueous solution and in the presence of DPC micelles, Pellegrini and coworkers observed fast conformational averaging on the NMR time scale. As anticipated, the two helical domains observed in aqueous solution − the N-terminal helix comprising residues 6–14, and the C-terminal helix comprising residues 19–23 − are extended (4–17 and 21–33, respectively) and stabilized in the presence of DPC micelles. A region of flexibility, which is centered around residues 15–16 in aqueous solution and around residues 18–19 in the micellar system, separates both helices (Fig. 2). Therefore, in solution, the two helical domains adopt a range of different spatial orientations, none of which corresponds to a tertiary structure in which helix − helix interactions can be observed (Pellegrini *et al.,* 1998b). This observation is in complete accord with conformational studies of lactam-containing PTHrP analogs (Mierke *et al.,* 1997), point-mutated and segment PTH-PTHrP hybrids (Peggion *et al.,* 1999; Schievano, 2000), and a model amphiphilic α-helix-containing PTHrP analog (Pellegrini *et al.,* 1997a).

Weidler *et al.* (1999) studied PTHrP-(1−34) by CD and NMR in what they define as near physiological solution (50 m*M* potassium phosphate, pH 5.1, 250 m*M* NaCl). According to their studies, PTHrP-(1−34) contains two helical domains, His^5-Leu^8 and Glu^{16}-Leu^{27}, which are connected by a flexible linker. Similar to Pellegrini and coworkers (1998b), who studied PTH-(1−34), they also did not detect any tertiary structure in PTHrP-(1−34).

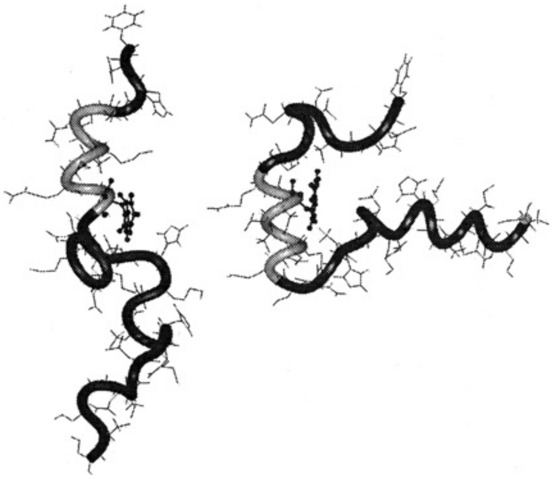

FIGURE 2 Ribbon diagram of two conformations of hPTH-(1–34) resulting from ensemble-based calculations. Averaging over these two-member ensembles fulfills all of the CD- and NMR-based experimental observations. The different locations and extents of the α-helices are highlighted in grey; the side-chain of Trp23, used to align the conformations, is shown as a ball-and-stick structure (Pellegrini *et al.,* 1998b).

The more hydrophobic and amphiphilic C-terminal sequence in PTHrP has a higher propensity for forming a helix than the N-terminal domain, as demonstrated by the higher percentage of TFE needed for nucleation of an N-terminal helix (Mierke *et al.,* 1997). Similar conclusions were reached by adding lipids (Epand *et al.,* 1985; Neugebauer *et al.,* 1992; Willis, 1994) or DPC micelles (Pellegrini *et al.,* 1997a, 1998b) to PTH- and PTHrP-derived sequences. Taken together, the conformational analyses yield a dynamic model for ligand−receptor binding. Binding is initiated by complementary hydrophobic interactions between the hydrophobic face of the amphiphilic C-terminal helical domain of the ligand, including the principal binding domain, and the hydrophobic membrane. This hydrophobic ligand − membrane interaction allows the propagation of the C-terminal helix and the formation of specific interactions with extracellular portions of the PTH receptor. In the membrane environment, nucleation of the N-terminal helix occurs either cooperatively with (in the antagonist) or independently of (in the agonist) the previously formed C-terminal helical domain. Consequently, in the case of PTH/PTHrP agonists, the flexibility around hinges 12–13 and 19–20 allows the membrane-induced "message domain" to be positioned correctly within the receptor. Specific message-receptor interactions leading to the conformational changes required for signal transduction can thus occur. In the case of PTH/PTHrP antagonists, which lack most of the "message" sequence, no conformational change in the receptor occurs and no signal transduction event is triggered. Absence of the critical hinge around positions 19–20, or a shift in register of the hinge region, results in reduced binding affinity and efficacy, as observed for the cyclic PTHrP analogs (Mierke *et al.,* 1997) and point-mutated PTH/PTHrP hybrids (Peggion *et al.,* 1999). This proposed dynamic model for ligand − membrane − receptor interaction has since been confirmed and extended by numerous studies (Gensure *et al.,* 2003; Castro *et al.*, 2005; Wittelsberger *et al.*, 2006a).

Piserchio and colleagues (2000b) studied bTIP39, the endogenous ligand for PTHR2, using both high-resolution NMR and CD in the presence of DPC micelles and computer simulation in a water/decane simulation cell. They reported a molecular architecture consisting of two stable α-helices, Ala^{5}-Leu^{20} and Leu^{27}-Val^{35}, separated by an unstructured region. This architecture is reminiscent of the structure of PTH-(1−34), with which TIP39 shares only limited sequence homology (Fig. 1). The N-terminal helix in TIP39, important for activation of PTHR2, has a spatial distribution of polar and hydrophobic amino acid residues almost identical to PTH, making it only moderately amphiphilic. Interestingly, in molecular modeling, Asp^{7} in TIP39 is homologous to Ile^{5} in PTH and His^{5} in PTHrP, both of which are crucial for ligand selectivity toward the PTHR2 (Behar *et al.,* 1996a; Gardella *et al.,* 1996a). Thus, the putative steric and repulsive interaction between His^{5} in PTHrP and H^{384} in the bottom of the narrow binding pocket of PTHR2, which is responsible for this receptor-ligand selectivity (Rolz *et al.,* 1999), is replaced by an attractive Coulombic interaction and smaller side-chain presented by Asp^{7} in TIP39. This complementarity may favorably accommodate the interaction between TIP39 and its cognate PTHR2. Compared to PTH, the N-terminal helix in TIP39 is longer by five residues (Leu^{16}-Leu-Ala-Ala-Leu^{20}), causing a shift in the relative location of the flexible region 21–26. The C-terminal helices in TIP39 and PTH-(1−34) are only slightly amphiphilic and share a lower degree of homology than the one observed for N-terminal helices. Some unique structural features related to the C-terminal portion of TIP39 may explain its weaker interaction with the N-ECD of PTHR2, and therefore its diminished role in recognition and binding (Hoare *et al.,* 2000). Compared to the C-terminal helix in PTH-(1−34), the amphiphilicity of the C-terminal helix of TIP39 is compromised by two factors: the presence of charged residues within the hydrophobic face and the location of Trp^{25} in the unordered region separating the N- and C-helices instead of within the C-terminal helix, as Trp^{23} is in PTH. These experimental and computational results reiterate the significance and relevance of studying structurally related, but functionally distinct, ligand−receptor systems to the PTH/PTHrP − PTHR1 system.

A much-debated issue is whether PTH and PTHrP fold into a tertiary structure, in which secondary structural elements interact specifically with each other to form a more stable and higher ordered structure. Cohen *et al.* (1991) suggested that in TFE, the amphiphilic helices located at the N and C termini of bPTH-(1−34) and hPTHrP-(1−34) interact to form a U-shaped tertiary structure, with the hydrophobic residues facing inward to form a hydrophobic core. As a result, the hydrophilic residues are oriented outward, exposing them to the polar solvent. However, in light of the lack of compelling spectroscopic evidence for long-range interactions between the two N- and C-terminal helices in both hPTH-(1−34) and PTHrP-(1−34) (Klaus *et al.,* 1991; Strickland *et al.,* 1993; Wray *et al.,* 1994), the notion of a U-shaped tertiary structure remains unsupported. Interestingly, Gronwald and coworkers (1996) reported that in aqueous TFE, the long-range proton-proton correlations (Val^{2}-to-Trp^{23} and Ile^{5}-to-Asn^{10}) between the two N-terminal helices (sequences 1–10 and 17–27) in the full-length, recombinant hPTH are dependent on interactions provided by residues in the middle and C-terminal portion of the molecule (sequences 30–37 and 57–62, respectively). They cautiously suggested that the molecule shows a tendency toward tertiary structure. It should be noted that in TFE, the low dielectric constant, which helps stabilize helices, is also supposed to shield the side-chains from hydrophobic interactions between the helices, and therefore, destabilizes alleged U-shaped tertiary structures.

Marx and coworkers (1995) suggested that hPTH-(1−37) assumes a U-shaped structure in aqueous solution containing a high salt concentration. However, their reported long-range, proton-proton correlations are limited to side-chains of Leu^{15} and Trp^{23} located close to the bend forming the putative U-shaped structure, leaving, therefore, too much flexibility to define a stable U-shaped structure. Others have also assigned a tertiary folded structure to PTH-(1−39), PTH-(1−34), and the osteogenic PTH sequence PTH-(1−31) (Chen *et al.,* 2000; Marx *et al.,* 2000). The same researchers identify a loop region around His^{14}-Ser^{17}, stabilized by hydrophobic interactions, and long-range proton-proton correlations between Leu^{15} and Trp^{23}, which are also found in hPTH-(1−37) and in N-truncated analogs hPTH-(2−37), -(3−47), and -(4−37) (Marx *et al.,* 1998).

Other studies of PTHrP analogs describe interactions between N- and C-terminal helical domains, in the presence of TFE, thus offering support for the U-shaped structure (Barden and Kemp, 1989, 1994, 1995, 1996; Barden *et al.,* 1997). Barden and Kemp mentioned the presence of a hinge at Arg^{19} − Arg^{20} in [Ala^{9}]PTHrP-(1−34)NH$_2$ and attributed to it a functional role in signal transduction. They also postulate long-range interactions between side-chains located on both sides of the turn, Gln^{16}-Arg^{19}, implicating the presence of a tertiary structure (Barden and Kemp, 1996). The question of tertiary structure in PTHrP-(1−34) was also addressed by Gronwald and coworkers (1997), who studied it in water and in 50% TFE. In the presence of TFE, they observed two stable α-helical regions spanning residues 3 to 12 and 17 to 33, which are connected by a flexible linker. Based on their CD and NMR study, Gronwald and colleagues concluded that there is no evidence of a stable tertiary structure in PTHrP-(1−34). Taken together, the current and prevailing view is that both PTH- and PTHrP-derived linear peptides in solution do not form an appreciable component of stable tertiary structure.

The lack of tertiary structure in PTHrP-(1−34) either in aqueous solutions or in the presence of TFE is not limited to the linear parent peptide (Barbier *et al.,* 2000; Maretto *et al.,* 1997; Mierke *et al.,* 1997). Similar conformational behavior was observed in a series of side-chain to side-chain-bridged mono- and bicyclic lactam-containing PTHrP analogs. These conformationally constrained analogs are obtained through cyclization of side-chain pairs, Asp^{13} to Lys^{17}, Asp^{22} to Lys^{26}, Lys^{25} (replacing Arg) to Glu^{29}, and Lys^{26} to Asp^{30}, located at the putative N- and C-terminal helical domains (Barbier *et al.,* 2000; Bisello *et al.,* 1997; Chorev *et al.,* 1991, 1993; Maretto *et al.,* 1997, 1998; Mierke *et al.,* 1997). This i- to -i + 4 side-chain to side-chain cyclization is known to stabilize helical structures in other peptide systems. Bioactivity in the agonist (1−34) and antagonist (7−34) series of lactam-containing analogs requires well-defined N- and C-helical domains that are linked by two flexible hinges located around residues 12–13 and 19–20 (Maretto *et al.,* 1997; Mierke *et al.,* 1997).

Two separate studies suggested that the bioactive conformation of PTH forms an extended helix (Condon *et al.,* 2000; Jin *et al.,* 2000). In a CD study, Condon and coworkers analyzed a series of lactam-containing PTH-(1−31) analogs that include the tricyclo (Lys^{13}Asp^{17},Lys^{18}Asp^{22}, Lys^{26}Asp^{30})-[Ala^{1},Nle^{8},Lys^{18},Asp^{17,22},Leu^{27}]hPTH-(1−31)NH$_2$, a highly potent ($EC_{50} = 0.14\,nM$) analog. This analog forms an extended helix − spanning residues 13–30 − in aqueous solution and is fully helical in 40% TFE. This tricyclic analog includes a lactam bridge, Lys^{18}-to-Asp^{22}, which engulfs Arg^{19}, a putative hinge site in the bioactive conformation. They concluded, therefore, that PTH binds to its cognate receptor in an extended helical conformation. A similar conclusion was reached by Jin and coworkers, who reported the crystal structure of hPTH-(1−34). The PTH crystallized in a slightly bent (residues 12–21), long helical antiparallel dimer (Fig. 3, see also color plate). In general, solid-phase structures of short peptides can be very much affected by the intermolecular packing forces stabilizing the crystal structure. In particular, formation of the dimer, as in the PTH crystal, can override other intramolecular interactions. In addition, there is no resemblance between the crystal environment and a membrane milieu in which the bimolecular ligand − GPCR interaction takes

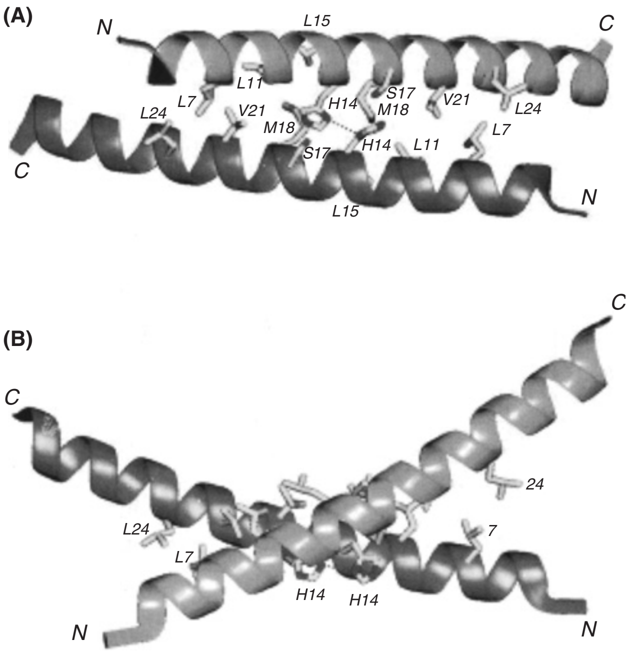

FIGURE 3 Overall structure of hPTH-(1–34) in the solid state. The monomeric chain is a slightly bent helix presented as green ribbons. At the crossing point of the amphiphilic helices in the dimer, His^{14} from each chain forms a hydrogen bond shown as a dotted line. The dimer interface is mainly hydrophobic. **(A)** and **(B)** are two different views of the dimer (Jin *et al.,* 2000).

place. We do not think that these studies contradict previous findings obtained with both PTH and PTHrP in solution, or with analogs more flexible than the tricyclic one. Conformations of the tricyclic analog of PTH in solution or PTH-(1−34) in the solid state may represent only a fraction of the ensemble of fast equilibrating conformations that can generate the bioactive conformation.

As we gain more knowledge about the complexity of receptor conformational states, the traditional search for the one bioactive ligand−receptor conformation must be reassessed. The new paradigm teaches that each ligand induces a specific series of conformational states that result in specific downstream signaling events. The "active state" for receptor triggering of β-arrestin-mediated signaling might be different from that for receptor activation of a G protein (Deupi *et al.*, 2007). Similarly, for receptors signaling through more than one G protein, as does PTHR1, the conformational switching involved in activation of one G protein (i.e., G_s) might be different from that activating another G protein (i.e., G_q) (Kenakin, 2003).

CONFORMATION-BASED DESIGN OF PTH/PTHrP ANALOGS

Restriction of conformational freedom of the ligand in a local or global manner may preclude a wide range of nonproductive conformations and will result in more potent analogs. Indeed, one of the goals of searching for the bioactive conformation of a ligand is to identify structural elements essential for its bioactivity and to devise ways to stabilize them. Furthermore, enhancement of complementary topological features in the ligand−receptor interface may also contribute to increased potency. For example, enhancement of the amphiphilicity of a helical segment may stabilize a favored bimolecular interaction and lead to a more productive receptor interaction. To this end, several studies have incorporated structural modifications that stabilize an element important in the putative bioactive conformation of several PTH analogs (Barbier *et al.*, 1997; Bisello *et al.*, 1997; Chorev *et al.*, 1991, 1993; Leaffer *et al.*, 1995; Mierke *et al.*, 1997; Surewicz *et al.*, 1999).

Much attention has been drawn to the amphiphilic nature of the C-terminal helix comprising residues 20–34 of hPTH-(1−34) and its role in receptor binding (Gardella *et al.*, 1993; Neugebauer *et al.*, 1992). A Lys27→Leu substitution in PTH-(1−34)NH_2 and PTH-(1−31)NH_2 improved the amphiphilic character of the C-terminal helical sequence and increased adenylyl cyclase activity over the corresponding nonsubstituted sequences (Barbier *et al.*, 1997; Surewicz *et al.*, 1999).

Vickery and coworkers (1996) reported a more extensive enhancement of the amphiphilicity of the C-terminal helix of PTHrP. Substitution in PTHrP-(1−34)NH_2 of the sequence 22–31 with a model amphipathic peptide (MAP; Glu^1-Leu-Leu-Glu-Lys-Leu-Leu-Glu-Lys-Leu-Lys^{10}), which is highly α-helical when incorporated into short peptides (Krstenansky *et al.*, 1989), generates $[(MAP_{1-10})^{22-31}]$ hPTHrP-(1−34)NH_2 (RS-66271). In this analog, important structural features, such as Leu^{24} and Leu^{27}, are maintained, and Ile^{22} and Ile^{31} are substituted conservatively by Leu. In aqueous buffer, RS-66271 displays eight- to ninefold higher helicity than the parent peptide. A detailed conformational analysis of RS-66271 in water, employing CD and ^1H NMR spectroscopy, confirmed the presence of an extensive helical structure encompassing residues 16–32 (Pellegrini *et al.*, 1997a). Nevertheless, the absence of a hinge element around Arg^{19}, considered to contribute to high biological activity, may explain the sixfold lower adenylyl cyclase activity and tenfold lower binding affinity in ROS17/2.8 cells of RS-66271, compared to the more flexible and less helical PTHrP-(1−34) (Krstenansky *et al.*, 1989, 1994). Importantly, the preservation of significant *in vitro* potency, despite the multitude of substitutions, validates the rationale behind the design of RS-66271.

It is generally accepted that PTH-(1−34) and PTHrP-(1−34) contain two helical domains spanning sequences 13–18 and 20–34 (Barden and Cuthbertson, 1993; Barden and Kemp, 1993; Strickland *et al.*, 1993; Wray *et al.*, 1994). Introduction of side-chain to side-chain cyclizations via lactam bond formation between residues that are four amino acids apart and located across a single helical pitch (residue i to residue i + 4) has been demonstrated to be an effective way to stabilize α-helical structure (Bouvier and Taylor, 1992; Danho *et al.*, 1991; Felix *et al.*, 1988a, 1988b; Madison *et al.*, 1990). Therefore, Chorev *et al.* (1991) undertook replacement of a potential ion pair participating in α-helical stabilization by a covalent lactam bridge in an attempt to further stabilize the helices in these regions. The initial application of this approach generated *c*[Lys^{13}-Asp^{17}]PTHrP-(7−34)NH_2, which was about tenfold more potent than the linear parent antagonist (K_b = 18 and 170 n*M*, K_i = 17 and 80 n*M*, respectively, in Saos2/B10 cells) (Chorev *et al.*, 1991). Rigidification of the C-terminal helix in *c*[Lys^{26}-Asp^{30}]PTHrP-(7−34)NH_2 did not improve antagonist potency (Bisello *et al.*, 1997), but a combination of two 20-membered lactam bridges, in both N- and C-terminal helices, generated *c*[Lys^{13}-Asp^{17},Lys^{26}-Asp^{30}]PTHrP-(7−34)NH_2, a potent (K_b = 95 n*M* and K_i = 130 n*M* in Saos2/B10 cells) (Bisello *et al.*, 1997), highly conformationally constrained, PTHrP-derived antagonist, and a valuable tool for conformational studies (Maretto *et al.*, 1997).

The same approach applied to the agonist PTHrP-(1−34)NH_2 yielded the mono- and bicyclic analogs *c*[Lys^{13}-Asp^{17}]PTHrP-(1−34)NH_2 and *c*[Lys^{13}-Asp^{17},Lys^{26}-Asp^{30}]PTHrP-(1−34)NH_2, which were equipotent to the linear parent compound (K_b = 3.2, 2.1, and 1 n*M*, K_m = 0.17, 0.22, and 0.57 n*M*, respectively, in Saos2/B10 cells) (Bisello *et al.*, 1997). A similar approach was also applied to signaling-selective analogs, hPTH-(1−31)NH_2, and the more potent [Leu^{27}]hPTH-(1−31)NH_2. Both of these analogs stimulate the adenylyl cyclase, but not the PLC/PKC signaling pathway (Barbier *et al.,* 1997). Whereas i-to-i+4 lactam bridge formation between Glu^{22} and Lys^{26}, as in *c*[Glu^{22}-Lys^{26},Leu^{27}]hPTH-(1−31)NH_2, results in about a fourfold increase in adenylyl cyclase activity compared to the linear parent peptide (EC_{50} = 3.3 and 11.5 n*M*, respectively, in ROS 17/2 cells), similar cyclization between Lys^{26} and Asp^{30} or i-to-i+3 lactam bridge formation between Lys^{27} and Asp^{30} results in cyclic analogs less potent than the corresponding linear parent peptides (Barbier *et al.,* 1997).

Interestingly, the higher adenylyl cyclase activity *in vitro* observed for *c*[Glu^{22}-Lys^{26},Leu^{27}]hPTH-(1−31)NH_2 compared to the linear peptide results in a higher anabolic effect on trabecular bone growth in ovariectomized rats (Whitfield *et al.,* 1997) and more effective protection than hPTH-(1−34) affords against loss of femoral trabeculae in the same animal model (Whitfield *et al.,* 1998).

The retention of full PKC activity (in ROS 17/2 cells) by the extensively N-terminally truncated linear fragment [Lys^{27}]hPTH-(20−34)NH_2 and the structurally related lactam-bridged analog *c*[Lys^{26}-Asp^{30}]hPTH-(20−34)NH_2 was consistent with the stabilization of the amphiphilic helix at the C terminus and implicated the helix as an important functional motif for binding to the PTHR1 (Neugebauer *et al.,* 1994). Taken together, these studies provide important insights regarding the structural nature of the hormones PTH-(1−34) and PTHrP-(1−34) and help to better characterize conformational features important for PTH binding and bioactivity.

PTH MINIMIZATION: SHORT AMINO-TERMINAL ANALOGS

Although amino-terminal fragments of PTH and PTHrP shorter than 1–27 were previously reported devoid of biological activity (Azarani *et al.,* 1996; Kemp *et al.,* 1987; Rosenblatt, 1981; Tregear *et al.,* 1973), the efforts of Gardella and coworkers focused on the isolated activation domain represented by the amino terminus, PTH-(1−14) (Carter and Gardella, 1999; Luck *et al.,* 1999; Shimizu *et al.,* 1999). In the search for small peptide and nonpeptide PTH-mimicking molecules as potential therapies for bone metabolic disorders, the marginally active PTH-(1−14) was used as the starting point for structural manipulations in an effort to optimize its activity.

Luck and coworkers (1999) reported that PTH-(1−14) is equipotent in stimulating increased cAMP levels (EC_{50} 100 *μM*) via the intact rat (r) PTHR1 and the *N*-terminal truncated receptor (rΔNt) missing N-ECD residues 26–181, both transiently expressed in COS-7 cells. In contrast, PTH-(1−34) is two orders of magnitude less potent in stimulating adenylyl cyclase via the rΔNt than through the intact rPTHR1 (Luck *et al.,* 1999). In addition, the “Ala scan” of PTH-(1−14) revealed that the first nine of the N-terminal residues forming the critical activation domain are involved in ligand−receptor interaction, rather than in an intramolecular interaction with the C-terminal domain PTH-(15−34) as suggested earlier (Cohen *et al.,* 1991; Gardella *et al.,* 1995; Marx *et al.,* 1995). This study reinforced the paradigm that the N-terminus of PTH interacts with binding determinants within the ECLs and the juxtamembranal portions of the TMs of the PTHR1.

Shimizu and coworkers (1999) identified some substitutions in the 10–14 sequence of the hormone that are not only compatible with function, but also result in more potent peptides. The peptides [$Ala^{3,10,12}$,Arg^{11}]PTH-(1−14) and [$Ala^{3,10}$,Arg^{11}]PTH-(1−11) are, respectively, 100- and 500-fold more active than PTH-(1−14). In addition, insertion of His, a “Zn^{2+} switch,” into some positions in the 10–13 sequence of PTH-(1−14) led to increases in ligand-stimulated cAMP levels in the presence of Zn^{2+} (Carter and Gardella, 1999). This result was interpreted by Carter and colleagues to suggest that the C-terminal portion of PTH-(1−14) contributes important interactions with the ECLs and TM domains. A particular ternary metal − ligand−receptor complex, in which the histidine residues in the ligand and the receptor participate in the coordination sphere around the Zn^{2+}, stabilizes these interactions. However, in the absence of demonstrable specific binding, it is unclear whether the extremely low levels of ligand-induced cAMP increases result from nonspecific interactions between PTH-(1−14) and PTHR1 or from interactions at sites different from the ones used by the 1–34 sequence.

Subsequently, introduction of homoarginine (Har) in position 11 of the (1−14) sequence was shown to increase potency by 40-fold (Shimizu *et al.,* 2001a). Next, the introduction of the sterically hindered and helix-promoting α-amino isobutyric acid (Aib) at positions 1 and 3 had an even larger impact (Shimizu *et al.,* 2001b): when introduced into the [$Ala^{3,12}$,Gln^{10},Arg^{11},Trp^{14}]PTH-(1−14) sequence ([M]PTH-(1−14)), potency increased by 100-fold. The resulting [$Aib^{1,3}$,M]PTH-(1−14), with an EC_{50} value of 1.1±0.1 nM, is ~100,000-fold more potent than native PTH-(1−14) and 2-fold more potent than PTH-(1−34). The shorter sequence [$Aib^{1,3}$,M]PTH-(1−11) displayed an EC_{50} value of 4±1 nM and is also fully efficacious. Finally, a study investigating the impact of other di-alkyl amino acids, 1-aminocycloalkane carboxylic acid (Ac_xc, x = 3, 5, or 6) or diethylglycine (Deg) yielded the most potent PTH-(1−14) and shorter length analogs to date: [Ac_5c^1,Aib^3,M]PTH-(1−X) (X = 14, 11, and 10; IC_{50}s = 80 nM, 260 nM, and 850 nM; EC50s = 1.7 nM, 3.1 nM, and 1.9 nM, respectively) (Shimizu *et al.,* 2004). A report by Barazza *et al.* (2005) confirmed that Ac_5c in either position 1 or 3 in combination with Aib at the respective other position further increases potency in the [Glu^{10},Arg^{11}]hPTH-(1–11) sequence. Using circular dichroism (CD) spectroscopy and nuclear magnetic resonance (NMR) combined with computer simulation studies, helix contents of the analogs were evaluated. Consistent with Lim *et al.* (2004; Lee, 2004}, the study concludes that helix stability of the short PTH analogs is necessary but not sufficient for bioactivity, and side-chains also play a role (Barazza *et al.*, 2005).

Additional work in the search for potency-enhancing N-terminal modifications was based on the revelation by Mierke and coworkers that the side-chains of residues 6 (Asn) and 10 (Asn or Gln) of the signaling domain (1–14) of PTH are located on the same face of the putative α-helix (Tsomaia *et al.*, 2004). Mutation of either position 6 or 10 to Ala in N-terminal (1–11) and (1–14) PTH analogs (Shimizu *et al.*, 2003) demonstrated a loss of activity that was partially recovered by introduction of the double mutation. When a Lys in position 6 was covalently bridged by a lactam bond to a Glu in position 10, the activity of the resulting cyclic analog was found to be six-fold higher compared to the linear peptide. Ala mutations in positions 6 and 10 had no effect on binding or activity of PTH-(1–34) when tested with wild-type PTHR1. However, Ala^6 abolished PTH-(1–34) signaling via PTHR-delNt, an effect reversed by introduction of Ala^{10}. High-resolution structures as determined by ^{1}H-NMR and NOE-restrained molecular dynamics simulations illustrate the structural differences between the linear and cyclic PTH-(1– 14) fragments, supporting the hypothesis that an α-helix is the preferred bioactive conformation of the N-terminal fragment of PTH (Tsomaia *et al.*, 2004).

Fiori *et al.* (2007) introduced a rigid dipeptide mimetic unit at select positions into the PTH-(1–11) sequence. Comparing activities with structural data obtained from CD spectroscopy and 2D-NMR studies, the authors suggest that the relative spatial orientation of the side-chains of Val^2, Ile^5, and Met^8, together with an intact N-terminal α-helix, are important determinants for activity.

PTH RECEPTORS

The physiological and pathophysiological activities of PTH and PTHrP are mediated predominantly by PTHR1. PTHR1 is encoded by a single-copy gene expressed primarily in kidney, intestine, and bone, the target tissues for PTH, and the PTH – PTHR1 interaction is essential for maintaining mineral ion homeostasis (Schipani *et al.*, 1993). The discovery of receptors for PTH and PTHrP, their functional properties, and biological importance are summarized in some excellent reviews (Juppner, 1995, 1999; Mannstadt *et al.*, 1999).

The expanding pharmacological evidence for actions of PTH on targets other than bone and kidney and the divergence of signaling pathways suggest the presence of a distinct subfamily of cognate receptors for PTH, PTHrP, and other related peptides. Homology-based screen and the exploration of molecular evolution of PTH receptors in various species yielded cDNAs encoding three distinct PTH receptor subtypes (Fig. 4) (Rubin and Juppner, 1999; Usdin *et al.*, 1995). The designation of PTHR2 and PTHR3 as PTH receptor subtypes is based on their high level of sequence homology with PTHR1, their interaction with PTH- and/or PTHrP-derived peptides, and their capacity to stimulate both PKA- and PKC-dependent signaling pathways. However, this designation does not reflect any physiological or functional relationship to the PTH/PTHrP – PTHR1 system. In addition, evidence is currently accumulating for the existence of a carboxyl-terminal PTH receptor responding to C-terminal fragments of PTH-(1–84) (Divieti *et al.*, 2001).

Studying the structure-function relationship of the PTHR1 is an important indirect approach to gaining insight into the ligand–receptor bimolecular recognition process and the signal transduction mechanisms. This approach, however, focuses on only one component of the hormone – receptor complex, the receptor, and therefore it is “blind” to the structural information of the ligand. In addition, many of the point mutated, truncated, and hybrid receptors can be affected by long-range structural consequences that are removed from the site of modification,

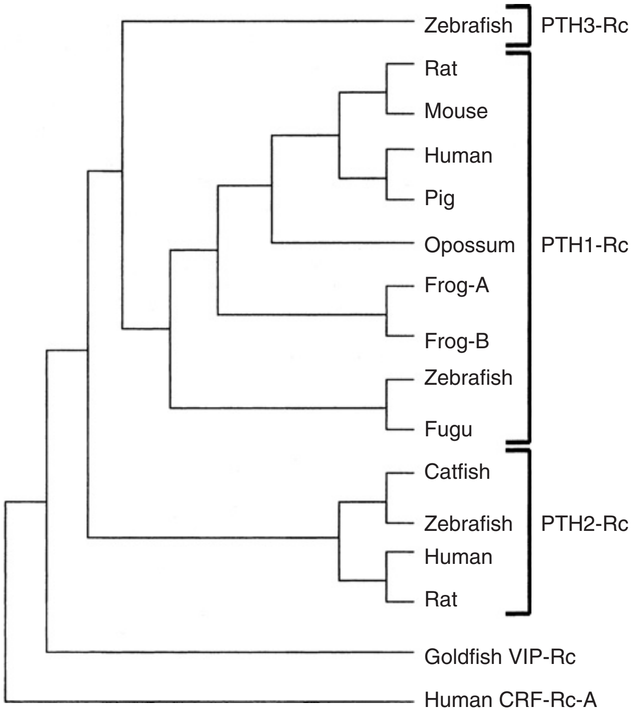

FIGURE 4 A phylogenic dendogram of PTH receptor subtypes. Analysis was reported by Rubin and Juppner (1999). VIP-Rc, vasoactive intestinal polypeptide receptor; CRF-Rc-A, corticotropin-releasing factor receptor A.

thus making the interpretation of structure-function relationships quite difficult.

PTH RECEPTOR TYPE 1

The ~87-kDa N-glycosylated PTHR1 is a member of the class II hepta-helical transmembrane domain G protein-coupled receptors. Class II comprises receptors recognizing peptide hormones ranging in size from 27 to 173 amino acid residues and it includes receptors for secretin, glucagon, calcitonin, growth hormone-releasing hormone, corticotropin-releasing hormone, vasoactive intestinal peptide, pituitary adenylyl cyclase-activating peptide, gastric inhibitory peptide, and glucagon-like peptide 1 (Juppner, 1994, 1995; Segre and Goldring, 1993). The putative hepta-helical structure, which defines the extracellular, TM, and cytoplasmic domains, was derived from structural homology studies (Abou-Samra *et al.*, 1992; Juppner *et al.*, 1991) and confirmed by epitope tag mapping of the extracellular and cytoplasmic domains (Xie and Abou-Samra, 1998). Class II GPCRs have no significant sequence identity (<12%) with other GPCRs. In addition, only 50 amino acid residues are strictly conserved, indicating their early emergence in evolution. The conserved residues are located predominantly in the N-ECD and the TM domains and must play an important structural and functional role.

The distinct features of the class II GPCRs include an N-ECD of ~160 residues, which is intermediate in length between those of the glycoprotein hormone (~400 residues) and aminergic receptors (~35 residues); a highly conserved pattern of 8 cysteines (6 in the N-ECD, 1 in the ECL1, and 1 in the ECL2); and multiple potential N-glycosylation sites located within the N-ECD.

PTHR1 homologues from different mammalian species, such as rat, opossum, pig, human, and mouse, are most divergent within the N-ECD, the ECL1, and the carboxyl-terminal intracellular domain (C-ICD) (Abou-Samra *et al.*, 1992; Juppner *et al.*, 1991; Kong *et al.*, 1994; Schipani *et al.*, 1993; Smith *et al.*, 1996). Evidently, the divergence in the response of different PTHR1 homologues to truncated sequences and analogs of PTH and PTHrP is instrumental in defining important functional domains within the PTHR1. For example, despite their 91% homology and similar apparent dissociation constant of ~10 n*M* for PTH-(1–34), rat and human PTHR1 have very different affinities for the antagonist PTH-(7–34) (K_d = 14 and 4385 n*M*, respectively) (Juppner *et al.*, 1994).

PTH RECEPTOR TYPE 2 AND TIP39

PTHR2, which has been cloned from rat and human cDNA libraries, selectively binds PTH, but not PTHrP (Usdin *et al.*, 1995, 1999a). *In situ* hybridization studies have identified a high level of expression of PTHR2 in brain and lower levels in the exocrine pancreas, epididymis, artrial and cardiac endothelium, vascular smooth muscle, lung, placenta, and vascular pole of renal glomeruli (Usdin *et al.*, 1995, 1999a). However, little is known about its physiological role in these tissues. While the tissue distribution, and particularly the lack of PTHR2 expression in kidney and bone, suggests a limited physiologic role in mineral metabolism, the distinct ligand specificity of PTHR2 has provided insight into the current model of PTH ligand–receptor interactions.

Usdin *et al.* (1999b) isolated a novel peptide from bovine hypothalamus, tuberofundibular peptide 39 (TIP39), whose binding affinity and stimulating activity for the PTHR2 are similar to those of PTH (Usdin *et al.*, 1995, 1999b). In contrast to PTH, TIP39 does not appreciably activate adenylyl cyclase in COS-7 cells transiently transfected with either human or rat PTHR1 (Usdin *et al.*, 1999b), but instead binds to them with moderate affinity (Hoare *et al.*, 2000). A homology search reveals that 9 out of the 39 residues of TIP39 are identical to bPTH, and most of them are located in the midregion of the molecule. The physiological role of the TIP39 – PTHR2 system remains to be established.

Several lines of evidence suggest that PTH is unlikely to be a physiologically important endogenous ligand for PTHR2. These include (1) different ligand rank order of intrinsic activity of a series of PTH analogs in the human and rat PTHR2, (2) considerably lower intrinsic activities and relative potencies of PTH-like ligands at the rPTHR2 than at the hPTHR2, (3) the partial agonist effect of PTH-based peptides when compared to bovine hypothalamic extracts (Hoare *et al.*, 1999a), and, last but not least, (4) the discovery that TIP39, a peptide distantly related to either PTH or PTHrP, is a potent and selective activator of the PTHR2 (Usdin *et al.*, 2000).

Although PTHR2 may not be the physiological target for PTH or PTHrP, its structural resemblance to PTHR1, its high-binding affinity, specific cross-linking, and effective coupling to PTH-induced intracellular signaling pathways make it an attractive target for exploring structure-function relations in the PTH/PTHrP-PTHR1 system. Analysis of the photoconjugates obtained upon cross-linking ^{125}I-Bpa^1-PTH and ^{125}I-Lys^{13}(*p*Bz$_2$)-PTH to hPTHR2 stably expressed in HEK293 cells (HEK293/BP-16, ~160,000 Rc/cell) revealed that both hPTHR1 and hPTHR2 use analogous sites for interaction with positions 1 and 13 (Behar *et al.*, 1999).

PTH RECEPTOR TYPE 3

Two PTH receptor alleles, one highly homologous to human PTHR1 and the other a novel PTHR3, have been cloned by genomic PCR from zebrafish (z) DNA.

Although these receptors exhibited 69% similarity (61% identity) with each other, neither of them exhibited as great a degree of homology with zPTHR2 (Rubin *et al.*, 1999). Zebrafish PTHR1 and zPTHR3 showed 76 and 67% amino acid sequence similarity with hPTHR1, respectively, but similarity with hPTHR2 was 63 and only 59% for both teleost receptors (Rubin and Juppner, 1999). Recombinant zPTHR3, transiently expressed in COS-7 cells, exhibited more efficient adenylyl cyclase activity when stimulated by [Ala^{29},Glu^{30},Ala^{34},Glu^{35},Tyr^{36}]fugufishPTHrP-(1−36)NH_2 and [Tyr^{36}]hPTHrP-(1−36)NH_2 (EC_{50} = 0.47 and 0.45, respectively) than by [Tyr^{34}]hPTH-(1−34)NH_2 (EC_{50} = 9.95 n*M*).In addition, zPTHR3 showed higher affinity to the PTHrP analogs than to the PTH analog. Finally, zPTHR1 activated the inositol phosphate (IP) pathway but zPTHR3 did not (Rubin and Juppner, 1999).

When these results are taken together, compared to the nondiscriminatory interaction of PTHR1s with PTH and PTHrP and the selectivity of PTHR2 for PTH, zPTHR3 emerges as the preferential target for PTHrP-derived agonists. Interestingly, some compelling findings suggest the presence of a PTHrP-selective receptor in rat supraoptic nucleus, which is distinct from PTHR1, whose activation leads to the release of Arg-vasopressin (Yamamoto *et al.*, 1997, 1998). We may speculate that since PTHR1 from all known species appear to have similar structural and functional properties, it is likely that the mammalian homologue of zPTHR3, when identified, will interact preferentially with PTHrP.

Receptor Chimera

PTH receptor subtype chimeras, deletion mutation, and point mutations have been used to explore the functional domains in the receptor involved in ligand binding and signal transduction (Gardella *et al.*, 1994; Juppner *et al.*, 1994; Lee *et al.*, 1994). Rat/human and rat/opossum PTHR1 chimeras revealed that the N-ECD plays an important role in binding of the amino-truncated PTH-(1−34)-derived peptides, such as the antagonist PTH-(7− 34) (Juppner *et al.*, 1994). Chimeras with the N-ECD of hPTHR1 have considerably higher binding affinity for PTH-(7−34), PTH-(10 −34), and PTH-(15−34) than the reciprocal chimera where the N-ECD is from the rPTHR1. In addition, deletion of the sequence 61–105 (encoded by exon E2) from the N-ECD did not affect the binding of either PTH-(1−34) or PTH-(7−34) (Juppner *et al.*, 1994). Therefore, this region, which is much more variable among the human, rat, and opossum receptor species than the rest of the N-ECD, does not contribute to the difference in binding affinity of PTH-(7−34) in the rat and human PTHR1. Interestingly, the ectopic regions of TM5 (residues S^{370} and V^{371}) and TM6 (residue L^{427}), which provide important interactions with the extreme amino-terminal residues of PTH and PTHrP, have been found to participate in the binding and signaling of [Arg^2]PTH-(1−34) (Gardella *et al.*, 1994). This analog is a weak partial agonist for cAMP stimulation through rPTHR1 and full agonist for cAMP stimulation through the opossum PTHR1 (Gardella *et al.*, 1991). Reciprocal specific point mutations of residues in rPTHR1 with residues from oPTHR1 (S370A, V371I, and L427T) increased binding affinity to the mutated rPTHR1 to the level observed for the wild-type opossum receptor, yet without affecting the binding of PTH-(1−34). Only one of these mutations in the rPTHR1 (S370A) conferred agonist activity to [Arg^2]PTH-(1−34) (Gardella *et al.*, 1994). The tolerance for the deletion of residues 61–105, which are located in the N-ECD, was utilized to replace it with an epitope tag derived from *Haemophilus influenza* hemagglutinin (HA) without affecting receptor functions, thus generating a powerful tool for monitoring receptor expression levels (Lee *et al.*, 1994). However, deletions of residues 31–47 near the amino terminus and residues 431–440 in the ECL3 were both detrimental to the efficient binding of PTH-(1−34) (Lee *et al.*, 1994).

Luck and colleagues reported that the amino-terminal fragment PTH-(1−14), which encompasses the principal activation domain, is equally potent in stimulating adenylyl cyclase in rPTHR1 and in the N-ECD-truncated rPTHR1 (Carter *et al.*, 1999b; Luck *et al.*, 1999). In contrast, PTH-(1−34) was ~100-fold weaker in potency with N-ECD-truncated rPTHR1 than PTH-(1−14). An alanine scan identified R^{186} in the PTHR1 as critical for the cAMP response only in the case of PTH-(1−14) but not for PTH-(1−34) (Luck *et al.*, 1999). Lack of photocross-linking of fully biologically active ^{125}I-Lys^{13}(*p*Bz_2)-PTH-(1−34) to [R186A/K]hPTHR1 mutants (Adams *et al.*, 1998) suggests that a contact site in the proximity of R^{186} contributes bimolecular interactions with PTH that are crucial for the signaling activity of PTH-(1−14). In addition, Carter and coworkers, carrying out Ala scan analysis and hydrophylic-to-hydrophobic substitutions in the 182–190 sequence of rPTHR1, identified by homolog-scanning mutagenesis strategy to be a candidate for a ligand-binding site (Lee *et al.*, 1995a), suggest that F^{184} and L^{187} are important deterninants of functional interaction with residues 3–14 in PTH (Carter *et al.*, 1999b).

Homolog-scanning mutagenesis (Cunningham *et al.*, 1989) is a powerful technique. It generates chimeric receptors by systematically replacing segments of the PTHR1 with homologous segments of other class II GPCRs and has the potential to maximize surface expression and minimize perturbation of receptor conformation. Exploring the extracellular domains of the rPTHR1 by substituting the corresponding segments of the homologous rat secretin receptor revealed that the ectopic end of TM1 and the carboxyl-terminal ends of ECL1, ECL2, and ECL3 are involved in ligand binding (Lee *et al.*, 1995a). In ECL3, two specific residues, W^{437} and Q^{440}, were identified as major contributors to agonist binding. Interestingly, these two mutations did not affect the binding affinity of PTH-(3−34), suggesting that these residues are involved in the interaction with the critical amino terminus of the hormone (Lee *et al.,* 1995a). Two chimeric receptors in which the entire amino-terminal domains of corticotropin-releasing receptor 1 and hPTHR1 were exchanged bound analogs of their cognate receptors with a specificity determined by the N-ECD (Assil *et al.,* 2001).

Although calcitonin (CT) and PTH share little sequence homology, their functional domains have a similar organization. In both hormones the N-terminal portions function as activation domains, whereas the C-terminal portions contain the principal binding determinants. Although similar in structure, CTR and PTHR1 class II receptor glycoproteins have only 42% homology and are selectively activated only by their respective ligands. Bergwitz *et al.* (1996) created reciprocal CT-Rc/PTHR1 chimeras in which the N-ECD was exchanged between the two receptors. Similarly, chimeric ligands were synthesized in which the activation and binding domains of each ligand were exchanged to create CT/PTH hybrid peptides. Using a COS-7 mammalian expression system to assess ligand binding and cAMP accumulation, it was demonstrated that the reciprocal hybrid ligands [CT-(1− 11)/PTH-(15−34) and PTH-(1−13)/CT-(12−32)] do not activate normal CT- or PTHR1; they can, however, activate their respective N-ECD-PTHR1/CT-Rc and N-ECD-CT-Rc/PTHR1 chimeras. This interaction was dependent on the cognate receptor's N-ECD binding the hybrid with the cognate C-terminal portion. These chimeric receptors were then activated by the amino-terminal portion of the ligand interacting with the membrane-embedded domains of the receptor and the associated ECLs.

Vilardaga *et al.* (2001a) have created and evaluated the functionality of 27 PTHR1/secretin receptor chimeras. Their data demonstrate that substitution of the C-terminal half (residues 105−186) of the N-ECD of PTHR1 with a secretin receptor homologous segment did not reduce affinity for PTH, but did abolish signaling in response to PTH. Further analysis revealed that residues 146−186 in the N-ECD of PTHR1 are critically important for receptor activation. The data obtained suggest the existence of one distinct binding site in the receptor's far N-terminal region (residues 1−62, site 1) that participates in high-affinity PTH binding, in addition to a second distinct binding site in the C-terminal half of the N-ECD (residues 146−186, site 2) that provides critical contacts for receptor activation. In the absence of site 1, higher concentrations of hormone are required to activate the receptor, whereas the absence of site 2 results in loss of signal transduction without the loss of high-affinity PTH binding. In the same study, a fundamental role in receptor activation was assigned to TM helices 3 and 6 (Vilardaga *et al.*, 2001a).

Another series of experiments confirms the important role of PTHR1 TMs in ligand recognition and receptor structure. Mutation of a single amino acid (N192I) in the TM2 of the secretin receptor to the corresponding residue in the PTH receptor produced PTH binding and functional signaling by the secretin receptor (Turner *et al.,* 1996a). The reciprocal mutation in the PTHR1 (I234N) produced a PTHR1 that was responsive to secretin. Neither mutation significantly altered the response of the receptors to their own ligands. These results suggest a model of specificity wherein TM residues near the extracellular surface of the receptor function as a selectivity filter that blocks access of the wrong ligands to sites involved in receptor activation (Turner *et al.,* 1996a).

The discriminatory domains in PTHR2, which allow response to PTH but not PTHrP, were studied using PTHR1/PTHR2 chimeras with reciprocal exchanges among N-ECD, ECLs, and portions of the TMs (Bergwitz *et al.,* 1997; Turner *et al.,* 1998). The chimeric receptor N-ECD-PTHR1/PTHR2 responded similarly to PTH and PTHrP (EC_{50} = 1.1 and 1.3 n*M*, respectively). However, this chimera had 100-fold higher apparent affinity for PTH than for PTHrP (Turner *et al.,* 1998). These findings suggest that in addition to the discriminatory role of the N-ECD, which predominantly affects binding, other domains of the PTHR2 may contain sites that restrict activation by PTHrP. These sites were located by generating PTHR2 mutants in which single or multiple nonconserved TM domain residues were mutated to the corresponding PTHR1 residues. Mutations within TM3 and 7 of PTHR2 (I244L in TM3 and C397Y, L399M and F400L in TM7) resulted in only partial recovery of affinity toward PTHrP. Turner *et al.* (1998) therefore concluded that the extracellular juxtamembrane portions of the TMs function as a selectivity filter or barrier that discriminates between Ile^5 and His^5 in PTH and PTHrP (Behar *et al.,* 1996a; Gardella *et al.,* 1996a), respectively, by accommodating the first and causing destabilizing interactions with the latter.

Bergwitz *et al.* (1997) arrived at similar conclusions employing PTH/PTHrP point hybrids, $[Trp^{23},Tyr^{36}]$- and $[Ile^5,Trp^{23},Tyr^{36}]PTHrP\text{-}(1-36)NH_2$. Whereas both analogs were equipotent and had similar affinities to the PTHR1, only the former was an antagonist of PTHR2 (Gardella *et al.,* 1996a). Probing the pharmacological properties of these analogs with PTHR1/PTHR2 domain, cassette, and point-mutated chimeras revealed that I^{244} at the ectopic portion of TM3, as well as Y^{318} near the carboxyl end of ECL2, provide functional interactions with position 5 of the ligands that are involved in the PTH/PTHrP specificity switch (Bergwitz *et al.,* 1997).

Clark *et al.* (1998) studied PTHR1/PTHR2 chimeras in which the N-ECD and ECL3 of the two receptors were interchanged. They found that both domains in both receptors interact similarly with PTH and contribute to the differential interaction with PTHrP. Introduction of the ECL3 of PTHR2 into PTHR1 increased PTH- and PTHrP-stimulated adenylyl cyclase activity and maintained high binding affinity to PTH but eliminated high-affinity PTHrP binding. Similarly, exchanging ECL3 in PTHR2 for the one from PTHR1 preserved high-affinity binding but reduced the response to PTH. Interestingly, Q^{440} in the ECL3 of PTHR1 is important for PTH-(1−34) binding (Lee *et al.,* 1995a) and is predicted to participate in the binding pocket that accommodates Val^2 in the ligand (Rolz *et al.,* 1999). The corresponding residue in PTHR2 is R^{394}. Introduction of the Q440R mutation in the ECL3 derived from PTHR1 and the R394Q mutation in the ECL3 derived from PTHR2 restored the function of ECL3 chimeric receptors. Moreover, simultaneous interchange of N-ECDs and ECL3s eliminated agonist activation but not binding for both receptors. Simultaneous elimination of the E2-coded sequence (residues 62–106) from the N-ECD of PTHR1 and introducing the Q440/R394 mutation into the ECL3 of the PTHR1 restored function in the PTHR2 chimera. Taken together, these results suggest that interaction between N-ECD and ECL3 in PTHR1 is important for PTHrP recognition. To achieve high-affinity binding of PTHrP to the mutated PTHR2, additional high-affinity interaction sites for PTHrP must be identified in PTHR1 and introduced to the PTHR2 (Clark *et al.,* 1998).

The reciprocal mutations of specific homologous domains and residues identify and delineate potential residues that are critical for local interactions with ligands of different pharmacological profiles and specificities and thus provide important insights into bimolecular ligand−receptor interactions. These studies have pointed to at least three critically important functioning domains on the extracellular surface of the PTHR1: (1) the N-ECD, which largely determines ligand-binding specificity by interactions with the C-terminus of PTH-(1−34), (2) the C-terminal region of the N-ECD, in particular residues 146−186, which plays a role in ligand orientation and subsequent receptor activation, and (3) the TM5/ECL3/TM6 region of the receptor, which interacts with the N-terminal activation domain in PTH. Taken together, receptor chimera-based studies indicate that class II GPCRs share a similar overall structure with multiple functionally important, ligand-specific domains. These domains are sufficiently different to permit synthetic hybrid ligands to bind and efficiently activate the complementary receptor chimeras.

Other Site-Directed Mutagenesis Studies

Mutagenesis has been instrumental in identifying polar residues within the hydrophobic TM domains of PTHR1 as important determinants of receptor function (Gardella *et al.,* 1996b; Turner *et al.,* 1996a). The polar residues R^{233} and Q^{451} located in TM2 and TM7, respectively, are highly conserved within class II GPCRs. Gardella *et al.* (1996b) found that mutating either R^{233} or Q^{451} resulted in reduced binding affinity and transmembrane signaling by the agonist but did not affect the binding of PTH-(3−34). These findings suggest that R^{233} and Q^{451} play important roles in receptor function by contributing to the interaction with the two critical N-terminal residues in PTH-(1−34) and thus affect affinity and signaling. Combining both mutations, as in R233Q/Q451K, restored the binding affinity of the agonist almost to the wild-type receptor level but was devoid of activation of PTH-mediated cAMP or inositol phosphate signaling pathways. These results strongly suggest that residues in TM domains 2 and 7 are linked functionally, are proximal to each other, as in the bacteriorodopsin, and are involved in agonist-induced conformational changes affecting coupling to G protein (Gardella *et al.,* 1996). Moreover, mutation of three residues (S^{227}, R^{230}, and S^{233}) predicted to be aligned on the same face of TM2 resulted in blunted PTH-(1−34)-stimulated adenylyl cyclase response and lower binding affinity for the agonist despite efficient cell surface expression (Turner *et al.,* 1996a). The same mutation at the corresponding sites in another member of the class II GPCRs, the secretin receptor, resulted in a similar reduction in adenylyl cyclase activity. Taken together, these studies led Turner and colleagues to propose that this ectopic region in TM2 participates in a signal transduction mechanism common to class II of GPCRs.

The Role of Cysteines in PTHR1

Cysteines are thought to play a critical role in the assembly and conformation of receptors. Cys→Ser mutations of any of the six cysteines in the N-ECD of PTHR1 severely impaired expression of the mutated receptor (Lee *et al.,* 1994). These six cysteines are highly conserved in class II GPCRs and therefore must contribute critically to receptor function. Mutations C281S or C351S, in the ECL1 and ECL2, respectively, resulted in a reduced level of cell surface expression and compromised binding affinity. The double mutant C281S/C351S, however, displayed significantly improved binding affinity, suggesting that these cysteines are involved in a disulfide bridge connecting the first and second ECLs as they are in rhodopsin and βAR (Lee *et al.,* 1994).

Elucidation of the distinct pattern of the three disulfide bridges formed by the six cysteines in the extracellular N-terminal domain of PTHR1 is a major accomplishment (Grauschopf *et al.,* 2000). Bacterial expression of the N-ECD in inclusion bodies was followed by oxidative refolding, which generated stable, soluble, monomeric, and functional protein (Grauschopf *et al.,* 2000). The N-ECD binds PTH-(1−34) with an apparent dissociation constant of 3–5 μM. Analysis of the disulfide bond pattern revealed the following pairwise arrangement: C^{131}-C^{170}, C^{108}-C^{148}, and C^{48}-C^{117} (Grauschopf *et al.,* 2000). This nonsequential pattern was validated by the structural elucidation of the N-ECD of the corticotropin-releasing factor receptor (CRFR), another member of the family B (class II) GPCRs, by Grace *et al.* (2004). They discovered a short consensus repeat (SCR) fold as the major structural element of the N-ECD of CRFR; it involves the six Cys's as well as a salt bridge and several other critical residues, all of which are conserved among family B GPCRs. It is therefore likely that the SCR motif is relevant to the entire family B of GPCRs (Grace *et al.*, 2004, 2007).

In addition to the six Cys's in the N-ECD and the two Cys's in ECLs 1 and 2, human PTHR1 has five more cysteines, distributed across the ICL1, ICL3, TM7, and the C-terminal intracellular domain (C-ICD). Their role in receptor structure and function was recently studied (Thomas *et al.*, 2007). A series of PTHR1 mutants was created with the cysteines either substituted by leucine or alanine or removed by introduction of an enzyme cleavage site. Assessment of expression levels and the biological function of the mutant receptors demonstrated that one position in particular, namely C217 of the ICL1, is critically important for transport as well as function of PTHR1.

Mutated Receptor-Based Genetic Disorders

Jansen's Metaphyseal Chondrodysplasia

Jansen's metaphyseal chondrodysplasia (JMC) is a rare form of short limb dwarfism associated with abnormalities in endochondral skeletal development, hypercalcemia, and hypophosphatemia, despite normal levels of PTH and PTHrP. Three missense mutations in the PTHR1 coding region, H223R, T410P, and I458R, have been discovered in patients with the disease (Schipani *et al.*, 1995, 1996, 1999). PTHR1 carrying any one of these mutations display constitutive, ligand-independent activation of the cAMP signaling pathway when tested *in vitro*.

The H223R, T410P, and I458R mutations are located at the cytoplasmic base of TM2, TM6, and TM7, respectively. In COS-7 cells transiently expressing the human I458R PTHR1, basal cAMP accumulation was approximately eight times higher than in cells expressing the recombinant normal receptor. Furthermore, the I458R mutant showed higher activation by PTH than the wild-type receptor in assays measuring the activity of downstream effectors, adenylyl cyclase, and PLC. Like the H223R and T410P mutants, the I458R mutant does not constitutively activate basal inositol phosphate accumulation. Interestingly, these mutations all occur at TM regions near the intracellular loops of PTHR1 that are hypothesized to interact with G proteins or other intracellular partners to trigger the subsequent signaling cascade.

These same mutations in PTHR1 have also been utilized to identify PTH and PTHrP analogs with inverse agonist activity. Two peptides, [Leu^{11},D-Trp^{12}]hPTHrP-(7 −34)NH$_2$ and [D-Trp^{12},Tyr^{34}]bPTH-(7−34)NH$_2$, which are highly potent antagonists for the wild-type PTHR1, exhibited inverse agonist activity in COS-7 cells expressing either mutant receptor (H223R or T410P) and reduced cAMP accumulation by 30–50% with an EC$_{50}$ of approximately 50 n*M* (Gardella *et al.*, 1996c). [D-Trp^{12},Tyr^{34}]bPTH-(7−34)NH2 was subsequently shown to selectively inhibit Gs/PKA activation while having no apparent activity for Gq/PKC stimulation (Gesty-Palmer *et al.*, 2006). Such inverse agonist ligands may be useful tools for exploring the different conformational states of the receptor, as well as leading to new approaches for treating human diseases with an underlying etiology of receptor-activating mutations.

Blomstrand's Chondrodysplasia

Blomstrand's osteochondrodysplasia (BC) is a rare lethal skeletal dysplasia characterized by accelerated endochondral and intramembranous ossification (Blomstrand *et al.*, 1985; Leroy *et al.*, 1996; Loshkajian *et al.*, 1997; Young *et al.*, 1993). The phenotype of BC is strikingly similar to PTHR1 knockout mice, which display prominent pathology in the growth plate (Lanske *et al.*, 1999). In both the human disease and the PTHR1-ablated mouse model, the growth plate is reduced in size because proliferating chondrocytes lack the normal columnar architecture as well as a greatly reduced zone of resting cartilage. This overall similarity of phenotype suggests that an inactivating mutation of PTHR1 is the underlying genetic defect in BC. To date, two types of inactivating mutations have been documented in BC patients (Karaplis *et al.*, 1998; Zhang *et al.*, 1998). The first is a single homozygous nucleotide exchange in exon E3 of the PTHR1 gene. This alteration introduces a P132L mutation in the N-ECD of the receptor (Zhang *et al.*, 1998). Proline 132 is conserved in all mammalian class II GPCRs. COS-7 cells expressing a GFP-tagged mutant receptor do not accumulate cAMP in response to PTH or PTHrP and do not bind radiolabeled ligand, despite being expressed at levels comparable to GFP-tagged wild-type PTHR1. Thus, while full-length PTHR1 is being synthesized, it does not bind the ligand and it is functionally inactive.

Another mutation in PTHR1 detected in BC patients results in the synthesis of truncated receptor fragments (Karperien *et al.*, 1999). Sequence analysis of all coding exons of the PTHR1 gene identified a homozygous point mutation in exon EL2 with one absent nucleotide (G at position 1122). This missense mutation produces a shift in the open reading frame, leading to a receptor truncated after amino acid 364 located in the ECL2. The mutant receptor, therefore, lacks TMs 5, 6, and 7.

Jobert *et al.* (1998) described a third point mutation in PTHR1 associated with BC. This mutation (G→A at nucleotide 1176) leads to the deletion of 11 amino acids (residues 373–383) in the TM5 of the receptor. The mutated receptor is well expressed in COS-7 cells, but does not bind PTH or PTHrP and fails to elevate cAMP and inositol phosphate in response to these ligands.

INTEGRATED STUDIES OF LIGAND–RECEPTOR INTERACTIONS

Two traditional and indirect approaches, one "ligand centered" and the other "receptor centered," have been pursued to further understand the ligand − PTHR interaction and each has made important contributions (see preceeding sections). The hormone-centered approach succeeded in mapping functional domains within the hormone that affect receptor binding and activation. In some cases, structural features responsible for biological properties have been identified down to the level of a single amino acid, which sometimes led to the development of important therapeutics. In the PTH/PTHrP field, the identification of the architecture of functional domains and the development of potent antagonists, partial agonists, and inverse agonists, signaling-selective analogs, and potent *in vivo* anabolic agents generate a very impressive list of accomplishments. However, this approach cannot be used to deduce the receptor domains that are in contact with the hormone across the interface. Furthermore, in some cases, modifying the primary structure of the hormone may result in altering the pattern of bimolecular interactions with the receptor. Some structural modifications of the hormone may directly alter its interaction with an important complementary structural feature of the receptor; others may affect bioactivity through either local or global conformational changes within the hormone that prevent formation of an optimal "bioactive conformation." In essence, the hormone-centered approach is "blind" to the structure of the receptor.

The "receptor-centered" approach has also succeeded in providing valuable insights. Point-mutated and chimeric PTHR − interspecies of PTHR1, such as rat with opossum receptor, or interhormone receptors, such as PTHR1 with calcitonin or secretin receptors − revealed the importance of specific receptor domains and single amino acids necessary for receptor function. However, analysis of the functional consequences that result from modifying the receptor structure alone cannot be used to identify unequivocally the interacting complementary structural elements in the hormone. Furthermore, one usually cannot distinguish R modifications that disrupt function as a result of local changes in an important "contact site" or a global conformational change. While local changes affect R interaction with a site in the hormone directly, global conformational changes lead to extensive modification of R topology, thereby altering interactions with the hormone indirectly. Hence, despite important contributions by both lines of investigation, conclusions drawn from the hormone-centered and receptor-centered approaches have inherent limitations and are inferential at best.

X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy are the tools of choice for analyzing the structure of bimolecular complexes. Unfortunately, membrane-embedded GPCRs are generally not amenable to either x-ray or NMR analysis because of their hydrophobic nature and conformational flexibility. Despite years of effort in several GPCR systems, to date only two GPCRs, the bovine retinal photoreceptor, rhodopsin (Palczewski *et al.*, 2000), and the β_2-adrenergic receptor-2 (β_2AR) (Rasmussen *et al.*, 2007; Cherezov *et al.*, 2007) have been crystallized and subjected to high-resolution X-ray structural analysis. The crystal structure available for rhodopsin is in its resting state; hence, the mechanism of switching from the "ground" to "activated" state has not been directly revealed. In addition, rhodopsin is categorically different from other GPCRs. Its ligand, 11-*cis* retinal, is "pre-bound" covalently; hence, the dynamics of ligand association and dissociation do not pertain. In addition, conformational changes in rhodopsin are induced by light. Therefore, the nature of ligand−receptor interactions for rhodopsin are fundamentally different from other GPCRs.

The β_2AR was crystallized while binding an inverse agonist. Using different approaches, one group determined the structure at a 3.4 Å/3.7 Å level (Rasmussen *et al.*, 2007); the other at 2.4 Å resolution (Cherezov *et al.*, 2007). The cytoplasmic ends of the TMs and connecting loops are well resolved, but the structure of the extracellular regions (which play an important role in hormone binding in the PTH system) are either less resolved or could not be determined. The overall structure is closely similar to rhodopsin (which is used as a template in molecular modeling of many GPCRs, including PTHR). Of note is the observation that when β_2AR co-crystallizes with a ligand, the receptor remains in monomeric form; hence, receptor dimerization does not appear necessary for G protein activation.

Photoaffinity labeling has emerged as an effective methodology for studying interactions of biological macromolecules with their ligands (Chowdhry and Westheimer, 1979; Dorman and Prestwich, 1994; Hazum, 1983; Hibert-Kotzyba *et al.*, 1995). The resultant photocross-linked conjugate can serve as a starting point for mapping "contact domains," and even "amino acid to-amino acid contact points" between a biologically active compound and an interacting macromolecule (Bitan *et al.*, 1999; Blanton *et al.*, 1994; Boyd *et al.*, 1996; Girault *et al.*, 1996; Hadac *et al.*, 1999; Ji *et al.*, 1997; Kage *et al.*, 1996; Keutmann and Rubin, 1993; Kojr *et al.*, 1993; Li *et al.*, 1995; McNicoll *et al.*, 1996; Phalipou *et al.*, 1999; Williams and Shoelson, 1993).

A number of laboratories, including our own, have utilized photoaffinity scanning (PAS) to map the bimolecular interface between a peptide hormone and its cognate GPCR. The approach has numerous stringent requirements, the fulfillment of which is crucial for success. Bioactive, specific, cleavage-resistant, photoactivatable, and radiolabeled PTH analogs must be designed and synthesized. A rich and stable source of functional wild-type or mutant PTHR, overexpressed preferentially in a homologous cellular background, must be developed and fully characterized pharmacologically. For purifying either the intact ligand−receptor conjugate or its ligand−receptor conjugated fragments, it may be advantageous to work with epitopetagged receptor. It may also be helpful to have antibodies to various receptor extracellular epitopes for use in purification and analysis. The PAS approach requires devising an analytic strategy with sequential chemical and enzymatic cleavages; theoretical digestion maps to allow unambiguous identification of hormone-binding sites within the PTHR; possibly the synthesis or expression of receptor domains that contain the identified contact sites for conformational studies; and the generation of site-directed mutated PTHRs, their expression and pharmacological characterization, and their use in the PAS technology to validate and/or delineate emerging results. Last but not least, this approach requires access to tools that will allow integrating cross-linking data with receptor mutagenesis data and eventually with conformational analysis and molecular modeling data to generate a unified, experimentally based model of the hormone−receptor complex.

In summary, PAS technology is a multidisciplinary, integrated, iterative, and labor-intensive approach. Nevertheless, it is currently the only direct method that yields the best approximation of the actual ligand−receptor complex. Because of the nature of this approach, however, it cannot yield molecular structures of the same resolution as those obtained by either x-ray crystallography or NMR analysis.

Photoreactive Analogs

Early efforts to generate a photoreactive, radiolabeled, and biologically active analog of PTH aimed to identify the receptor as a distinct molecular entity (Coltrera *et al.*, 1981; Draper *et al.*, 1982; Goldring *et al.*, 1984; Wright *et al.*, 1987). All of these studies used poorly characterized ligands containing nitroarylazide-based photophores and reported molecular masses ranging between 28 and 95 kDa for the hormone − receptor complex. Shigeno *et al.* (1988a, 1988b) carried out a careful synthesis and characterization of the nitroarylazide-based photoligand and identified it as [$Nle^{8,18}$,Lys^{13}(N^{ε}-(4-N_3-2NO_2-phenyl),Tyr^{34}]PTH-(1–34)NH_2, a fully active analog in ROS 17/2.8 cells. Using this photoaffinity ligand, they were able to identify in the same cells a plasma membrane glycoprotein corresponding to the PTH receptor that had the apparent molecular mass of 80 kDa.

Introduction of the arylketone-based photoaffinity scanning methodology (Adams *et al.*, 1995; Bisello *et al.*, 1999; Han *et al.*, 2000; Nakamoto *et al.*, 1995; Suva *et al.*, 1997) into the field of calciotropic hormones and their corresponding receptors enabled a series of investigations on contact points between ligand and PTHR1 (Adams *et al.*, 1998; Behar *et al.*, 1999, 2000; Bisello *et al.*, 1998; Greenberg *et al.*, 2000; Suva *et al.*, 1997; Zhou *et al.*, 1997; Gensure *et al.*, 2001; Gensure *et al.*, 2003; Wittelsberger *et al.*, 2006a). Advantages of the benzophenone moiety as a photophore over the aryl azide moiety are numerous. A partial list includes the high efficiency of cross-linking (only a small amount is lost to hydrolysis), and very little nonspecific cross-linking. Photoactivation is carried out at a wavelength greater than 330 nm, in which proteins are less susceptible to photodegradation. In addition, there is excellent compatibility with the solid-phase peptide synthesis methodology. Furthermore, synthesis, purification, and biological evaluation can be conducted in the laboratory under normal ambient light conditions.

Radioiodination is the tagging method of choice because of its high specific radioactivity translating into high sensitivity of detection of the radiolabeled conjugated ligand−receptor complex and the fragments derived from it. Therefore, successful PAS analysis requires maintaining the connectivity between the radiotag and the photophore throughout the controlled degradation of the conjugated ligand−receptor complex. Modifications in PTH-(1−34), which include $Met^{8\text{ and }18} \rightarrow Nle^{8\text{ and }18}$, $Lys^{13,26,\text{and }27} \rightarrow Arg^{13,26,\text{ and }27}$, and $Trp^{23} \rightarrow$ 2-naphthylalanine^{23} (Nal), render the ligand resistant to the various chemical and enzymatic cleavage agents (i.e., CNBr, lysyl endopeptidase (Lys-C) and BNP-skatole, cleaving at the carboxyl side of Met, Lys, and Trp, respectively).

The fundamental requirement in any photoaffinity cross-linking study is that the photoreactive analogs have the same pharmacological profile as the parent peptide hormone. It will therefore be safe to assume that they share similar bioactive conformations and generate topochemically equivalent ligand−receptor complexes. The photoreactive benzophenone-containing analogs of PTH and PTHrP were designed specifically for PAS studies aimed at investigating the bimolecular interactions of the activation and binding domains of PTH and PTHrP with either PTHR1 or PTHR2 subtypes.

Identification of Contact Sites using Benzophenone-based Photoreactive Groups

Using the PAS methodology, contact sites have been identified for positions 1, 11, 13, 15, 18, 19, 21, and 27 in PTH and positions 1, 2, 19, 23, 27, 28, and 33 in PTHrP (Adams *et al.*, 1995; Behar *et al.*, 2000; Bisello *et al.*, 1998; Carter *et al.*, 1999a; Greenberg *et al.*, 2000; Zhou *et al.*, 1997; Wittelsberger *et al.*, 2006a; Gensure *et al.*, 2001; Gensure *et al.*, 2003). Two different photophores were used in different studies: *p*-benzoylphenylalanine (Bpa) (Behar *et al.*, 2000; Bisello *et al.*, 1998; Carter *et al.*, 1999a; Wittelsberger *et al.*, 2006a; Gensure *et al.*, 2001; Gensure *et al.*, 2003) and the Lys(N^{ε}-*p*-benzoylbenzoyl) [Lys(N^{ε}-$p$$Bz_2$]

(Adams *et al.*, 1995; Behar *et al.*, 2000; Greenberg *et al.*, 2000; Zhou *et al.*, 1997). The former has the benzophenone moiety attached to the peptide backbone through a β-carbon, whereas the latter is presented on a relatively long side-chain removed by six atoms from the backbone. These different modes of presentation of the benzophenone moiety may play a limited role in selecting cross-linking sites.

Photocross-linking of ^{125}I-[Bpa^x,Nle^{8,18},Arg^{13,26,27},Nal^{23},Tyr^{34}]bPTH-(1−34)NH$_2$ (Bpa^x-PTH) to the human PTHR1 stably overexpressed (~400,000 Rc/cell) in human embryonic kidney cell line 293 (HEK293/C21) typically generates an 87-kDa photoconjugate (Bisello *et al.*, 1998; Wittelsberger *et al.*, 2006a). Chemical digestions by CNBr and BNPS-skatole, which cleave at the carboxyl end of Met and Trp, respectively, and enzymatic digestions by endopeptidases Lys-C, Glu-C, or Arg-C, in addition to endoglycosidase F/N-glycosidase F (Endo-F), which deglycosylates aspargines at consensus glycosylated sites, generate an array of fragments. These radioiodinated fragments are characterized by SDS-PAGE, and the apparent molecular weights obtained are compared with the theoretic digestion restriction map of the photoconjugated receptor. Although the resolving power of PAGE is limited, the combination of consecutive cleavages (e.g., Endo-F followed by Lys-C followed by CNBr) carried out in reversed order (e.g., Lys-C followed by BNPS-skatole and BNPS-skatole followed by Lys-C) is extremely powerful. It generates a reproducible pattern of fragments delimited by specific end residues and the presence or absence of glycosylation sites. Comparing the putative digestion map of the PTHR1 with experimentally obtained fragment sizes identifies the sequence of the smallest radiolabeled ^{125}I-Bpa^x-PTH—PTHR1-conjugated fragment.

Using this approach, two Met residues, 414 and 425, present at the midregion and the extracellular end of TM6, emerged as potential cross-linking sites for ^{125}I-Bpa^1-PTH. Contact between residue 1 in PTH and M^{414} requires the N terminus of PTH to protrude into the 7 helical and hydrophobic TMs bundle. In contrast, contact with M^{425} can be achieved while the N terminus is dipping only superficially into the TM bundle.

These biochemical methods are often supplemented by molecular biology to provide additional resolving power to the PAS method. In the case mentioned earlier, transient expression of two point-mutated hPTHR1, [M414L] and [M425L], generated fully active receptors in COS-7 cells (Bisello *et al.*, 1998). ^{125}I-Bpa^1-PTH lost its ability to photocross-link to [M425L] but not to [M414L], thus suggesting that M^{425} is the putative contact site for position 1 in PTH.

Behar *et al.* (2000) reported that radioiodinated PTHrP-based agonist [Bpa^1,Ile^5,Tyr^{36}]PTHrP-(1−36) NH$_2$ (^{125}I-Bpa^1-PTHrP), which carries a photophore at the same position as the photoreactive PTH analog ^{125}I-Bpa^1-PTH, forms a contact with M^{425} in hPTHR1. This exciting finding confirms that the functional and conformational similarity between PTH and PTHrP extends to a common contact site for the N-terminal residue in the ligand. The location of this site at M^{425} in the ectopic portion of TM6 supports the prevailing view that these two hormones interact very similarly, if not identically, with the PTHR1. Unlike Bpa^1-PTH and Bpa^1-PTHrP, which have a single cross-linking site (M425), the radiolabeled antagonist Bpa^2-PTHrP (^{125}I-[Bpa^2,Ile^5,Arg^{11,13},Tyr^{36}]PTHrP-(1−36)NH$_2$) cross-links to both M425 and a proximal site within receptor region [415−425] (Behar *et al.*, 2000). In contrast, Bpa^2-PTH, a full agonist, cross-links to M425 only. This investigation provides strong support for a fundamental difference in the binding mode of PTH agonists versus antagonists.

Using similar PAS techniques, the cross-linking site for radiolabeled [Nle^{8,18},Lys^{13}(N$^{\varepsilon}$-p(3-I-Bz)Bz),Nal^{23},Arg^{26,27},Tyr^{34}]bPTH-(1−34)NH$_2$ [Lys^{13}(pBz$_2$)-PTH] was identified as Arg186 at the C-terminal end of the N-ECD (Adams *et al.*, 1998; Zhou *et al.*, 1997). Then, a site in the first extracellular loop (L261) was found to contain the contact site of [Lys^{27}(pBz$_2$)-PTH (Greenberg *et al.*, 2000), suggesting that the extracellular loops contribute to the specificity of the PTH − PTHR1 interaction. A study by Gardella and coworkers using PTHrP analogs containing Bpa at positions 23, 27, 28, and 33 in the C-terminal half of PTHrP found all contact points to lie within the N-ECD, namely regions [33−63], [96−102], [64−95], and [151−172], respectively (Gensure *et al.*, 2001). The difference in contact point between Lys^{27}(pBz$_2$)-PTH and Bpa^{27}-PTHrP is striking; however, the discrepancy may be attributed to differences in the hormone used, the photolabile moiety, or the receptor species used in the experiment.

A more recent study found that all contact points from the midregion of PTH lie within the C-terminal domain of the N-ECD: PTH analogs containing Bpa in positions 11, 15, 18, and 21 cross-linked to receptor regions [165−176], [183−189], [190−298], and [165−176], respectively (Wittelsberger *et al.*, 2006a). The contacts were used as distance restraints in molecular dynamics simulations and resulted in a substantial refinement of the PTH−PTHR1 model (Fig. 5). Specifically, (1) the overall receptor-bound conformation of the hormone is not extended, but bent; (2) helix [169−176] of the N-terminal extracellular domain (N-ECD) of the receptor is redirected toward the heptahelical bundle; and (3) the hormone traverses between the top of transmembrane (TM) helices 1 and 2, rather than between TM7 and TM1 (Fig. 5). The results point to a critical role of the midregion of PTH to fix, by extensive contacts within region [165−189] of the receptor, the entry of the hormone into the heptahelical bundle between TM1 and TM2. This anchorage orients the amino terminus into position to activate the receptor.

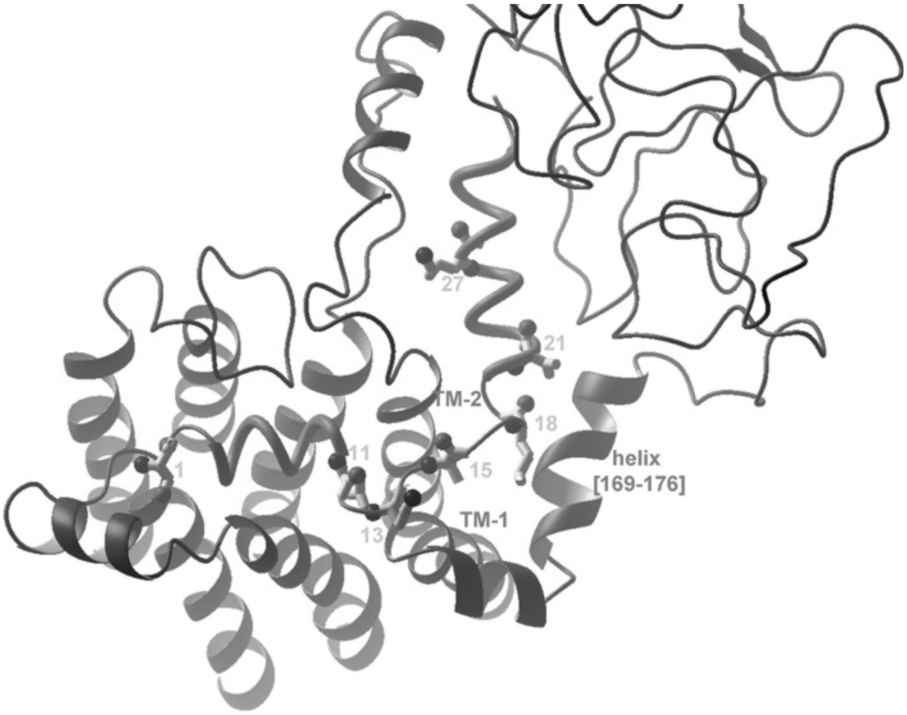

FIGURE 5 Experimentally derived model of the PTH–PTHR1 complex (Wittelsberger et al., 2006). Extracellular view of the receptor (green) illustrating interactions with PTH (orange), including PTH's N-terminal activation domain, midregion, and C-terminal binding domain. Side-chains at positions for which cross-linking sites were identified are displayed (yellow). The largest part of the N-ECD (residues 1–168), is shown for reference only (no structural features are implied).

REACHING THE RESOLUTION LIMIT OF BPA-BASED PHOTOAFFINITY CROSS-LINKING

In the course of defining contact sites for Bpa^{11}-PTH and Bpa^{21}-PTH using engineered Met-containing mutants of PTHR1, Wittelsberger *et al.* (2006a) observed a low MW band in the CNBr-digestion pattern of cross-linked conjugates that corresponded in size to free ligand, suggesting that cross-linking to the methyl group of methionine occurred. It is known that insertion of a Bpa radical can occur at the $\varepsilon -$ methyl of Met, and that subsequent CNBr treatment results in hydrolysis at that site, releasing a thiocyanomethyl derivative of the ligand (Kage *et al.*, 1996).

This finding instigated undertaking of a "Met scan" around position 176 of the receptor (Wittelsberger *et al.*, 2006b). Met was introduced individually at ten positions: 163, 168, 169, 171−176, and 183 of the N-ECD of PTHR1. Introduction of Met at 170 was not attempted because removal of the native Cys results in poor receptor expression (Lee *et al.*, 1994). The observations made after cross-linking of mutant receptors with both Bpa^{11}- and Bpa^{21}-PTH followed by CNBr treatment were striking: cross-linking occurs to M*et al*ong a wide range of the receptor domain, positions 163−173 (Fig. 6), a span of 11 amino acids. Importantly, when Met is incorporated at a site further away (e.g., position 183), cross-linking to Met does not occur. The range of "Met-reactivity" can be demarcated: Bpa^{21}-PTH cross-links to Met in positions 163 up to 173, but positions 174 and 175 are "out of reach." The study demonstrates a pronounced preferential reactivity of Bpa for Met over other amino acids. The region PTHR-(163−173) extends over 16 Å in the model (Fig. 6). The "Met-reactive" reach of positions 11 and 21 in PTH overlap considerably, despite being ten residues (13 Å) apart in the ligand (Fig. 6). The phenomenon is referred to as the "magnet effect" of Met (Wittelsberger *et al.*, 2006b). These data imply that when Met in the receptor is reported as the cross-linking site for a ligand, one needs to consider the possibility that such contact sites might be shifted or broader than actual owing to the presence of Met.

Another limitation of Bpa lies in its size and rotational freedom. The reactive radius of the benzophenone group was approximated as a sphere with a radius of 3.1 Å centered on the ketone oxygen (Dorman *et al.*, 1994). In Bpa, rotation around the $C\alpha - C\beta$ bond defined by angle χ_1 allows for significant increases in accessible distances (Fig. 7).

To define appropriate distance restraints for experimentally determined contact sites in molecular modeling, a model system was created (Wittelsberger *et al.*, 2008). The solution structure of PTH-(1−34) was used as determined by NMR (Pellegrini *et al.*, 1998b); Bpa was then introduced at a centrally located position replacing Arg^{20} (Fig. 8).

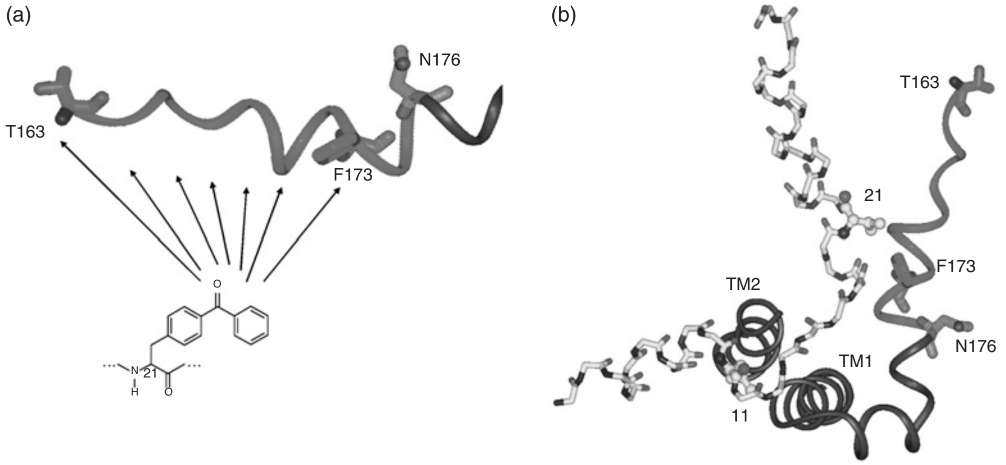

FIGURE 6 The "magnet effect" of Met. The "Met-reactive" region for Bpa^{11}-PTH and Bpa^{21}-PTH is colored light green. (A) The PTHR-(163–173) region has a 16 Å span. (B) Residues 11 and 21 in the ligand (yellow) are 13 Å apart, yet are "Met-reactive" within the same domain of PTHR (light green) (Wittelsberger et al., 2006b).

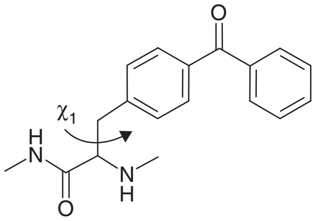

FIGURE 7 Structure of *p*-benzoylphenylalanine (Bpa) with the dihedral angle χ_1 denoted.

Next, the side-chain of Bpa^{20} was rotated from $\chi_1 = -60°$ to $\chi_1 = 60°$ to $\chi_1 = 180°$. For each of these three states, the distances from the reactive carbonyl group of Bpa to the side-chain of select residues toward the C-terminus (Lys^{27}) and toward the N-terminus (Asn^{10}) of the hormone were measured. As illustrated in Fig. 8, the distances between Bpa^{20} and K^{27} range from 7.0–15.5 Å; the distances between Bpa^{20} and Asn^{10} range from 8.1−17.2 Å. Similar conclusions can be drawn for the interactions between Bpa and the receptor. With the same exact ligand-binding mode, depending on the χ_1 rotational state of Bpa, a wide range of different amino acids of the receptor would be available for cross-linking; the distance variation from the reactive carbonyl in Bpa is approx. 10 Å. The study therefore concludes that it is imperative to use a distance constraint of at least 10 Å for experimentally determined contact points in MD simulations of ligand−receptor complexes, resulting in limited resolution.

Benzophenone-based photoaffinity scanning (PAS) was applied extensively in generating the model of PTH − PTHR1 because it provides direct information about sites of bimolecular contact. Although it lacks the resolution of x-ray crystallography, PAS provides "snapshots" of interactions by fixing contacts through covalent bond formation. An ensemble of contacts identified provides the set of constraints essential for molecular modeling. When PAS is combined with other approaches, an informative model of a hormone − Rc complex may emerge. In the PTH − PTHR system, however, we and others have taken the cross-linking methodology, based on Bpa photoactivation, toward its limit of resolution. Even new photolabile moieties, such as the trifluoromethyldiazirinphenylalanine-based Tdf group developed by Escher and colleagues (Fillion *et al.*, 2006), which lacks the Met selectivity of Bpa, still suffer from the inherent resolution limit of photoaffinity labeling, not to mention formidable challenges in synthesizing the photolabile moiety.

The Bpa-based technology does remain an excellent approach for the first rounds of investigations into a ligand−receptor system to obtain general landmarks and key contact regions.

Disulfide Capture

"Site-specific disulfide capture" or "Cys-trapping" is well suited to the PTH − PTHR system because it is most productively applied to systems in which a first-generation map of the ligand−receptor interface already has been generated. The approach applied to other GPCRs (Buck *et al.*, 2005a,b), is based on engineering a single Cys residue into a receptor domain postulated to be crucial for ligand-binding or activation. After demonstrating that the Cys-containing receptor mutant retains biological function, it can be used to capture Cys-containing peptide analogs of the cognate ligand for structural studies.

In this approach, one cysteine mutation is introduced into a specific site within the ligand and one in a region of the receptor predicted to interact with the specific mutated residue in the ligand. If these cysteines are in close proximity,

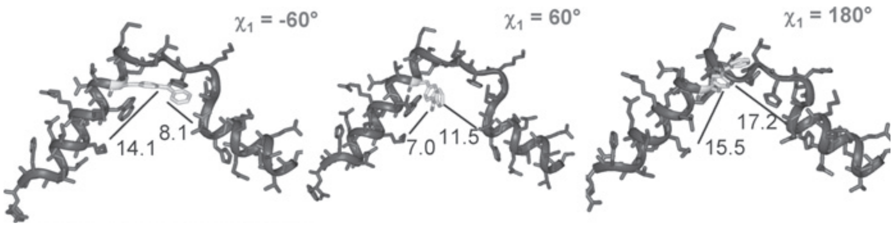

FIGURE 8 Model system based on the NMR structure of PTH-(1–34) (green). Distances (in Å) between the reactive carbonyl group of Bpa (yellow) and the side-chains of Asn^{10} and Lys^{27} are indicated in blue for the three staggered rotamers ($\chi_1 = -60°$, 60°, and 180°) (Wittelsberger *et al.*, 2008).

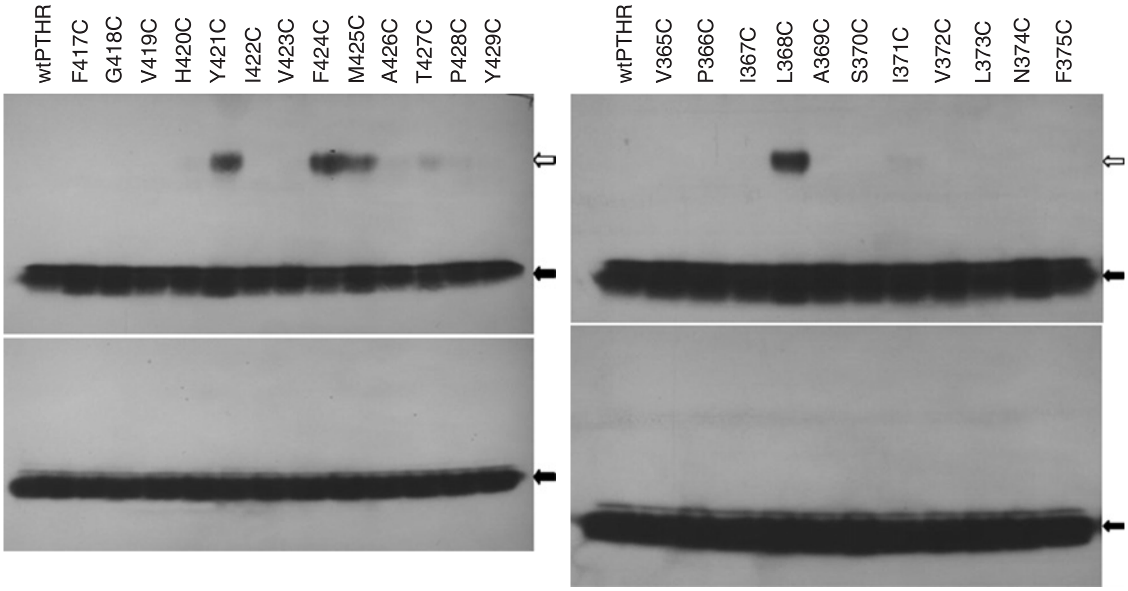

FIGURE 9 Disulfide-trapping of Cys^1-PTH to PTHR mutants with introduced cysteines in TM6 (left) and TM5 (right). Cells expressing the various mutant receptors were incubated with ^{125}I-labeled Cys^1-PTH and subsequently analyzed by SDS-PAGE in the absence (upper panel) or presence (lower panel) of reducing agent (β-mercaptoethanol). The white arrow indicates Cys^1-PTH cross-linked to receptors and the black arrow indicates the uncross-linked peptide.

upon ligand binding they form a disulfide bond. Because the geometry governing disulfide bond formation is more constrained than Bpa cross-linking, this novel approach can be employed to generate a more refined molecular model of the PTH − PTHR1 complex. Using a PTH analog containing a cysteine at position 1, Monaghan *et al.* (2008) probed a total of 24 positions in the top regions (toward the extracellular surface) of TM5 and TM6 of PTHR1: 11 positions in TM5 and 13 in TM6. Selection was based on the model of the PTH − PTHR1 complex (Wittelsberger *et al.,* 2006a). Four sites to which cross-linking occurred were identified (Fig. 9). Importantly, previous PAS studies, using a PTH analog with Bpa at position 1, only identified a single site of interaction.

SDS-PAGE analysis consistently showed that the Y421C, F424C, and L368C mutant receptors cross-link the peptide more effectively than the M425C PTHR1, as judged by the intensity of the cross-linked bands following autoradiography (Fig. 9). This suggests that in the natural binding state, position 1 of the ligand comes closer to Y421, F424, and L388 (or spends more time in their vicinity) than it does to M425. Although M425 remains a contact point, the predominance of Bpa^1-PTH interacting with M425, assumed in a previous report (Bisello *et al.,* 1998), no doubt reflects the preference of the Bpa moiety for methionine residues. When applied as constraints to the model of the PTH − PTHR1 complex, the new sites of cross-linking identified by the disulfide-trapping procedure allowed for a more detailed map of the receptor contacts of the N-terminus of PTH (Fig. 10).

In the case of the PTH − PTHR complex, the disulfide-trapping approach is heavily reliant on the previous successful application of Bpa PAS. For the platform built on the earlier method, it would not be possible to guide the placement of the Cys pairs. In addition,

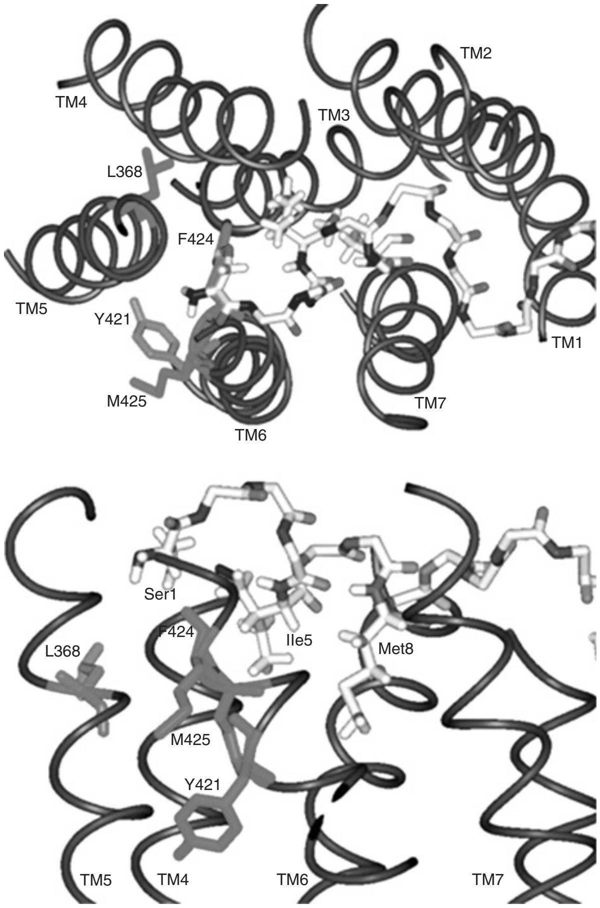

FIGURE 10 Model of the interaction of position 1 of PTH with PTHR obtained by Molecular Dynamic simulations. The four residues of PTHR that cross-linked to Cys^1-PTH are shown in green; the backbone of the ligand is shown in yellow (nitrogen = blue, oxygen = red). The side-chains of Ile^5 and Met^8 of the peptide are displayed for orientation. The top panel depicts a view from above the receptor; the lower panel depicts a side view.

prerequisite to application of the method is the availability of biologically active Cys-substituted hormone analogs and functional Cys-substituted receptor mutants.

One of the major advantages of the disulfide-trapping approach is that, unlike Bpa-mediated PAS, it allows for the simultaneous identification of numerous residues within the receptor that are in close proximity to a defined position in the ligand being investigated. Previous work with Bpa^1-PTH identified only a single residue, M425, of PTHR1 that was proximal to the N-terminus of the hormone (Bisello *et al.,* 1998): one ligand yields one contact. In contrast, the disulfide-trapping methodology amplifies the utility of each ligand probe: each probe can be used in a series of experiments to map the surface of interaction on the receptor. For example, in the investigation described earlier, 24 potential contact sites within TM5 and TM6 of PTHR1 were interrogated with a single PTH peptide, Cys^1-PTH. As a result of this scan, three additional points of interaction between position 1 of PTH and PTHR1 were defined. Based on the earlier-described demonstration, we are confident that disulfide-trapping can be applied successfully to other putative regions of PTH–PTHR interaction, and that a further refined model will emerge.

NMR Studies

Of course, there are clear advantages to obtaining data from direct structural studies of ligand–Rc interactions. The corticotropin-releasing factor (CRF) receptor (CRF-Rc) belongs to the same GPCR family B (class II) as PTHR1. An NMR study of the N-ECD of CRF-Rc identifies a short consensus repeat (SCR) motif as a characteristic structural determinant and suggests a hormone-binding site (Grace *et al.,* 2004). It also provides support for a two-step binding and activation sequence for the CRF-Rc. Recently, NMR analysis of the same protein in complex with the antagonist astressin reveals that the hydrophobic face of the amphipathic C-terminal segment of the ligand is in contact with the SCR motif, as well as the presence of characteristic intermolecular hydrogen bonds and a salt bridge (Grace *et al.,* 2007).

Mierke and coworkers analyzed the conformation of PTH while bound to the N-terminal extracellular domain (N-ECD) of PTHR1 (unpublished). A soluble form of the N-ECD of PTHR1 was previously expressed from insect cells using a baculovirus infection system (Monaghan, 2007). When PTH is added to the purified protein, transferred NOEs (trNOE) experiments demonstrate the formation of a C-terminal helix in the ligand during the interaction with protein. Saturation transfer difference (STD) experiments were carried out to determine the topological orientation of PTH-(1–34) while bound to the N-ECD. The data obtained identify the two aromatic residues, Trp-23 and Phe-34, to be buried deep in the N-ECD (unpublished).

Detection of Conformational Changes in the Receptor by Disulfide Cross-Linking

Photoaffinity cross-linking and other structural studies have provided valuable information about the nature of the ligand–receptor bimolecular complex. However, these approaches do not provide insights into the dynamics of receptor activation. The nature of the receptor “switch” from basal to activated state, triggered by binding of an agonist, is still not well understood for the vast majority of GPCRs, including PTHR1. The transmembrane helices (TMs) are likely to play a key role in conveying agonist-induced changes in conformation from the extracellular regions of the receptor to the cytoplasmic surface that interacts with G protein. TM3 and TM6 have been shown to play a role in PTHR1 activation (Vilardaga *et al*., 2001a) and to be involved in conformational changes critical for

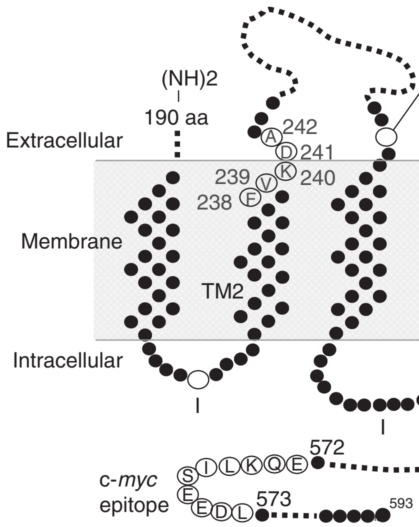

FIGURE 11 PTHR1 template and sites of mutation. Shown are amino acid residues A242, D241, K240, V239, F238 in TM2 and F447 in TM7 that were mutated to Cys (Thomas et al., 2008). Also shown is the location of two FXa cleavage sites in ICL3. For detection by Western blot, a c-myc tag was inserted between residues 572 and 573 of the C-ICD. The endogenous cysteines are shown as open circles (O).

G_s activation (Vilardaga *et al*., 2001b). The extracellular-facing region of TM2 of PTHR1 has been implicated in ligand binding (Gensure *et al*., 2003; Wittelsberger *et al*., 2006a), and TM7 has been suggested to interact with TM2 during the ligand binding and activation steps (Rölz *et al*., 1999; Gardella *et al*., 1996b).

The "*in situ* disulfide cross-linking strategy" (Ward *et al*., 2002; Zeng *et al*., 1999) is one approach to detect conformational changes in TM domains and was successfully applied to several GPCRs. A pair of cysteines is substituted into receptor regions (mostly TM regions) postulated to play a role in receptor activation (Han *et al*., 2005a, 2005b; Huang *et al*., 2005). Spontaneous formation of a disulfide bridge between TM helices indicates that the Cys's are positioned in proximity when the receptor is in its basal state. Agonist-dependent disulfide linkage indicates an agonist-induced conformational change that brings the two residues replaced by Cys's in close proximity. Likewise, formation of a disulfide bond in the ligand-free state that is reduced/abolished in the presence of agonist indicates a conformational change that brings the two respective positions further apart. In some instances, disulfide linkage generates a constitutively active Rc, indicating that a conformational change is promoted that "locks" the Rc in its active state. Of course, the system can be used to assay other ligands, such as antagonists or inverse agonists, and should allow for the detection of subtle differences in the conformational changes induced by different classes of ligands. For the muscarinic (M_3) acetylcholine Rc, a class I GPCR, agonist-dependent disulfide cross-linking has been demonstrated, indicating that Rc interaction with an agonist (but not an antagonist) changes the relative positions of two TM helices (TM3 and TM7), bringing them sufficiently close to form a linkage not possible in the "ground" state (Han *et al*., 2005b). Recently these investigations were extended to inverse agonists of the M_3 Rc; distinct conformational changes were detected compared to those associated with agonist docking (Li *et al*., 2007).

As a template for the design of several double Cys-containing mutants, Thomas *et al*. (2008) constructed PTHR1 with a c-myc epitope tag inserted at the C-terminal tail (between residues 572 and 573) and Factor Xa (FXa) cleavage-sites in ICL3. Ordinarily, cleavage by FXa generates two fragments of receptor. However, if an engineered disulfide bond forms, these fragments remain held together covalently despite FXa cleavage. In the first study, Cys was introduced along the extracellular facing region of TM2 (positions 238 − 242) and into one position toward the extracellular facing end of TM7 (position 447) (Fig. 12). Of five double-Cys mutants tested, A240C/F447C and A242C/F447C formed a disulfide bond after treatment with agonist, whereas in one case (mutant F238C/F447C), a disulfide linkage present in the ground state was disrupted by agonist. With mutant D241C/F447C, no disulfide bond formation was observed in both the presence and absence of agonist. The fifth mutant, (V239C/F447C), was inactive and therefore not tested.

The data nicely fit the model of the PTH−PTHR1 interaction (Wittelsberger *et al*., 2006a). The model predicts that the extracellular ends of TM2 and TM7 are in close proximity, with F447 projecting toward TM2 (Fig. 13). The

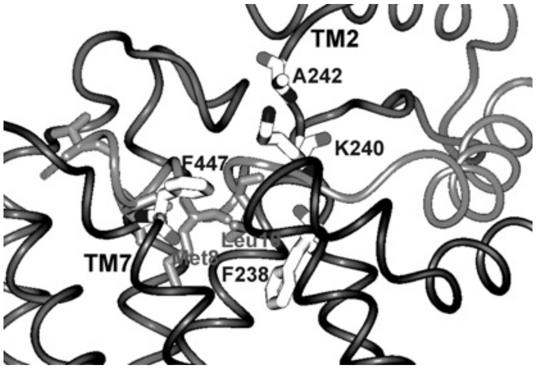

FIGURE 12 Expanded view of the PTH–PTHR1 complex illustrating the close proximity of the residues in TM2 (238, 240, 242) to TM7 (447) (yellow side-chains). Upon binding of PTH (light green), Met^8 and Leu^{10} of the hormone interfere with the interaction between F447 and F238. In contrast, hormone binding does not disrupt, and may even enhance, the interaction between F447 and K240. D241 is not shown because it faces away from the reader in this figure.

distances between F447 and 238, 240, or 242 in the model are consistent with the experimental results that demonstrate that there is potential to form disulfide bonds. The model also suggests that the binding of PTH would interfere with the F447−F238 association; Met-^{8} and Leu-^{10} of PTH project down toward the receptor core, in between TM2 and TM7. K240 and A242 face the same direction (toward TM7), whereas D241 faces in the opposite direction. This explains why K240 and A242 are proximal enough to F447 to form disulfide bonds (<7 Å) when substituted with cysteine. The side-chain of position 241 is pointing away and therefore unable to form a disulfide bond with a cysteine at position 447 of TM7 (Thomas *et al*., 2008).

The investigation reveals that hormone docking results in K240 and A242 moving toward F447, while F238 moves away from F447. According to the molecular model, K240, D241, and A242 are part of a loop just outside TM2 (Fig. 13), whereas F238 is still part of TM2. Association

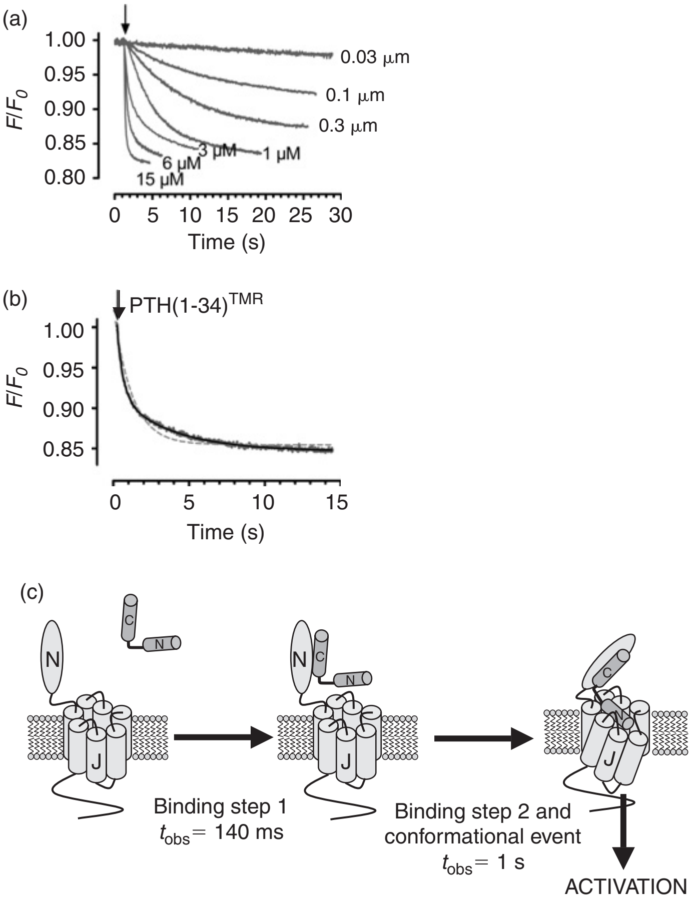

FIGURE 13 Association time courses of PTH(1–34)$^{\mathrm{TMR}}$ with $^{\mathrm{GFP}}$N-PTHR. (A) Kinetics of PTH(1–34)TMR binding to $^{\mathrm{GFP}}$N-PTHR recorded by decreases in the fluorescence emission of the GFP moiety. Measurements were recorded in a single cell at 20 °C with various concentrations of PTH(1–34)$^{\mathrm{TMR}}$. (B) Comparing fits of one-component (dashed line) and two-component (solid line) model for the time course of binding of PTH(1–34)$^{\mathrm{TMR}}$ (3 μM). The two-components model accurately describes the binding response. (C) Mechanistic model for PTHR activation. Upon PTH(1–34) binding there are two rates of association, a faster one that corresponds to agonist binding to the receptor N-domain and is strictly concentration-dependent, followed by a slower binding step to the receptor J-domain that is coupled to receptor activation (Castro, 2005).

between the cytoplasmic ends of TM2 and TM7 has been suggested to be responsible for the ligand-free activation of constitutively active receptors (Rolz *et al.*, 1999). These data with the F238C/F447C mutant suggest that the opposite movement occurs at the other end of the helices, i.e., movement of TMs at the extracellular side of the receptor is away from each other.

The system will likely also enable the detection of differences in conformational changes induced by different classes of ligands (i.e., agonist vs. antagonists).

Fluorescence Resonance Energy Transfer (FRET) Studies

Vilardaga and coworkers devised a complementary approach utilizing Fluorescence Resonance Energy Transfer (FRET) to study the hormone–receptor interaction (Castro *et al.*, 2005). By recording FRET between tetramethyl-rhodamine in PTH-(1−34) and GFP in the N-ECD of PTHR1, the binding event was measured in real time in living cells. The association time course between PTH-(1−34) and PTHR was fitted to a two-step binding process where the agonist initially binds the receptor with a fast time constant ($\tau \approx 140\,\text{ms}$) and then with slower kinetics ($\tau \approx 1\,\text{s}$). The fast and slow phases were assigned to hormone association to the receptor N-ECD and the J domain (comprising the 7 TM helices and the connecting extracellular loops), respectively. In addition, a PTHR sensor, PTHR-CFP_{IC3}/YFP_{CT}, was constructed by introducing enhanced cyan fluorescent protein into ICL3 and enhanced yellow fluorescent protein into the C-terminal tail of PTHR1 (Vilardaga *et al.*, 2003). The sensor produces a FRET signal in the basal state that is reduced when agonist binds, presumably owing to conformational changes taking place during receptor activation. Data from both FRET systems indicate that the slow PTH-(1−34) binding step to the J-domain coincides with a conformational switch in the receptor (Castro *et al.*, 2005).

Recently, these studies have been extended to PTHrP (Dean *et al.*, 2008). Despite displaying a binding affinity comparable to PTH-(1−34), PTHrP-(1−36) was found to bind to PTHR1 more slowly and to dissociate more rapidly than PTH. Addition of hPTH-(1−34) to HEK293 cells expressing PTHR-CFP_{IC3}/YFP_{CT} induced a rapid ($t_{1/2} = 0.7\,\text{sec}$) reduction (~13%) in the FRET signal that remained suppressed for quite awhile. In contrast, PTHrP-(1–36) induced a relatively slow FRET response ($t_{1/2}$ = 2–5 sec), and the signal decayed immediately upon buffer exchange. Combined with data from a detailed pharmacological analysis, the authors suggest that the differences in the kinetics of PTH and PTHrP binding and dissociation derive from differences in the ability of the two hormones to bind to R°, a G protein-uncoupled state. These differences in the interaction with PTHR1 may explain important differences in the biological profile of the hormones, e.g., duration of action, differences in stimulation of bone resorption, and endocrine versus paracrine roles.

Experimentally Based Molecular Modeling

Over the years, an experimentally based model of the PTH–PTHR1 complex has steadily evolved as new bimolecular contact sites were identified and additional conformational data on receptor domains and hormone were generated (Bisello *et al.*, 1998; Piserchio *et al.*, 2000a; Rolz *et al.*, 1999; Wittelsberger *et al.*, 2006a) and unpublished work. Initially, the model was constructed combining hydrophobicity profile analysis with a search of the Brookhaven Protein Data Bank (PDB) employing the Basic Logic Alignment Search Tool (BLAST) to first identify and then refine the location of the TM helices (Altschul *et al.*, 1990; Kyte and Doolittle, 1982). Identification of the TM domains of the PTHR1 is in good agreement with respect to those identified in peptides containing TM helical regions as determined by high-resolution NMR in micellar system (Mierke *et al.*, 1996; Pellegrini *et al.*, 1997b, 1998b). Arrangements of the TM heptahelical bundle in rhodopsin and bacteriorhodopsin (Grigorieff *et al.*, 1996; Henderson *et al.*, 1990; Pebay-Peyroula *et al.*, 1997; Schertler *et al.*, 1993; Schertler and Hargrave, 1995) were used as templates for the initial arrangement of the putative TM helical domains of the PTHR1 and optimized to account for hydrophobic moment toward the membrane environment, helix-helix, helix-core, and helix-membrane interactions (Pellegrini *et al.*, 1997b).

Unlike the high structural similarity in the arrangement of the heptahelical TM domains bundle (Baldwin, 1993), the cytoplasmic and ectopic domains of GPCRs are extensively variable, and no *a priori* structural model is available. For family B GPCRs including PTHR1, a SCR motif might be the dominant structural element of the N-ECD (Grace *et al.*, 2004, 2007). The extracellular and intracellular loops are constrained to some extent by the TM helical domains to which they are attached. Additional constraints are imposed by the three disulfide bridges at the extracellular N-terminus (Grauschopf *et al.*, 2000) and the disulfide bridge connecting the first and second ECL. All of these cysteines are highly conserved in the family B (class II) GPCRs of which PTHR1 is a member.

A homology search with BLAST (Altschul *et al.*, 1990) has identified the conformational preferences of the C-terminal portion of the N-ECD proximal to TM1 of PTHR1 and PTHR2 and ECL3 of PTHR1 (Bisello *et al.*, 1998; Rolz *et al.*, 1999). These homology searches suggest that the ECL3 adopts a helical conformation at T^{435}-Y^{443} (Rolz *et al.*, 1999) and that the juxtamembrane portion of N-ECD in PTHR1 and PTHR2 contains amphipathic helices K^{172}-M^{189} and L^{129}-E^{139}, respectively (Bisello *et al.*, 1998; Rolz *et al.*, 1999). Unfortunately, such homology searches may not always result in the assignment of a distinct secondary structure to a specific receptor sequence.

Mierke and Pellegrini (1999) modeled the receptor and receptor−ligand complex in a H_2O/decane/H_2O (40 Å each) simulation cell that mimics the membrane milieu. The molecular simulation is carried out in multiple steps in which the heptahelical bundle and/or the cytoplasmic and extracellular domains are allowed to move freely. PTH, in its membrane-associated conformation, is then added to the receptor model, applying the ligand−receptor distance constraints derived from the cross-linking experiments, and additional simulations are carried out. At this stage, additional constraints obtained via site-directed mutagenesis and chimera receptor studies can be incorporated to enhance the modeling procedure.

One way to identify conformational features of the cytoplasmic and ectopic domains of the GPCR is by synthesizing these receptor fragments and examining them by NMR in a membrane mimetic system. Adding small portions of the corresponding TM(s) to the otherwise flexible receptor-derived termini or loops provides an anchor(s) that partially reproduces the native orientation of the receptor domain relative to the membrane-mimicking milieu.

The conformational features of the following PTHR1 domains have been characterized: the third intracellular loop (ICL3), the C-terminal juxtamembrane portion of the N-ECD, and the ECL3 (Bisello *et al.,* 1998; Mierke *et al.,* 1996; Pellegrini *et al.,* 1997; Piserchio *et al.,* 2000a). These peptides were studied in a micellar system that mimics the cellular membrane and generates a micelle-water interface resembling the membrane-water interface. ICL3 was constructed as a 29 amino acid peptide with Cys residues in positions 1 and 28 (Pellegrini *et al.,* 1997b). Side-chains of the two cysteines were bridged by an octamethylene linker to maintain the putative ~12-Å distance between two consecutive TM domains (Schertler *et al.,* 1993; Schertler and Hargrave, 1995). Analysis of this constrained peptide revealed interesting conformational features that allow insight into ICL3 − G protein interactions (Pellegrini *et al.,* 1997b).

More relevant to the ligand−receptor bimolecular interactions are analyses of the two ectopic domains that photocross-link to Lys^{13} and Lys^{27} in PTH-(1−34) (Adams *et al.,* 1998; Bisello *et al.,* 1998; Greenberg *et al.,* 2000). The sequence PTHR1[172–189], which contains the contact sites for positions 13, 15, and possibly 11 and 21 of PTH (Adams *et al.,* 1998; Zhou *et al.,* 1997; Wittelsberger *et al.,* 2006a), was subjected to a combination of homology search and molecular dynamics calculations using a two-phase simulation cell consisting of H_2O and CCl_4 to mimic a membrane-water interface (Bisello *et al.,* 1998; Pellegrini *et al.,* 1998a). These analyses suggest that the segment R^{179}-E-R-E-V-F-D-R-L-G-M^{189} forms an amphipathic α-helix whose axis is parallel to the membrane surface and points away from the heptahelical bundle (Fig. 14, see also color plate) (Bisello *et al.,* 1998; Pellegrini *et al.,* 1998a). ^{1}H-NMR analysis of the synthetic peptide hPTHR1[168–198] in the presence of micelles was carried out in combination with distance geometry and a molecular dynamics simulation (Pellegrini *et al.,* 1998a). The analysis identifies three helical segments: 169–176, 180–188, and 190–196. The C-terminal helix, hPTHR1[190–196], corresponding to the ectopic portion of the first TM helix, is hydrophobic and embeds perpendicularly into the micelle. The other two α helices, [180–188] and [169–176], lie on the

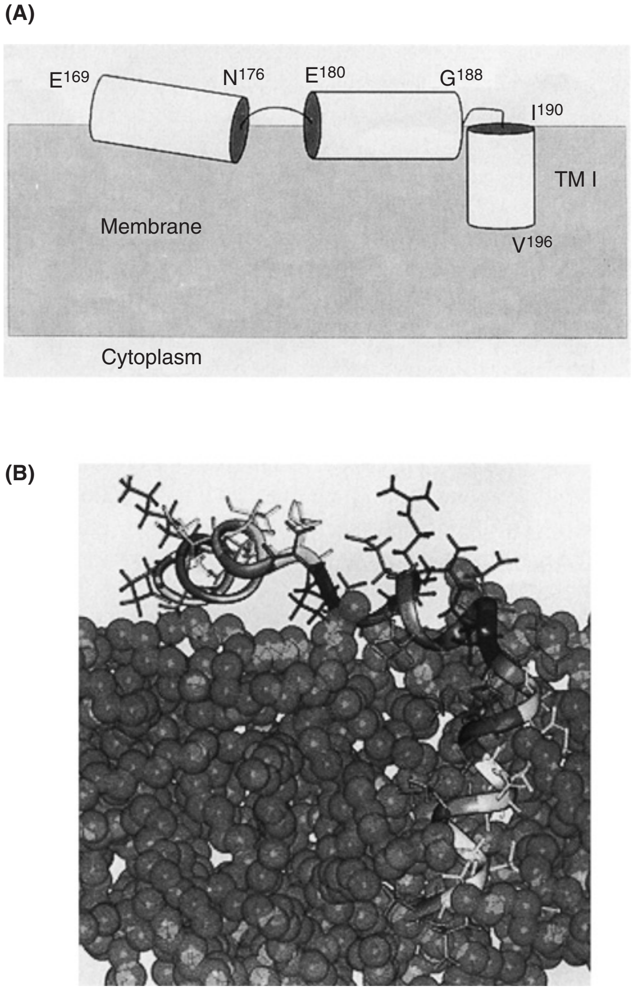

FIGURE 14 Structural features and topological orientation of PTHR1[168–198], which consists of the juxtamembrane portion of N-ECD and the ectopic portion of TM1 (Pellegrini *et al.,* 1998a). **(A)** Schematic presentation of the experimentally determined conformation, which consists of three α-helices, two of which have been determined to lie on the surface of the membrane; the third, at the top of TM1, is membrane-embedded. **(B)** Molecular simulation of this peptide in a water/decane simulation cell used to refine the structure obtained in the presence of dodecylphosphocholine (DPC) micelles used in the NMR study. Decane molecules are shown in green as CPK space-filling spheres. The peptide molecule is colored according to hydrophobicity (blue, polar, red, hydrophobic). (See also color plate.)

membrane surface (Fig. 14). Polar residues in the linker, E^{177} and R^{179}, and in the middle helix, R^{181}, E^{182}, D^{185}, and R^{186}, are exposed to the solvent, whereas the hydrophobic residues, F^{173}, F^{184}, and L^{187}, project toward the hydrophobic membrane (Pellegrini *et al.,* 1998a). It is plausible that the positively charged Lys^{13} in PTH participates in stabilizing the Coulombic interaction with the negatively charged E^{182} and D^{185} located at the solvent-exposed hydrophilic phase of the receptor sequence. Nevertheless, this may not be an essential interaction, as analogs in which the ε-amino on Lys^{13} is blocked by acylation maintain high affinity and efficacy.

Combining the putative TM bundle obtained in the modeling studies with the experimentally derived conformation of the synthetic hPTHR1[168–198] establishes a partial PTHR1 model that can be used to dock hPTH-(1−34) in its experimentally derived putative bioactive conformation (Pellegrini *et al.,* 1998b). This "*in silico*" experiment resulted in the first generation of an experimentally based model of the PTH − hPTHR1 complex (Bisello *et al.,* 1998). Using contact sites identified by PAS studies for positions 1, 13, and 27 as a docking cue placed the C-terminal amphiphilic helix of the ligand parallel to the membrane-aligned portion of the receptor-derived peptide. This positioning allows the formation of complementary Coulombic interactions between the polar residues in the C-terminal helix, comprising the principal binding domain of the ligand and the polar residues E^{177}, R^{179}, R^{181}, E^{182}, D^{185}, and R^{186} in the receptor-derived peptide. Interestingly, this docking procedure brings only M^{425}, and not M^{414}, into sufficient proximity to permit cross-linking to position 1 in PTH. Therefore, these observations are in complete agreement with the results obtained through cross-linking studies (Bisello *et al.,* 1998).

Based on the contact site between position 27 in PTH and L^{261} in the ECL1 of PTHR1 (Greenberg *et al.,* 2000), the synthetic peptide hPTHR1[241–285], composed of ECL1 and a few residues from the ectopic portions of TM2 and TM3 at the N- and C-termini of the loop, respectively, was subjected to detailed conformational analysis. The studies included high-resolution NMR in the presence of dodecylphosphocholine micelles followed by distance geometry calculations and molecular dynamic simulations. The receptor fragment was found to contain three α-helical segments: [241–244], [256–264], and [275–284] (Fig. 15, see also color plate) (Piserchio *et al.,* 2000a). The first and last helices correspond to the ectopic portions of TM2 and TM3, respectively. Hydrophobic amino acids corresponding to the ectopic portion of the TMs are more strongly associated with the lipid micelle and may serve as membranal anchors. Moreover, all of the hydrophobic residues in the partially ordered central helical portion (terminated by the unique helix-breaking sequence P^{258}-P-P-P^{261}) project toward the lipid surface (Piserchio *et al.,* 2000a).

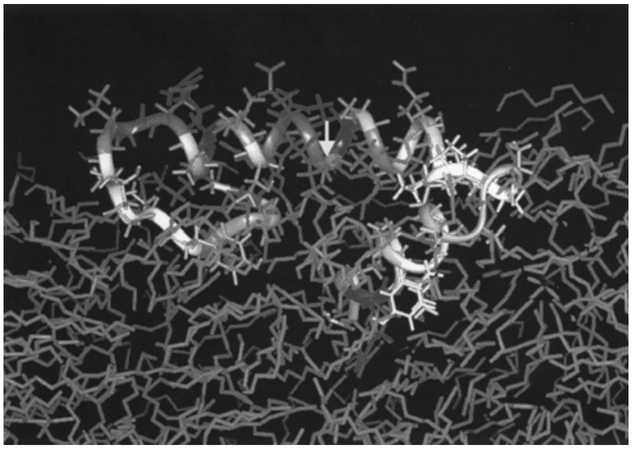

FIGURE 15 Structure of PTHR1-(241–285) (comprising the ECL1) from the molecular dynamics simulation in a water/decane simulation cell. Peptide residues are colored according to side-chain hydrophobicity (blue, polar; red, hydrophobic). Decane molecules are depicted in green; water molecules are not displayed for clarity. Residues belonging to TM2 and TM3 are embedded into the decane phase. The yellow arrow indicates the location of L^{261}, the cross-linking site to Lys^{27} in PTH-(1–34) (Piserchio et al., 2000a).

The emerging structure of ECL1 suggests an antiparallel organization of the two amphiphilic helices: the C-terminal helix in PTH, which includes Lys^{27}, and the [256–264] helical portion in ECL1, containing the contact site L^{261} (Pellegrini *et al.,* 1998b). The two helices are oriented with their hydrophobic faces interfacing the membrane and their polar faces exposed to the solvent and are capable of forming numerous intermolecular interactions. These additional findings were integrated into the PTH–PTHR1 model and placed the C-terminal helix of PTH in between the ECL1 helix and the C-terminal juxtamembrane helix of the N-ECD of hPTHR1 (Piserchio *et al.,* 2000a).

Finally, incorporation of the contact sites identified for midregion residues 11, 15, 18, and 21 (Wittelsberger *et al.,* 2006a) as distance restraints in MD simulations of the hormone–receptor interaction resulted in a significant refinement of the model of the PTH–PTHR1 complex compared to previous generations, as depicted in (Fig. 5). Most significantly, the hormone must adopt a loop structure in the midregion in order to bring both Leu^{11} and Val^{21} in close proximity to PTHR1[165−176]. The N-terminal α-helix of PTH (residues 2–10) is maintained during the simulation, as are the contacts between the N-terminus of the ligand (particularly Leu^5 and Met^8 of PTH) with the PTHR1 (Rolz, 1999). The contact point between Ser^1 in PTH and Met425 of the receptor is also maintained. Another change involves the α-helical region [169−176] in the N-ECD of the PTHR1. During the MD simulations, this α-helix is found to fold underneath the C-terminal helix of the ligand. The driving force for this arrangement

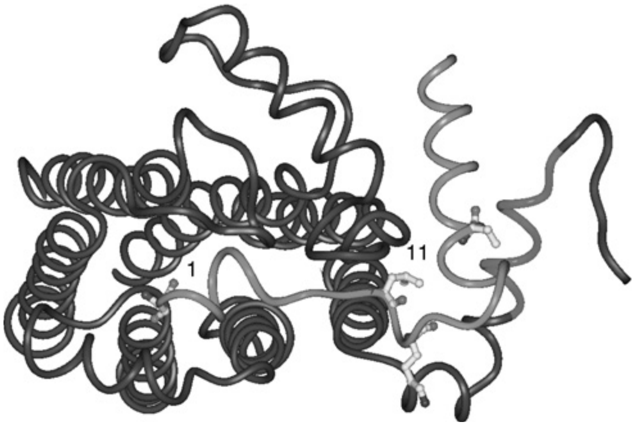

FIGURE 16 Model of the PTH ligand–receptor complex obtained from MD simulations using experimentally determined contact points with tighter distance constraints of 8 Å (Wittelsberger et al., 2008). The ligand is shown in orange. The receptor region [165–176] containing the cross-linking site of Bpa^{11}-PTH is colored light green. The N-terminal helix of the hormone is distorted.

is a contact between this region of the receptor and residue 11 of PTH. These findings suggest a broad role for the N-ECD in determining ligand binding, involving interactions with not only the C-terminus of PTH, but also with the midregion of the ligand. During the MD simulations, an almost 90-degree bend of the two α-helices of PTH is observed (Fig. 5). This arrangement is the result of simultaneous contacts between the hormone midregion with the proximal N-ECD of the PTHR1, and position 27 of PTH with Leu-261 in the center of ECL-1 (Greenberg *et al.*, 2000).

A distance restraint of 14 Å is typically applied to the experimentally identified contact points during the simulation. Distance restraints are applied to the Cβ atom at the site of Bpa incorporation and to the side-chain of the appropriate residue across the interface in the receptor. This large distance seemed appropriate to allow some flexibility of Bpa fitting into the receptor during the cross-linking experiment, because native ligand is used in the refinement protocol, not the Bpa-containing analogs. To validate the choice of the distance restraint, we recently conducted a study comparing the outcome from MD simulations using 14 Å versus a tighter restraint of 8 Å for the 7 experimentally determined contact points between PTH and PTHR1. Using 14 Å, the structure obtained corresponds to the model depicted in Fig. 5, whereas the outcome of the 8 Å simulation can be summarized in Fig. 16. When the tight distance constraints are accommodated, the structural features of the ligand are distorted. The N-terminal α-helix, running from Val^2 to Gly^{10}, is reduced to a centrally located loop of helix, attempting to simultaneously fulfill the cross-linking points of Ser^1 to M425 (top of TM7) and Leu^{11} to L174 (proximal N-terminus, located in a second α-helix). In contrast, when using 14 Å constraints during the simulations (Fig. 5), the helical nature of the N-terminus of PTH is maintained throughout the entire Val^2 to Gly^{10} region.

As they emerged, the experimentally derived models of the complex between PTH/PTHrP ligands with both PTHR1 and PTHR2 were studied in detail (Adams *et al.*, 1998; Bisello *et al.*, 1998; Zhou *et al.*, 1997) to identify interresidue contacts within the heptahelical TMs (Rolz *et al.*, 1999). Some insights can explain ligand specificity (Behar *et al.*, 1996a; Gardella *et al.*, 1996a), the consequences of some site-directed mutations (Bergwitz *et al.*, 1997; Clark *et al.*, 1998; Gardella *et al.*, 1996b; Lee *et al.*, 1995a; Turner *et al.*, 1998), the constitutive activity of JCM-mutated receptors (Schipani *et al.*, 1995, 1997), the consequences of cross-linking studies (Bisello *et al.*, 1998), some structure–activity relations in the ligand (Cohen *et al.*, 1991; Rosenblatt *et al.*, 1976), first results on dynamics of TM movement during receptor activation (Thomas *et al.*, 2008), and some aspects of signal transduction. For example, there is a loss of affinity for PTH-(1−34) following mutations W437A/L/E or Q440A/L in PTHR1, an effect much reduced for PTH-(3−34) (Lee *et al.*, 1995a). The model positions both Q^{440} and W^{437} on the same face of ECL3, both projecting toward the center of the TM bundle (Rolz *et al.*, 1999). The side-chains of these residues participate in forming the hydrophobic pocket that accommodates Val^2 in the ligand, providing stabilizing interactions by shielding it from the extracellular aqueous environment. Mutating Q^{440} and/or W^{437} to any smaller or a more polar residue will compromise the binding pocket for Val^2 by exposing it to water. The model also attempts to explain position 5 in PTH and PTHrP as a receptor-subtype specificity switch (Behar *et al.*, 1996a; Gardella *et al.*, 1996a). It suggests that in PTHR1, the Ile^5 side-chain is accommodated by a hydrophobic pocket. The bottom of this pocket is composed of hydrophobic residues at the ectopic end of TM3 (A^{284} and V^{285}) and TM7 (F^{447}), and it is large enough to accommodate either Ile^5 or His^5 (Fig. 17). In PTHR2, owing to the presence of F386 and F401 located at the ectopic ends of TMs 6 and 7, respectively, the binding pocket for residue 5 is reduced in size compared to the same pocket in PTHR1. It therefore cannot accommodate His^5-containing PTHrP or hybrid ligands and discriminates against them (Fig. 17) (Rolz *et al.*, 1999). These authors also encourage the use of their model as a tool for predicting the pharmacological consequences of specific mutations such as receptor-subtype specificity-reversing mutations.

The quality of any model, namely its capacity to realistically represent ligand–receptor interactions and predict the nature of the interface, is based primarily on data and procedures used in its construction. It is important to avoid overinterpretation of any model and remember the assumptions and approximations used in its construction. Likewise, extrapolations derived from any model must be tested in order to validate the model's predictive potential.

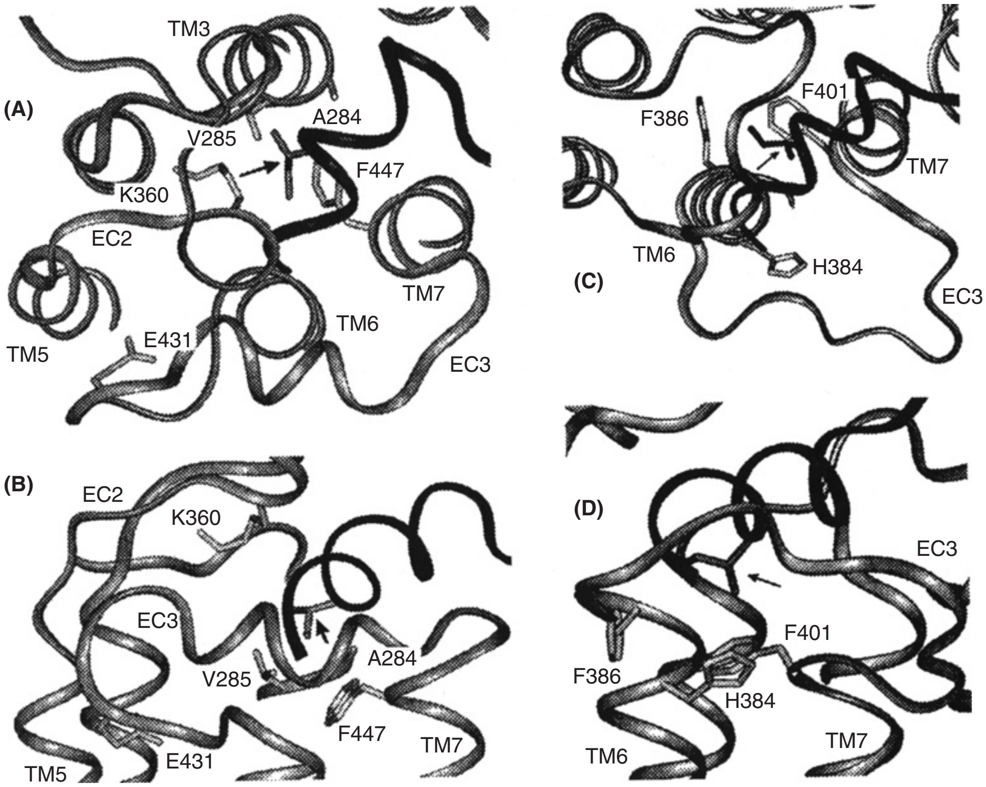

FIGURE 17 Illustration of the binding pocket for Ile^5 of PTH in PTH1- and PTHR2 (Rolz *et al.*, 1999). In PTHR1 Ile^5, the side-chain (indicated by black arrow) is accommodated in a hydrophobic pocket made up of the ectopic portions of TM3 (A284 and V285) and TM7 (F447). Top **(A)** and side **(B)** views of this pocket in PTHR1. The receptor and the ligand are depicted as ribbons in grey and black, respectively. In PTHR2, the binding pocket for residue 5 in the ligand is more limited in size compared to the same pocket in PTHR1 owing to the presence of F386 and F401 in the ectopic portions of TM6 and TM7, respectively. Top **(C)** and side **(D)** views of this pocket in PTHR2. In addition, in PTHR1 the presence of E432 in the ectopic portion of TM6, at the entrance to the binding pocket of His^5, can attract His^5 by favorable Coulombic interaction. However, in PTHR2 the presence of H384, at the bottom of the binding pocket for residue 5, will destabilize the interaction with an incoming side-chain of His^5-containing ligand.

In summary, the model of the PTH–PTHR1 complex should be regarded as a steadily evolving "working" platform that might help explain new experimental findings and that will be modified and/or refined as new experimental results are incorporated.

Ligand-Tethered hPTHR1

Shimizu *et al.* (2000a) reported what seems to be the absolute integration of ligand and receptor entities. Borrowing from the protease-activated thrombine receptor system (Kawabata and Kuroda, 2000), they generated constitutively active, ligand-tethered hPTHR1s. The receptors were constructed by truncating the N-ECD from E^{182} to the N terminus (yielding ΔN-ECD-PTHR1), adding a Gly$_4$ spacer (Gly$_4$-ΔN-ECD-PTHR1), and linking it to N-terminal fragments of PTH (varying in size from 9 to 11 residues). Transient expression of the ligand-tethered receptor PTH-(1−9)-G$_4$-ΔN-ECD-hPTHR1 in COS-7 cells resulted in tenfold higher basal cAMP levels compared to the wild-type hPTHR1 control. Tethering the extended and more potent [Arg^{11}]PTH-(1−11) resulted in 50-fold higher basal cAMP levels than those seen with the wild-type hPTHR1. Interestingly, like in PTH-(1−14) (Luck *et al.*, 1999), where Val^2, Ile^5, and Met^8 are the most critical residues for activation, these residues were also the most critical ones for the constitutive activity of the [Arg^{11}]PTH-(1−11)-G$_4$-ΔN-ECD-hPTHR1 (Shimizu *et al.*, 2000a).

The elegance of this study lies in devising a unique way to specifically "immobilize" the principal activation domain of the ligand in the proximity of the contact sites critical for receptor activation. The high effective molarity of the tethered ligand minimizes the role of binding affinity in bimolecular interactions compared to free ligand, thus allowing the identification of residues within the tethered ligand essential for induction of activity. However, the accessibility to the ligand tethered receptor system requires the employment of molecular biology and therefore it is most applicable to tethered ligands composed of coded amino acids. In addition, stringent requirements for the efficient expression of tethered ligand−receptors in a relevant cellular background may turn out to be major obstacles in practicing and extending this approach in the future. It remains to be demonstrated whether the tethered ligand−receptor system is a source for identifying structural constraints that can contribute to the refinement of the experimentally based ligand−receptor model and to rational drug design. The elimination of most of the entropic component from the ligand−receptor interaction may generate contact interactions and produce activation mechanisms that differ from those involved in the interaction with a diffusible ligand.

The autoactivation mechanism of ligand-tethered PTHR1 was validated by molecular modeling (Monticelli *et al.,* 2002). The model found the N-terminal α-helix of tethered PTH-(1−11) to interact with ECL3, specifically with the side-chains of the hydrophobic residues Leu^5 and Met^8 buried deep into well-defined pockets in the central core of the TM helical bundle, consistent with the requirement for these amino acids to be involved in receptor activation. The authors postulate that the improved signaling properties of $[\text{Arg}^{11}]$PTH-(1−11) over wild-type PTH-(1−11) is owing to a stable hydrogen bond between Arg^{11} and E444, at the beginning of TM7.

$$\begin{tikzcd}
R + L_{(3-34)} + L_{(1-14)} \arrow[r, shift left, "K_{(3-34)}"] \arrow[d, shift left] & RL_{(3-34)} + L_{(1-14)} \arrow[l, shift left] \arrow[d, shift left] \\
RL_{(1-14)} + L_{(3-34)} \arrow[u, shift left, "K_{(1-14)}"] \arrow[r, shift left, "\alpha K_{(3-34)}"] & RL_{(3-34)}L_{(1-14)} \arrow[l, shift left] \arrow[u, shift left, "\alpha K_{(1-14)}"']
\end{tikzcd}$$

FIGURE 18 Model for simultaneous binding of ^{125}I-PTH-(3–34) and PTH-(1–14) to PTHR1, where R is the receptor; L(3–34) is ^{125}I-PTH-(3–34); K(3–34) is the equilibrium association constant for ^{125}I-PTH-(3–34); K(1–14) is the equilibrium association constant for PTH-(1–14); and α is the cooperativity factor defining the effect of L(1–14) occupancy on the receptor-binding affinity of L(3–34) and reciprocally the effect of L(3–34) occupancy on the receptor-binding affinity of L(1–14) (Hoare *et al.,* 2001).

MULTIPLE CONFORMATIONAL RECEPTOR STATES: AN EMERGING PARADIGM

Like several other class II GPCRs (Beyermann *et al.,* 2000; Holtmann *et al.,* 1995; Juarranz *et al.,* 1999; Stroop *et al.,* 1995), PTHR1 can be devided into two functional domains. One consists of the large N-ECD (N domain) that has been proposed to provide most of the principal binding interactions with the ligand (Bergwitz *et al.,* 1996; Juppner *et al.,* 1994). The rest of the receptor, which includes the ECLs, TMs, and the ICLs designated as the juxtamembrane domain (J domain), is involved in ligand interactions that lead to activation and signal transduction (Bergwitz *et al.,* 1996; Gardella *et al.,* 1994; Juppner *et al.,* 1994; Turner *et al.,* 1996). A similar two functional domain architecture is found in the ligands, PTH and PTHrP; the 15–34 sequence includes the principal binding domain (Caulfield *et al.,* 1990; Rosenblatt *et al.,* 1980), and the 1–14 sequence includes the activation domain for intracellular signaling (Bergwitz *et al.,* 1996; Gardella *et al.,* 1991; Luck *et al.,* 1999; Shimizu *et al.,* 2000a, 2000b; Takasu *et al.,* 1999a).

It is now generally accepted that the interaction between hormone and PTHR1 proceeds in at least two sequential steps (Bisello *et al.,* 1998; Juppner *et al.,* 1994; Luck *et al.,* 1999; Mannstadt *et al.,* 1999; Castro *et al.,* 2005): A first step is an interaction between the C-terminal segment of PTH-(1−34) and the N-ECD (and possibly extracellular loops) that contributes mainly to binding affinity. The second step is the activating interaction between the N-terminal segment of the hormone with sites within the heptahelical bundle. Recently, a third functional interaction was suggested to represent a critical middle step in the PTH–PTHR1 interaction: residues from the midregion of PTH-(1−34) form extensive contacts with sites at the C-terminal end of the N-ECD that guide the N-terminus of PTH into the binding groove of the TM bundle (Wittelsberger *et al.,* 2006a).

In the course of studying the pharmacology of $[(\text{MAP}_{1-10})^{22-31}]$ hPTHrP-(1−34)NH_2 (RS-66271), a PTHrP analog in which sequence 22−31 was replaced by a model amphipathic peptide, the discrepancy between its low binding affinity compared to that of PTH-(1−34), and its PTH-(1−34)-like *in vivo* and *in vitro* activities (Frolik *et al.,* 1999; Krstenansky *et al.,* 1994; Vickery *et al.,* 1996), was explained by differential binding affinity to G protein-coupled and uncoupled receptor states. According to Usdin and coworkers, RS-66271 binds with high affinity ($\text{IC}_{50} = 16\,\text{p}M$) to the G protein-coupled PTHR1, but with much lower affinity ($\text{IC}_{50} > 100\,\text{n}M$) to the uncoupled receptor (Hoare and Usdin, 1999). Therefore, if the PTHR1 population in the intact cell is predominantly uncoupled from G protein, the binding affinity of RS-66271 will be markedly low (Hoare *et al.,* 1999b; Hoare and Usdin, 1999, 2000). hPTH-(1−34), on the other hand, was shown to bind with high affinity ($\text{IC}_{50} < 10\,\text{n}M$) to the PTHR1, whether or not it is coupled to G protein (Hoare *et al.,* 1999b). Interestingly, His^5 in RS-66271, which has been implicated previously in specifying the signaling and binding of PTHrP to PTHR1 but not to PTHR2 (Behar *et al.,* 1996a; Gardella *et al.,* 1996a), is also implicated as a determinant in G protein-coupled versus -uncoupled PTHR1 selectivity (Hoare *et al.,* 2001). Replacement of His^5 in RS-66271 with Ile reduced selectivity toward the high-affinity G protein-coupled PTHR1 by 17-fold and increased the affinity to the uncoupled receptor by 160-fold. The ability of $[\text{Ile}^5]$RS-66271 to restore adenylyl cyclase activation in N-ECD-truncated PTHR1, in which RS-66271 failed to stimulate cAMP production, suggests that the residue in position 5 affects receptor selectivity through interactions with the ECLs and the ectopic portions of the TMs (Hoare *et al.,* 2001).

Hoare and coworkers (2001) reported that [$Ala^{3,10,12}$,Arg^{11}]rPTH-(1−14)NH_2 [PTH-(1−14)] and PTH-(3−34) show allosteric binding behavior on PTHR1 (Fig. 18). The former binds predominantly through the J domain, partially inhibits ^{125}I-PTH-(3−34) binding, and the interaction is stabilized by G protein coupling. The latter interacts almost exclusively with the N domain, only partially inhibits PTH-(1−14)-stimulated adenylyl cyclase activity, and the interaction is GTPγS insensitive. The higher binding affinity of agonist ligands to RG versus the R state of the receptor implies different conformations of the receptor in both states. At the uncoupled state, PTH-(1−14) and the PTH-(3−34) bind almost independently of each other. The negative cooperativity between the binding of ^{125}I-PTH-(3−34) and PTH-(1−14) is significantly greater in the RG state than in the uncoupled one. Moreover, agonist binding to the RG state is pseudo-irreversible. PTH-(3−34) inhibits PTH-(1−14)-stimulated cAMP accumulation, increases EC_{50} by 18-fold, and reduces E_{max} in a noncompetitive manner.

The study suggests that the receptor is in an "open" conformation in the uncoupled state, permitting simultaneous binding of both PTH-(3−34), the N domain-interacting ligand, and PTH-(1−14), the J domain-interacting ligand. At the coupled state, for which agonist has higher affinity than for the uncoupled state, the receptor is in a more "closed" conformation, preventing simultaneous access of both ligands to their preferred binding sites and trapping the ligand within the coupled receptor. Consistent with these results, the modified N-terminal fragment ^{125}I-[$Aib^{1,3}$,Nle^{8},Gln^{10},$homoarginine^{11}$,Ala^{12},Trp^{14},Tyr^{15}]rPTH-(1–15)NH_2, ^{125}I-[$Aib^{1,3}$,M]PTH-(1–15), a PTH analog interacting with the receptor predominantly through the J domain, was also found to selectively bind to a G protein-coupled, active-state PTHR1 conformation (Dean *et al.*, 2006b).

The finding that agonists bind with higher affinity to the RG complex than to the uncoupled receptor, whereas antagonists bind with similar affinities to the two states (Hoare *et al.*, 2001; Teitelbaum *et al.*, 1982), is in agreement with studies of type I GPCRs (Lefkowitz *et al.*, 1993). It suggests that agonists enhance G protein activation by stabilizing the RG state.

Bisello *et al.* (2002) showed that PTHrP analogs modified at position 1 induced selective stabilization of an active G protein-coupled receptor conformation that resulted in lack of β-arrestin recruitment to the cell membrane, sustained cAMP signaling, and absence of ligand–receptor complex internalization. Molecular modeling comparing the conformational states induced by agonist versus the position 1-modified analogs showed a significant difference in the location of ICL3.

Likewise, Bisello *et al.* (2004) showed that PTHR2 activity is regulated in an agonist-specific manner. When comparing PTH-(1−34) and TIP39-induced PTHR2 desensitization and internalization, they find that TIP39 induces β-arrestin and protein kinase C mobilization and receptor internalization, whereas PTH-(1−34) does not. Furthermore, PTH-(1−34) stimulation of cAMP activity was brief and resensitization occurred rapidly, whereas cAMP stimulation by TIP39 was sustained and partly desensitized for a prolonged period. Specific PTHR2 − ligand interactions therefore mediate distinct post-activation processes.

Dean *et al.* (2008) recently reported differences in the binding/activation mechanism between PTH and PTHrP. They find that despite displaying a binding affinity comparable to PTH-(1−34), PTHrP-(1−36) was found to bind to PTHR1 more slowly and to dissociate more rapidly than PTH. PTH-(1−34) produces greater cumulative cAMP signaling than PTHrP. The cAMP signaling response capacity of PTHrP-(1−36) in cells decayed more rapidly than did that of PTH-(1−34) ($t_{1/2}$ = ~1 vs. ~2 h). These differences were suggested to derive from differences in the ability of the two hormones to bind to R°, a G protein-uncoupled state (defined as a receptor conformation that can bind ligand with high affinity in the presence of GTPγS). The R° conformation is a putative preactive state for RG, the conformation primed to couple with a G protein. Such conformational flexibility may explain important differences in the biological profile of the hormones, e.g., duration of action, differences in stimulation of bone resorption, and endocrine versus paracrine roles (Dean *et al.*, 2008).

Furthermore, it was recently shown that PTHR1, like some other GPCRs (Azzi *et al.*, 2003; Charest *et al.*, 2007; Seta *et al.*, 2002; Shenoy *et al.*, 2006; Wei *et al.*, 2003), can be activated in a G protein-independent manner (which gives rise to the suggestion to change their name to 7TM receptors). Gesty-Palmer *et al.* (2006) show that PTH can stimulate the mitogen-activated protein kinase ERK1/2 through distinct signal transduction pathways: an early G protein-dependent pathway involving PKA and PKC, as well as a late G protein-independent pathway mediated through β-arrestins. These findings imply the existence of distinct active conformations of the hPTHR1 responsible for the two pathways, which can be stimulated by unique ligands. The signaling selective analogs [Bpa^{1},Ile^{5},$Arg^{11,13}$,Tyr^{36}]PTHrP-(1−36), PTHrP-(2−36), and [Trp^{1}]PTHrP-(1−36) (Bisello *et al.*, 2002; Gesty-Palmer *et al.*, 2006) stabilize a receptor conformation that selectively activates Gs/PKA, but is unable to recruit β-arrestin. The receptor conformation induced by [D-Trp^{12},Tyr^{34}]PTH-(7−34)NH_2 is unable to activate G proteins, but is able to recruit β-arrestin (Gesty-Palmer *et al.*, 2006).

As proposed for most GPCRs in general (Kenakin, 2003; Kobilka *et al.*, 2007), the evolving paradigm contends that different stages during receptor activation stabilize different receptor conformations and that unique ligand-specific receptor conformations are differentially activating distinct signaling pathways.

SUMMARY AND OUTLOOK

With PTH-(1−34) as the first truly bone anabolic agent approved to treat osteoporosis, understanding the interactions between hormone and PTHR1 as well as mechanisms of receptor activation has become ever more important. Much information was gathered over the past decade from photoaffinity cross-linking experiments, structure-functional studies, and molecular modeling, and the currently available model of the PTH–PTHR1 complex provides significant detail on both ligand and receptor structure.

At the same time, the understanding that the receptor exists in multiple conformational states, depending on ligand, allosteric effector, or even signaling pathway, adds a new level of challenge (and opportunity) to investigations of the PTH system and GPCR research in general. The biggest impact in the future might come from techniques such as cysteine cross-linking and time-resolved single cell fluorescence approaches that can be used to examine the dynamics of triggering receptor activation. The detection of ligand-specific differences in the stabilization of specific conformations and therefore activation of distinct signaling pathways is crucial. The elucidation of the structural detail of conformational changes is still in the early stages.

The field of GPCR research is advancing quickly, with a second (in addition to rhodopsin) GPCR recently crystallized, with structural information on the N-ECD of class II GPCRs available, with new activation pathways discovered, and discoveries from other GPCR systems. PTH ligand–receptor interactions are therefore likely to remain a fertile research field in the next decades.

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Chapter 30

Cellular Actions of Parathyroid Hormone

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Parathyroid hormone (PTH) is essential for the maintenance of calcium homeostasis through direct actions on its principal target organs, bone and kidney, and through indirect actions on the gastrointestinal tract. PTH acts directly on the skeleton to promote calcium release from bone and on the kidney to enhance calcium reabsorption. The indirect effects of PTH on the gastrointestinal tract lead to greater calcium absorption through its actions to facilitate the conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D. Actions of PTH at these three sites (the kidney, the skeleton, and the gastrointestinal tract) result in restoration of the extracellular calcium concentration. When the hypocalcemic signal for parathyroid hormone release returns to normal, calcium ion continues to regulate the release of PTH. In the setting of a hypercalcemic signal, not because of abnormal secretion of PTH (i.e., primary hyperparathyroidism): parathyroid hormone secretion is inhibited. The resulting physiological events associated with reduced concentrations of PTHlead to reduced calcium mobilization from bone, a reduction in renal tubular reabsorption of calcium, and, by virtue of reducing the conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D, to reduced absorption of dietary calcium. Hence, in hypercalcemic states, control of parathyroid hormone secretion by calcium reduces serum calcium levels.

PTH helps to regulate phosphorus metabolism. An increase in phosphorus leads to a reduction in the circulating calcium concentration. The resultant increase in PTHleads to phosphaturia, a classical physiological effect of the hormone and restoration of the serum phosphorus concentration (Chapter 20).

Cellular responsiveness to PTH occurs via receptor-mediated activation of intracellular events and several major biochemical pathways. Details of the initial steps by which a change in extracellular calcium is sensed by the parathyroid cell and the mechanisms by which PTH binds to its receptor, as well as the induction of several different messenger systems, are covered in other chapters. This chapter focuses on the cellular effects of PTH on the skeleton.

CELLULAR ACTIONS OF PARATHYROID HORMONE ON BONE

The emergence of mouse genetics during the last 18 years, in combination with a plethora of *in vitro* studies, has generated a wealth of knowledge regarding the effects and mechanisms of actions of parathyroid hormone (PTH) on bone cells. This information has helped to enhance our understanding of the pharmacologic and physiologic actions of PTH.

BRIEF HISTORICAL TRAIL

Anabolic actions of parathyroid gland extract were first described in young rats, guinea pigs, kittens, and rabbits as early as the 1930s (Pugsley and Selye, 1933; Burrows, 1938; Jaffe, 1933; Parsons, 1972). At the time, the responses were thought to reproduce some of the pathologic processes associated with primary hyperparathyroidism in which an early destructive phase was followed by a reparative phase of bone formation, secondary to parathyroidectomy (Heath, 1996). When hPTH(1–34) was first synthesized in the early 1970s (Potts *et al.*, 1995), the early studies suggesting an anabolic potential for bone were revisited. Small clinical trials in the 1970s and early 1980s were impressive and suggested that PTH did have potential as a bone-forming agent (Cosman and Lindsay, 1998a; Dempster *et al.*, 1993). The common thinking at the time was that PTH was primarily a catabolic hormone as seen classically in the disorder of PTH excess, primary hyperparathyroidism. It was not surprising then that these early studies suggesting an

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opposite metabolic effect induced by the same hormone would be viewed with considerable skepticism. The apparent paradox that contained in one molecule both anabolic and catabolic actions was resolved over the ensuing several decades. The key to insight was appreciation of the fact that continuous versus intermittent and high dose versus low dose would dictate the predominant response to PTH. It is now recognized that continuous exposure to high levels of PTH is associated with catabolic effects whereas exposure to low doses of PTH intermittently is associated with anabolic effects. It has been extensively demonstrated that intermittent exposure of the skeleton to PTH is anabolic in mice and rats. This form of delivery gives a pulse of PTH that is cleared from the circulation within 2–3 hours (Frolik *et al.*, 2003; Bellido *et al.*, 2005; Lindsay *et al.*, 1993). This anabolic response of the skeleton to repeated cycles of systemic PTH elevation is associated with increased bone formation on the three surfaces of bone (cancellous, endocortical, and periosteal) and of both the appendicular (peripheral) and axial (central) skeleton. PTH as an anabolic agent for osteoporosis represents a new therapeutic class for osteoporosis. Clinical trials with the foreshortened aminoterminal form, namely PTH(1–34) have demonstrated efficacy to reduce vertebral and nonvertebral fractures in postmenopausal women. How PTH serves this anabolic role is now thought to be because of actions on all three bone cell types: the osteoblast, osteoclast, and osteocyte.

PTH Regulation of Osteoblasts and their Osteoprogenitors

Stimulation of bone formation at endocortical surfaces is a universal species-independent hallmark of the anabolic effect of PTH (Cosman and Lindsay, 1998b; Dempster *et al.*, 1993; Whitfield *et al.*, 1998; Oxlund *et al.*, 1993). Intermittent PTH rapidly increases mineralizing surface and mineral apposition rate in cancellous bone of animals and human subjects indicating stimulatory actions on osteoblast number or function. Because increases in mineral apposition rate are larger than changes in mineralizing surfaces and, at least in rodents, correlate with increased osteoblast numbers, it has been proposed that increased osteoblast numbers account for the bone-forming properties of the hormone (Jilka, 2007). The ability of PTH to affect cell numbers by regulating proliferation, differentiation, and survival of osteoblasts and osteoblast progenitors has been, and still is, a major view of PTH's actions on this cell type.

Effects of PTH on the Proliferation of Osteoblast Progenitors

The impact of PTH on the proliferation of osteoblast progenitors has been studied *in vivo* in rodents. In young rats, in which proliferating cells are abundantly available adjacent to the growth plates and in cortical endosteum of the metaphyses and the periosteal diaphyseal surfaces (Kimmel and Jee, 1980; Kember, 1960): PTH targets cells in the G_1/S phase of the cell cycle (Onyia *et al.*, 1995; Young, 1962; Young, 1964; Roberts, 1975). Similarly, short-term exposure to PTH suppresses proliferation of osteoblastic cells derived from the primary spongiosa of growing rats (Qin *et al.*, 2005; Onyia *et al.*, 1997b). The effects of PTH in young rats suggest possible roles in skeletal development, accretion of peak bone mass, and increased bone formation. In PTH-treated adult rats exposure to [^{3}H-] thymidine, which labels dividing cells, indicated that the percentage of labeled osteoblasts derived from labeled osteoblast progenitors is not affected. However, unlabeled, nonproliferative bone surface cells are activated (Dobnig and Turner, 1995). Daily administration of PTH to adult or aged mice does not affect the number of mesenchymal stem cells as measured by the number of colony-forming unit-osteoblasts (CFU-OB) present in femoral marrow isolates (Jilka *et al.*, 1999; Sakai *et al.*, 1999; Knopp *et al.*, 2005). SAMP6 mice, a strain with low CFU-OB number, gives an anabolic response to PTH that is equivalent to that of control mice (Jilka *et al.*, 1999). Collectively, these lines of evidence suggest that in cancellous bone, PTH does not affect the replication of uncommitted osteoblast progenitors but suppresses proliferation of committed osteoprogenitors.

In vitro studies further support the hypothesis that the actions of intermittent PTH on the osteoblast cell cycle are specific to the differentiation/developmental stage. Anti-proliferative effects have been reported in osteoblastic cell lines or cultures of primary cells. At the cellular level, several mechanisms of inhibition of proliferation by PTH occur. Topoisomerase IIα, a marker of proliferation (Feister *et al.*, 2000): decreased expression of the cell cycle marker H4, increased expression of the cell cycle inhibitors p27^{Kip1} and p21^{Cip1} (Onyia *et al.*, 1995; Qin *et al.*, 2005) and caused a lack of stimulation of thymidine incorporation (Young, 1962; Young, 1964; Onyia *et al.*, 1995; Onyia *et al.*, 1997a). Concomitant with the increase in cell cycle inhibitors, PTH also attenuates the expression of cyclin D1, which is required for cell cycle progression (Qin *et al.*, 2005; Datta *et al.*, 2005). In UMR-106 cells, PTH blocked entry of cells into S phase of the cell cycle, thereby increasing the number of cells in G_1; and cell proliferation was inhibited as a consequence of an increase in p27^{Kip1} (Onishi and Hruska, 1997). The effect of PTH on osteoblast proliferation was also examined in pre-confluent cultures of calvaria cells derived from transgenic mice expressing green fluorescent protein (GFP) under the control of a collagen promoter. In these mice, the emergence of a GFP signal marks the transition of osteoprogenitors into preosteoblasts. Transient exposure of pre-confluent cultures to PTH reduced the proportion of S-phase cells but increased the proportion of cells in Go/G_1 and G_2 + M phases (Wang, Liu, and Rowe, 2007). Thus, although transient exposure to PTH has no effects on the replication of uncommitted progenitors, it appears to enhance their commitment to an osteogenic fate. In agreement with this observation, histomorphometric studies have consistently shown early increases in bone-forming surfaces, consistent with the stimulation of differentiation of osteoprogenitors, rather than proliferation of osteoblasts (Cosman and Lindsay, 1998a; Dempster *et al.*, 1993; Onyia *et al.*, 1997b; Leaffer *et al.*, 1995; Hodsman and Steer, 1993; Meng *et al.*, 1996).

In contrast to the anti-mitotic effects of transient PTH exposure, proliferative effects of the hormone have also been reported. PTH promotes proliferation in bone marrow or calvaria-derived osteoblastic cultures (Swarthout *et al.*, 2001; Cole, 1999; Cornish *et al.*, 1999; Miao *et al.*, 2001; Ishizuya *et al.*, 1997; MacDonald, Gallagher, and Russell, 1986): in periosteal cells (Scutt *et al.*, 1994) and in an *in vivo* model of ectopic bone formation from bone-marrow-derived stromal cells (Datta *et al.*, 2007). In periosteal cells, cyclic exposure to PTH has a biphasic effect to enhance then suppress proliferation of osteoblast progenitors (Ogita *et al.*, 2007). With the exception of the *in vivo* model and the experiments on periosteal osteoblasts, all *in vitro* studies were performed under prolonged (6 to 24 hours) treatment with PTH; and as such they may represent the consequences of continuous rather than intermittent administration of the hormone. Low doses of PTH also promoted proliferation of osteoblasts in a PKC-dependent manner (Swarthout *et al.*, 2001). Alternatively, proliferation of differentiating osteoblasts may be differentially affected by PTH: proliferation is enhanced in early but suppressed in later stages of osteoblast differentiation. Finally, proliferative actions of PTH may be specific to bone compartments (as in the periosteum) or required in other settings as intermittent PTH promotes mesenchymal cell proliferation in the early stages of fracture repair in rodents (Nakazawa *et al.*, 2005).

Effects on PTH on Osteoblast Differentiation

It is generally accepted that differentiation and proliferation are inversely associated. Differentiation requires exit from the cell cycle and, as a result, proliferation is attenuated as differentiation proceeds. Consistent with its lack of an effect on the replication of uncommitted progenitors and its anti-mitotic actions on differentiating osteoblasts, PTH may first induce the commitment of cells to the osteoblastic lineage and then promote their differentiation by inducing an exit from cell cycle. The ability of PTH to promote commitment to osteoblast differentiation was initially demonstrated using a fibroblast osteoprogenitor colony-forming assay (CFU-f) with bone marrow stromal cells of neonatal or young rats (Ishizuya *et al.*, 1997; Nishida *et al.*, 1994). Bone-marrow-derived osteoblastic cultures established from ovariectomized rats treated with intermittent PTH showed increased ability to form mineralized nodules (Valenta *et al.*, 2005). In general, evidence from *in vitro* studies suggests that under conditions that simulate administration of PTH in an intermittent fashion, the hormone stimulates osteogenic cell proliferation and osteoblastic lineage commitment in bone-marrow-derived as well as in periosteal cells (Dempster *et al.*, 1993; Miao *et al.*, 2001; Locklin *et al.*, 2003; Midura *et al.*, 2003). Moreover, PTH upregulates the expression of several Wnt family members and Wnt signaling, suppresses the Wnt antagonist DKK1 in osteoblast progenitors and mature osteoblasts, and requires the Wnt receptor secreted frizzled related protein 1 (SFRP1) for its bone anabolic actions (Bikle *et al.*, 2002; Kulkarni *et al.*, 2005; Bodine *et al.*, 2004; Guo *et al.*, 2005; Barnes *et al.*, 2005). PTH also rapidly and transiently stimulates the expression and activity of the osteoblast-specific transcription factor Runx2 as well as the expression of alkaline phosphatase and type I procollagen in cultured bone marrow or calvaria cells (Locklin *et al.*, 2003; Ishizuya *et al.*, 1997). *In vivo*, a single injection of PTH upregulates the expression of collagen I within 6 hours and increases bone matrix synthesis within 24 hours in young growing rats (Onyia *et al.*, 1995; Onyia *et al.*, 2000). Consistent with a PTH-induced increase in the number of differentiating osteoblasts, intermittent PTH enhances ossicle development from bone-marrow-derived stromal cells implanted into immunocompromised mice (Pettway *et al.*, 2005).

In vitro, prolonged exposure to PTH appears to exert an attenuating effect on osteoblast differentiation: It inhibits the expression and synthesis of matrix proteins, including collagen I, osteocalcin, and alkaline phosphatase activity, regardless of whether exposure is for a few hours or several days in differentiated osteoblasts (Tetradis *et al.*, 1998; Bogdanovic *et al.*, 2000; Clohisy *et al.*, 1992; Howard *et al.*, 1981; Dietrich *et al.*, 1976; Kream *et al.*, 1993b; Raisz and Kream, 1983b; Raisz and Kream, 1983a; Tetradis *et al.*, 1997; Tetradis *et al.*, 1996). It is well established that PTH regulates gene expression of osteoblasts, supporting synthesis of matrix proteins required for new bone formation (Parfitt, 1976a) and proteins associated with matrix degradation and turnover (Parfitt, 1976b). Prolonged exposure to PTH in cultured bone cells altered several nuclear matrix proteins (Bidwell *et al.*, 1998). Some of these appear to be architectural transcription factors and some, such as NMP4 (a nuclear matrix protein) and NP (a soluble nuclear protein): bind directly to the regulatory region of the rat type I collagen α(I) promoter in the presence of PTH (Bidwell *et al.*, 1998; Alvarez *et al.*, 1998). The expression of matrix metalloproteinase-13 (MMP-13): which is responsible for degrading components of extracellular matrix, is stimulated by PTH through a PKA-dependent pathway that leads to removal of histone deacetylase 4 (HDAC4) from the MMP-13 promoter (Shimizu *et al.*, 2007). These experiments suggest that the profile of gene expression, protein activity, and pro-differentiating actions of PTH are subject to the duration of the exposure. *In vitro* data may predict *in vivo* data associated with continuous exposure to PTH. Following continuous infusion of PTH in adult rats, osteoblasts are associated with fibrosis rather than new bone matrix (Dobnig and Turner, 1997; Kitazawa *et al.*, 1991). The transcriptional signature of intermittent versus continuous PTH administration was examined *in vivo* in the rat. In the intermittent treatment, many genes encoding signaling mediators, transcription factors, cytokines, and proteases/protease inhibitors were regulated rapidly and cyclically with each PTH injection. Genes associated with skeletal development showed a slowly accruing pattern of expression. With continuous treatment, the expression of some genes was sustained while others showed a biphasic response, decreased then increased expression (Li *et al.*, 2007a).

An interesting observation in studies of the anabolic effects of PTH has been the consistent finding that PTH upregulates expression of both matrix-degrading proteins, such as matrix metalloproteinases and ADAMTS-1, and cytokines associated with regulating matrix degradation and turnover, such as interleukin (IL)-6 and IL-11 (Onyia *et al.*, 1995; Onyia *et al.*, 1997a; Clohisy *et al.*, 1992; Greenfield *et al.*, 1995; Greenfield, Horowitz, and Lavish, 1996; Huang *et al.*, 1998; McClelland *et al.*, 1998; Winchester *et al.*, 1999; Elias, Tang, and Horowitz, 1995; Miles *et al.*, 2002). In response to PTH, ADAMTS-1 increases the three-dimensional growth of human primary osteoblasts through type I collagen processing (Rehn *et al.*, 2007). This action involves inhibiting migration of cells on a planar substrate but enhancing growth in a collagen scaffold.

Intermittent PTH may promote osteoblastogenesis by a recently identified mechanism that involves downregulation of the expression of the transcription factor PPARγ. PPARγ exerts a tonic, suppressive effect on osteoblast differentiation by promoting commitment of mesenchymal cells to the adipocytic lineage at the expense of the osteoblastic lineage. *In vitro*, short and transient exposure of human mesenchymal stem cells to PTH, inhibited adipocyte formation by an insulin and troglitazone-containing medium shown to induce adipocytic differentiation (Chan *et al.*, 2001). *In vivo*, the anabolic effect of intermittent PTH was associated with decreased number of adipocytes in the bone marrow. Similar to the expression profiling of genes related to osteoblast differentiation, PTH-induced inhibition of adipogenesis may depend on the duration of treatment. The anti-adipogenic effects of PTH were not seen in cells exposed to a continuous PTH treatment (Rickard *et al.*, 2006).

Effects on Osteoblast Survival

Dose response studies indicate that intermittent PTH administration to adult mice decrease the prevalence of osteoblast apoptosis in the secondary spongiosa of distal femur. The decrease in osteoblast apoptosis was positively correlated with increased bone mineral density in both the spine and hind limbs (Jilka *et al.*, 1999; Bellido *et al.*, 2003). In the same report, osteoblast apoptosis was not affected by six days of continuous PTH infusion or by two days of hormone elevation caused by a calcium-deficient diet. Expression of a constitutive active PTH1 receptor (PTHR1) in cells of the osteoblastic lineage decreased osteoblast apoptosis concomitantly with increasing the number of proliferating osteoblasts (Calvi *et al.*, 2001). In contrast to these studies, an increase in osteoblast apoptosis in the primary spongiosa was observed following intermittent PTH administration to mice (Stanislaus *et al.*, 2000). However, this increase was transient and paradoxically associated with decreased activity of caspases 2, 3, and 7 measured in the femoral metaphysis; a site including both primary and secondary spongiosa. A transient increase in apoptosis in proliferating cells and osteocytes of young rat metaphyses was recorded during the initial response to PTH (Stanislaus, Devanarayan, and Hock, 2000). Intermittent PTH was also reported to increase osteoblast apoptosis in cancellous bone of transiliac biopsies obtained from postmenopausal women (Lindsay *et al.*, 2007). The number of apoptotic osteoblasts was positively correlated with increases in bone formation rate. Such conflicting observations may result from differences in the skeletal site used to measure apoptosis, or in the susceptibility of osteoblast subsets to the hormone. A subset of osteoblasts may be highly susceptible to apoptosis. Additionally, in some instances a pro-apoptotic action of PTH on osteoblasts may be secondary to its matrix-degrading properties. Parathyroid hormone may induce upregulation of matrix metalloproteinases (McClelland *et al.*, 1998; Zhao *et al.*, 1999b) leading to matrix degradation and osteoblast retraction through a calpain-dependent, proteolytic modification of the osteoblast cytoskeleton (Murray *et al.*, 1997). The consequence of cell retraction and detachment because of matrix degradation is apoptosis of cells that are unable to reattach. Even so, the transient increase in osteoblast apoptosis and upregulation of matrix metalloproteinases can still be consistent with mechanisms activating bone turnover. One consequence of matrix-activation-degrading enzymes may be that reconditioned bone surface can serve as an attractant for newly differentiating osteoblasts to increase bone-forming surfaces (anabolic action) or as an attractant for differentiating osteoclasts to continue resorption of old surfaces (catabolic action). Reattachment of detached osteoblasts may delay or inhibit their apoptosis.

The majority of *in vitro* studies suggest that short-term exposure to PTH protects rat, murine, and human osteoblastic cells from a variety of pro-apoptotic stimuli (Bellido *et al.*, 2003; Jilka *et al.*, 1998; Chen *et al.*, 2002). In contrast, long-term exposure to PTH stimulates apoptosis of the multi-potential C3H10T1/2 osteoblastic cell line (Chen *et al.*, 2002). Mechanistic studies using cultured osteoblastic cells indicate that the anti-apoptotic action of PTH is initiated by rapid cAMP-mediated activation of PKA. Downstream of PKA the anti-apoptotic pathway was two-pronged: it involved activation of survival proteins in the cytoplasm and survival genes in the nucleus (Bellido *et al.*, 2003). In the cytoplasm, PKA phosphorylated and inactivated the pro-apoptotic protein Bad. In the nucleus it increased transcription the survival gene Bcl-2, cAMP response element-binding protein (CREB); and Runx2. The upregulation of Runx2 expression was short-lived as the transcription factor was targeted for proteasomal degradation by the Smurf1 E3 ligase (ubiquitin-protein isopeptide ligase): and, also accounted for the short-lived anti-apoptotic effect of PTH in cultured osteoblastic cells. On the other hand, continuous PTH caused sustained reduction in Runx2 levels. Factors that control Runx2 expression in response to PTH may also be involved in the anti-apoptotic actions of the hormone. p21^{Cip1} expression increased with PTH treatment and contributed to the increase in Runx2. In addition, p21^{Cip1} may have a separate, Runx2-independent pro-survival effect as it can prevent activation of caspases and block the pro-apoptotic effects of apoptosis signal-regulating kinase 1 (Gartel and Tyner, 2002) or promote survival of osteoblastic cells (Bellido *et al.*, 1998).

Differential regulation of Runx2 expression and degradation by intermittent versus continuous PTH administration influences both the survival and pro-differentiating actions of the hormone and as such is at least one of the mechanisms that may explain its anabolic versus catabolic profile. Differences in the profile of responses to intermittent versus continuous exposure to PTH in cells of the osteoblastic lineage are still being identified and have yet to be completely clarified. Recently, regulation of gene expression by PTH was used as a basis for investigating the molecular mechanism of its dual actions in bone: anabolic with intermittent versus catabolic with continuous administration of the hormone (Onyia *et al.*, 2005; Li *et al.*, 2007a). Gene array approaches indicate two gene expression patterns that can be placed in two groups according to the dynamics of their changes. Under the influence of intermittent treatment, many genes encoding signaling mediators, transcription factors, cytokines, and proteases/protease inhibitors are regulated rapidly and cyclically with each PTH injection (Li *et al.*, 2007a). With continuous treatment, some genes are regulated within six hours and their levels are sustained while others show a biphasic response: a decrease then increase later.

Independent of the phenomena that determine osteoblast fate in response to PTH, be that proliferation, differentiation, function, or apoptosis, or combinations of them all, the signaling cascades and their interaction with pathways elicited by autocrine and paracrine factors are clearly under dynamic controls.

PTH REGULATION OF OSTEOCYTES

It is now appreciated that osteocytes, the most abundant cells in bone, are principal target cells for PTH. The PTH1 receptor (PTH1R) has been detected in osteocytes *in vivo* (Lee *et al.*, 1993; Langub *et al.*, 2001). Receptor sites that appear to have specificity for carboxyl-terminal regions of PTH(1–84) and other circulating PTH fragments were present in osteocytic cells derived from clonal, conditionally transformed, calvaria cells (Divieti *et al.*, 2001). The response of osteocytes to mechanical stress *in vivo* is enhanced by PTH(1–33) and may even require circulating PTH. When PTH is rendered absent (i.e., after total parathyroidectomy): such effects are no longer demonstrable (Plas *et al.*, 1994; Chow *et al.*, 1998). An anabolic dose of intermittent PTH(1–34) administered to mice, or short-term exposure to PTH, prolonged osteocyte survival (Jilka *et al.*, 1999; Bellido *et al.*, 2003). This effect together with the anti-apoptotic actions of the hormone was, at a minimum, a critical contributor to the PTH-induced increase in bone mass. In contrast to the anti-apoptotic actions of PTH(1–34): PTH(1–18): and carboxyl-terminal fragments of PTH [PTH(34–84)], promoted apoptosis *in vitro*, in osteocytic cells lacking a functional PTH1R. Carboxyl-terminal fragments of PTH are secreted from the parathyroid glands or produced by proteolytic cleavage of PTH(1–84) in peripheral tissues. It is therefore possible that PTH1R and receptors for carboxyl-terminal fragments of PTH exert opposite effects on osteocyte survival. Osteocytes may be able to integrate signals between these two types of PTH receptors in response to changes in circulating levels of intact PTH and carboxyl-terminal fragments of PTH. In addition, carboxyl-terminal fragments of PTH upregulate the expression of connexin 43, a gap-junction protein important in mediating intercellular communication in osteocytes (Ziambaras *et al.*, 1998). The functional significance of this observation is not known. Perhaps carboxyl-terminal fragments of PTH produced by the parathyroids during hypercalcemia promote communication of osteocytes to other bone cells to limit osteoclast recruitment and thereby exert negative feedback on bone resorption. Such speculations on actions of carboxyl terminal fragments of PTH although interesting have to be regarded with caution because a bona fide receptor for this portion of the PTH molecule has not yet been cloned, despite several decades of intense effort by several laboratories.

More recently, PTH has been found to regulate the expression of sclerostin (SOST), which antagonizes the pro-osteoblastogenic actions of bone morphogenetic proteins (BMPs) and LRP5 and 6 (Poole *et al.*, 2005; Van Bezooijen *et al.*, 2004; Semenov, Tamai, and He, 2005; Ellies *et al.*, 2006). SOST may not act as a classical BMP antagonist predominantly blocking Smad phosphorylation, but use alternative signaling pathways or interfere with unknown BMP-induced factors, cofactors, or other pathways. SOST was identified as a potent osteocyte product that negatively regulates bone formation *in vitro*. In murine models and in patients with the bone overgrowth disorders sclerosteosis and van Buchem disease, the *Sost* gene is inactive, thus relieving the anabolic pathway of tonic inhibition by SOST (Winkler *et al.*, 2003; Van Bezooijen *et al.*, 2004; Wergedal *et al.*, 2003; Brunkow *et al.*, 2001). Continuous infusion of PTH to mice for four days decreased the mRNA and protein expression of SOST 80–90% in vertebral bone and in osteocytes, respectively (Bellido *et al.*, 2005). In contrast, a single injection of PTH caused a transient 50% reduction in Sost mRNA at two hours, but four daily injections had no effect on Sost mRNA or protein. Similarly, in a short-term mouse calvaria model of local bone formation five days of intermittent PTH application stimulated bone formation and Sost expression. On the other hand, local bone formation was robustly decreased four hours after the last local PTH administration, whereas it was unchanged from control levels after the follow-up period, suggesting a tight control of Sost expression by PTH. Interestingly, the profile of PTH-induced changes in Sost expression was different from PTH-induced regulation of the osteocalcin gene (Keller and Kneissel, 2005). A decrease of Sost mRNA levels was observed in the cortical bone of skeletally mature rats within six hours after the single systemic subcutaneous injection of PTH(1–34): confirming rapid regulation of SOST by PTH (Keller and Kneissel, 2005). Suppression of Sost by PTH represents a novel mechanism for hormonal control of osteoblastogenesis mediated by osteocytes. PTH may promote osteoblastogenesis directly by stimulating Wnt and BMP signaling in osteoblast progenitors or indirectly by controlling SOST production in osteocytes. In support of this idea, overexpression of a constitutively active PTHR1 receptor gene specifically in osteocytes, suppressed Sost expression, and increased bone mass in transgenic mice (O'Brien *et al.*, 2006).

Hence, the increased production of osteoblasts needed for increased bone turnover in primary hyperparathyroidism may result from such an indirect effect of PTH on early stages of osteoblastogenesis. Mechanisms underlying a potential sustained SOST inhibition after long-term intermittent PTH treatment, as opposed to transient inhibition following single application, remain to be determined.

PTH REGULATION OF LINING CELLS

By electron microscopy, PTH has been shown to rapidly stimulate differentiation of nonproliferating osteoprogenitors lining quiescent bone surfaces (Roberts, 1975). Intermittent PTH administration to rats increased the number of osteoblasts lining the surface of cancellous bone (Leaffer *et al.*, 1995). This increase was accompanied by a reciprocal reduction in the number of lining cells that covered the surface of quiescent bone. Following cessation of hormone administration, osteoblast number declined while lining cells increased. Based on this evidence, it was proposed that intermittent PTH caused lining cells to revert to their osteoblast phenotype, thus providing a potential explanation for the rapid appearance of osteoblasts on previously quiescent bone surfaces in response to PTH. However, the extent to which the few lining cells contribute to generate the number of osteoblasts required to produce the increased osteocyte number and density observed in mice receiving daily PTH injections remains to be explored (Jilka *et al.*, 1999). Moreover, the reciprocal changes in osteoblast and lining cell number could also be explained if lining cells were replaced by osteoblasts that overfill the resorption cavity and migrate onto adjacent quiescent surfaces as suggested by evidence from studies in humans (Lindsay *et al.*, 2006; Ma *et al.*, 2006). Lining cell activation has also been proposed to explain the PTH-induced increase in osteoblasts when remodeling is low or absent, such as in the periosteal surface. A better understanding of the biosynthetic properties of lining cells, and their response to PTH, is needed before the role of these cells as mediators of PTH can be accounted.

PTH Regulation of Osteoclasts and their Osteoprogenitors

Barnicott (1948) first recognized the resorptive properties of PTH. In this study, remnants of parathyroid glands were laid on the inverted underside of calvaria and reimplanted under the skulls of mice. Significant bone resorption in the calvaria resulted. This work laid the foundation for *in vitro* assays utilizing rat and mouse bones in which resorption was induced routinely by PTH (Raisz, 1963; Raisz, 1965). PTH indirectly enhances bone resorption and release of calcium from bone surfaces by activating osteoclasts. This is despite lack of evidence for direct binding of ^{125}I-bovine PTH(1–84) to avian osteoclasts (Teti, Rizzoli, and Zallone, 1991) and for activation of resorption via a direct effect on the osteoclast (Mears, 1971; Murrills *et al.*, 1990). In bone, PTH receptors are found primarily on osteoblasts (Luben, Wong, and Cohn, 1976; Partridge *et al.*, 1981; Majeska and Rodan, 1982): and apparently osteoclasts do not possess PTH receptors (Suda *et al.*, 1996). Thus, the effect of PTH on osteoclasts is most likely indirect. *In vivo*, PTH induced a transient increase in apoptosis of osteoblasts (Stanislaus, Devanarayan, and Hock, 2000) and upregulation of osteoblast matrix metalloproteinases (McClelland *et al.*, 1998; Zhao *et al.*, 1999a). This is consistent with PTH-mediated mechanisms activating bone turnover in which protein synthesized by osteoblasts activates osteoclasts. Evidence that bisphosphonates attenuate the anabolic effect of intermittent PTH in animal models and humans is consistent with the involvement of osteoclasts (Black *et al.*, 2003; Finkelstein *et al.*, 2003; Ettinger *et al.*, 2004; Delmas *et al.*, 1995; Wronski *et al.*, 1993): but such inhibition was not seen in other studies (Liu *et al.*, 2007; Cosman *et al.*, 1998; Cosman *et al.*, 2005; Ma *et al.*, 2003). Indeed, both OPG and alendronate prolonged the duration of the anabolic effect in ovariectomized mice, apparently by preventing the increased bone resorption that occurs in the later stages of treatment (Samadfam, Xia, and Goltzman, 2007).

In genetically modified mice in which collagenase is unable to cleave collagen I, daily PTH injections over the skull did not induce osteoclast-mediated resorption but did occur in control animals (Zhao *et al.*, 1999a). *In vitro*, osteoclast-like cells in culture failed to respond to PTH unless cocultured with stromal or osteoblast-like cells (Kanzawa *et al.*, 2000; Suda *et al.*, 1999; Horwood *et al.*, 1998; McSheehy and Chambers, 1986; Fuller, Owens, and Chambers, 1998; Fuller *et al.*, 1998).

Stromal cells and osteoblastic lineage cells regulate osteoclast differentiation through cell–cell contact by controlling the synthesis of osteoprotegerin (OPG/OCIS) and the ligand for the receptor activator of NF-*k*B (RANKL/ODF/TRANCE/OPGL) (Kanzawa *et al.*, 2000; Suda *et al.*, 1999; Horwood *et al.*, 1998). These two secreted proteins compete for binding to the osteoclast progenitor receptor activator NF-*k*B(RANK): a TNF receptor family member (Suda *et al.*, 1999). If RANKL binding to RANK predominates, as seen following PTH treatment of cultured osteoblast-like osteosarcoma cells transfected with the PTH1 receptor, osteoclast progenitors differentiate into osteoclasts in the presence of M-CSF (Suda *et al.*, 1999; Fuller, Owens, and Chambers, 1998; Fuller *et al.*, 1998). Intermittent PTH provoked a transient increase in RANKL (Ma *et al.*, 2001) that may result in a transient increase in osteoclastic resorptive activity. In addition, PTH downregulated OPG expression in a variety of bone cell lines (Kanzawa *et al.*, 2000; Lee and Lorenzo, 1999). PTH stimulated osteoclast formation in part by increasing the RANKL/OPG ratio in osteoblasts (Khosla, 2001; Ma *et al.*, 2001; Buxton, Yao, and Lane, 2004). Activation of the cAMP/PKA–CREB pathway was required for the effects of PTH on RANKL and OPG (Fu *et al.*, 2002; Lee and Lorenzo, 2002; Kondo, Guo, and Bringhurst, 2002). Reciprocal regulation of OPG and RANKL expression by PTH preceded its effects on osteoclast formation by 18–23 hours. These effects were more pronounced in primary bone marrow cells than in calvaria bone organ cultures or MC3T3-E1 cells (Lee and Lorenzo, 1999). Recently, evidence for a direct role of PTH on RANKL expression and osteoclastogenesis *in vivo* was obtained using mice lacking a distant transcriptional enhancer of the RANKL gene that confers responsiveness to PTH (Fu, Manolagas, and O'Brien, 2006). Elevation of PTH levels in these animals, induced by a calcium-deficient diet, failed to upregulate RANKL expression in bone. Moreover, PTH was unable to stimulate osteoclast formation in primary cultures of bone marrow cells derived from these mutant mice.

In vivo studies of young rats treated once daily with hPTH(1–34) for three days showed an altered ratio of mRNA for osteoprotegerin and RANKL (Onyia *et al.*, 2000): but it is not known if the magnitude of these changes translates into relevant function. Changes in resorption associated with once-daily treatment of PTH are rarely detected in rat models *in vivo*. In humans and animals with osteonal bone skeletons, increased resorption in response to PTH in cortical bone occurs, but only after the increase in surface bone formation, which occurs during the first remodeling period (sigma) (Cosman and Lindsay, 1998a; Hodsman and Steer, 1993; Mashiba *et al.*, 2001; Hirano *et al.*, 1999; Hirano *et al.*, 2000; Burr *et al.*, 2001). This increase in new bone-forming surfaces appears to offset the increase in resorption that precedes new osteonal formation in intracortical bone.

As suggested by *in vitro* data (Kanzawa *et al.*, 2000; Suda *et al.*, 1999; Horwood *et al.*, 1998): increased resorption plays a key role in the mechanisms activated when PTH is given by continuous infusion and occurs within the first three days of infusion in young rats. In humans and dogs, continuous infusion of PTH was associated with histomorphometric and biochemical data consistent with the early induction of resorption and increased bone turnover (Malluche *et al.*, 1982; Cosman *et al.*, 1991; Cosman and Lindsay, 1998a). In rats, resorption results in loss of bone mass despite a small increase in bone-forming surfaces (Watson *et al.*, 1999; Hock and Gera, 1992; Tam *et al.*, 1982). In a pathologic condition of continuously elevated PTH levels, primary hyperparathyroidism (PHPT): catabolic actions associated with the osteoclastogenic properties of PTH have been linked to its ability to regulate the expression of RANKL and OPG and to increase the RANKL/OPG ratio *in vitro* and *in vivo* (Lee and Lorenzo, 1999; Khosla, 2001; Buxton, Yao, and Lane, 2004; Huang *et al.*, 2004; Ma *et al.*, 2001; Onyia *et al.*, 2000). Early data in human subjects also argue for RANKL as a key intermediate in the catabolic actions of PTH. In subjects with PHPT who underwent successful parathyroid surgery, Stilgren *et al.* analyzed mRNA in transiliac bone biopsies. They showed a positive correlation between RANKL levels and both bone formation and resorption markers (Stilgren *et al.*, 2004). Moreover, the ratio of RANKL to OPG declined after surgery. Concomitant with the decrease in RANKL/OPG expression was an increase in bone mass. In cross-sectional studies, PTH and serum OPG levels were shown to be negatively correlated with each other (Szulc *et al.*, 2001). A similar negative correlation was observed in a study of osteoporotic women treated with PTH and in women with early breast cancer (Seck *et al.*, 2001). These observations in human subjects suggest that PTH exerts its catabolic effect on bone by stimulating RANKL-mediated bone resorption.

Upregulation of RANKL and osteoclastic activity are also noted during the anabolic actions of intermittent PTH. Gene array analysis in bone from rats indicated a fivefold increase and cyclic stimulation of RANKL expression in the anabolic (intermittent) regime (Li *et al.*, 2007a; Onyia *et al.*, 2005). A moderate but sustained stimulation was associated with continuous infusion of the hormone. OPG levels also increased with the intermittent PTH regime, but the increases lagged considerably behind the increases in RANKL resulting in a transient stimulation of the RANKL/OPG ratio. In contrast, in the continuous treatment, OPG levels decreased with time maintaining an elevated RANKL/OPG ratio. In addition to its effects on osteoblasts, a cyclic upregulation of bone resorption, versus a moderate but sustained elevation of RANKL/OPG, may contribute to the anabolic actions of intermittent PTH. RANKL may also stimulate the secretion of an osteoblastogenic factor from osteoclasts (Martin and Sims, 2005). Osteoclast activation following continuous infusion of PTH may also be related to the shift in matrix protein synthesis by osteoblasts to a more fibroblast-like profile (Dobnig and Turner, 1997): which would result in an extracellular matrix (ECM) feedback signal to activate increased bone turnover.

PTH rapidly and dramatically stimulated the expression of monocyte chemoattractant protein-1 (MCP-1) in femurs of rats receiving daily injections of the hormone or in primary osteoblastic cells (Li *et al.*, 2007b). Studies with the mouse monocyte cell line RAW 264.7 and mouse bone marrow indicated that osteoblastic MCP-1 can recruit osteoclast monocyte precursors and facilitate RANKL-induced osteoclastogenesis and fusion. Thus, in addition to stimulation of RANKL production, upregulation of MCP-1 levels may contribute to the osteoclastic/bone remodeling activity that accompanies the anabolic effects of PTH.

When hPTH(1–34) was infused at 40 μg/kg/day in young rats and compared to a PTH injection of 40 μg/kg/day, there was upregulation of c-fos and IL-6 mRNA expression following injection and equivalent upregulation of c-fos but no alteration of IL-6 mRNA levels following infusion (Liang *et al.*, 1999). These responses were detected in the femur metaphysis, which is enriched for osteoblasts, but not in the cortical diaphysis, which contains predominantly hematopoietic and stromal cells. In primary cultures of bone marrow stromal cells from either metaphysis or diaphysis of rat femurs, PTH increased both c-fos and IL-6 expression suggesting that, *in vivo*, there are site-specific regulatory factors controlling the profile of genetic responses, in addition to those associated with different treatment regimens. Confounding their interpretation is a study in which serum IL-6 was increased in mice infused with PTH or rats in which PTH was locally injected over calvaria to mimic a resorption model. Blocking IL-6 release was associated with a decrease in resorption markers, with no change in biochemical markers of formation (Grey *et al.*, 1999; Pollock *et al.*, 1996). There were no measures of bone mass to ascertain if this altered profile was associated with a catabolic or anabolic effect, and no comparison was made to intermittent, once-daily administration of PTH.

Another potential osteoclastogenic mechanism of PTH may involve recently recognized effects of the hormone on Wnt signaling; as well as unappreciated actions of β-catenin on osteoclastogenesis. It was recently shown that mice harboring a gain-of-function mutation of β-catenin in osteoblasts exhibited decreased bone resorption resulting from stimulation of OPG and suppression of RANKL expression (Glass *et al.*, 2005). Additionally, Wnt/β-catenin signaling in osteoblasts upregulated RANKL expression and inhibits osteoclastogenesis *in vitro* (Spencer *et al.*, 2006). As PTH has been shown to interact with Wnt signaling components (reviewed later in this chapter): it is possible that it promotes osteoclastogenesis by opposing the effects of endogenous Wnt/β-catenin-mediated transcription on RANKL and/or OPG. More studies along this line will be needed to elucidate a potential role of PTH–Wnt interactions in osteoclastogenesis.

One limitation of these gene-profiling studies has been our lack of knowledge of how the balance of increased activation frequency may favor formation (anabolic effect of intermittent PTH) or resorption (catabolic effect of continuous PTH). A mathematical model that assumes a longer delay in osteoclast activation (because of a requirement for signals from the osteoblast to osteoclast progenitors) than the delay required for osteoblast differentiation argues that osteoblast function will predominate with intermittent PTH, whereas resorption will be greater with continuous PTH (Kroll, 2000). The importance of interval duration of PTH administration emphasized in this theoretical model has some support from preliminary data. In rats treated with PTH, six injections in one hour once daily, or infusion for one hour once daily, induced an equivalent bone gain to that of one injection/day, whereas six injections over eight hours or continuous infusion for longer than one hour/day resulted in bone loss (Dobnig and Turner, 1997; Turner *et al.*, 1998). Studies in humans have previously shown in PHPT the ability of PTH to act both as a catabolic and as an anabolic hormone. Moreover, as mentioned earlier in this chapter, both intermittent and continuous PTH infusion activates the Wnt signaling pathway to stimulate the recruitment of osteoblast progenitors. The catabolic and anabolic cell signaling properties of PTH appear to be independent as illustrated further in studies by Kalinowski *et al.* in which the skeletal anabolic response to intermittent PTH exposure was maintained in mice with defective osteoclast formation because of transplantation with RANKL-deficient hematopoietic cells (Lorenzo *et al.*, 2006).

The current concept thus is that PTH utilizes both catabolic (OPG-RANKL) and anabolic (Wnt and BMP) pathways. Chronically elevated levels of PTH are more likely to lead to RANKL-associated processes while intermittent administration of PTH is more likely to lead to LRP5-independent Wnt-associated processes. However, it is also clear that chronically elevated levels of PTH (e.g., PHPT) or intermittent exposure to PTH (e.g., treatment for osteoporosis) is also associated with evidence for counterbalancing actions. It follows then that under physiologic conditions, PTH will provide dual anabolic and catabolic skeletal actions that are nicely balanced. On the other hand, the stimulatory effect of PTH on bone resorption may represent a two-edged sword in the setting of anabolic therapy. Intermittent PTH may increase release of osteogenic growth factors from the bone matrix or from osteoclasts, but this effect may be counterbalanced by increased resorption, particularly in later stages of the therapy. The negative impact of increased resorption may be overcome by administering PTH for short periods of time in a cyclic fashion to maximize the bone-forming effects and minimize the pro-resorptive effects (Cosman *et al.*, 2005; Iida-Klein *et al.*, 2007). Such approaches have theoretical appeal but remain to be conclusively demonstrated.

MOLECULAR MECHANISMS AND MEDIATORS OF PTH ACTION: SIGNALING CASCADES TRANSCRIPTION FACTORS, GROWTH FACTORS, AND HORMONES

General Aspects

The actions of PTH (and PTHrP) are mediated by binding of the hormone to its G protein coupled receptor, referrer to as PTH receptor 1 (PTHR1) (Potts, 2005). Activated PTHR1 stimulates G_{as}-mediated activation of adenyl cyclase, which in turn promotes cyclic AMP (cAMP) production and subsequent activation of protein kinase A (PKA). Alternatively, PTHR1 stimulates G*aq*-mediated activation of protein kinase C (PKC). Finally, PTH also activates β-arrestin with a dual outcome: a) subsequent activation of extracellular regulated kinases (ERKs): and b) a negative feedback mechanism involving desensitization of PTHR1-induced cAMP signaling (Gesty-Palmer *et al.*, 2006; Gensure, Gardella, and Juppner, 2005).

Cytoplasmic Signaling: The cAMP/PKA and PKC Pathways

In vitro studies have suggested that cAMP is a key signaling pathway activated by PTH; although its relative importance *in vivo* is not well understood. cAMP binds to the regulatory subunit of PKA, which releases the active catalytic subunits of the enzyme. The catalytic form of PKA phosphorylates proteins on serine residues in the specific recognition sequence R-X-X-S, which often causes changes in the target proteins' structure and function. PTH also activates phospholipase $C\beta$ by $G\alpha_q$ leading to the formation of diacylglycerol (DAG): which activates protein kinase C (PKC) and 1,4,5-inositol triphosphate (IP3) (Civitelli *et al.*, 1988; Babich *et al.*, 1991): resulting in increased intracellular free Ca^{2+} (Reid *et al.*, 1987). Activation of calcium and protein kinase C (PKC) signal transduction pathways require higher doses of PTH *in vitro* than those required to activate the cAMP-PKA pathways. Thus, it has been proposed that cAMP is the key pathway by which the anabolic responses of PTH are effected (Whitfield *et al.*, 1998; Civitelli *et al.*, 1990; Li *et al.*, 2007a; Potts, 1995). Proving this suggestion, gene expression profiling of intermittent versus continuous PTH administration to rats' bone suggested that in both regimes the PKC pathway played a limited role in mediating PTH's dual effects while the PKA pathway appeared to predominate (Frisch, 2000; Howe and Juliano, 2000). Several PTH-induced genes that are primary response genes regulating signaling or transcription in osteoblasts are cAMP dependent. Those include c-fos (Clohisy *et al.*, 1992): collagenase (Civitelli *et al.*, 1989; Scott *et al.*, 1992): IL-6 (Greenfield *et al.*, 1995): type I collagen (Kream *et al.*, 1993a): Nurrl (Tetradis, Bezouglaia, and Tsingotjidou, 2001): Nur77 (Tetradis *et al.*, 2001): NOR-1 (Pirih *et al.*, 2003): E4bp4 (Ozkurt, Pirih, and Tetradis, 2004): RGS-2 (Tsingotjidou *et al.*, 2002): and ICER (Tetradis *et al.*, 1998; Pirih *et al.*, 2003). More recently, the cAMP-inducible coactivator PPARγ coactivator-1α (PGC-1α) was shown to synergize with the orphan nuclear receptor Nurr1 to induce target gene transcription in osteoblasts in response to PTH (Nervina *et al.*, 2006). Additionally, RAMP3 (Receptor Activity Modulating Protein-3): which modifies calcitonin receptor function, is also a cAMP-dependent target of PTH in osteoblasts (Phelps *et al.*, 2005).

In fibroblasts, cAMP signaling activates PKA-controlled cell growth by abrogating signaling required for detachment of cells, inhibiting both progression through the cell cycle and apoptosis (Bretschneider, Vasiev, and Weijer, 1997; Hofer *et al.*, 2000; Thomas, 2000). If valid for osteoprogenitors, this could provide mechanisms by which PTH promotes differentiation but not proliferation of bone cells. However, regarding the mechanistic basis for the differential effects of intermittent versus continuous PTH treatment on bone, the magnitude of changes in cAMP and the PKA pathway cannot explain the paradoxical actions of PTH to inhibit and stimulate bone formation and to spare or stimulate osteoclast-mediated resorption. Concepts on how waves of differing amplitude and frequency of cAMP and Ca^{+2} signaling traverse a cell and may be amplified in neighboring cells by activation of intracellular calcium-sensing receptors are examined for the relevance to signal transduction in bone cells in response to PTH (Steddon and Cunningham, 2005). Compounds that increase (calcimimetics) or decrease (calcilytics) the sensitivity of the G-protein-coupled calcium-sensing receptor to calcium are currently being explored as means for regulating the response of bone (and other cell types) to endogenous serum levels of PTH (Stanislaus, Devanarayan, and Hock, 2000; Liang *et al.*, 1999).

c-fos and c-jun AP-1 Family Members

As one of the cAMP-dependent mechanisms, the upregulation of c-fos and the role of the immediate early gene comprising the AP-1 fos and jun family members have been widely studied. *In vivo*, PTH [as either hPTH(1–84) or hPTH(1–34)] rapidly and transiently increased c-fos, fra-2, junB, and c-jun significantly above baseline levels in the metaphyses and whole tibia of young and adult mice at doses as low as 1 μg/kg (Sabatakos *et al.*, 2000). Although other AP-1 members, δfosB (Jochum *et al.*, 2000) and fra-1 (Stanislaus, Devanarayan, and Hock, 2000): regulate bone formation, PTH did not regulate their expression in young rats (Stanislaus, Devanarayan, and Hock, 2000). The regulation of c-fos in bone has the greatest magnitude of change of the PTH-regulated AP-1 family members and is time- and cell-dependent (Kano *et al.*, 1994; McCauley *et al.*, 1997; Clohisy *et al.*, 1992). *In situ* hybridization of young rat bone showed that bone-lining cells expressed an increased amount of c-fos within 15 to 60 minutes of exposure to hPTH(1–84) (0.2 mg/kg): followed by stromal cell and osteoclast expression after 1 to 2 hours. *In vitro*, constant exposure to PTH (1–34) and PTHrP(1–34) induced c-fos gene expression rapidly in bone cell lines (Fiol *et al.*, 1994; Tyson *et al.*, 2002). The effect of PTH on c-*fos* expression is mediated predominantly by the actions of cAMP acting through the major cAMP response element (CRE) within the c-*fos* promoter. The major CRE is bound by CREB and CREB is phosphorylated at serine 133 in response to PTH treatment. Most likely, a second phosphorylation event, at serine 129 by GSK-3 (Clohisy *et al.*, 1992): is required before full transcriptional activity in response to PTH can occur. In addition to c-fos, PTH increases expression of c-*jun* in osteoblasts both rapidly and transiently as is typical of immediate-early genes (Civitelli *et al.*, 1989; Scott *et al.*, 1992). Heterodimerization of Fos with its partner Jun can lead to increased expression of many genes possessing an AP-1 site within their promoters. The presence of AP-1 binding sites in the collagenase-3 promoter and the fact that the AP-1 element is required for the PTH induction of collagenase-3 expression (Johnson, Spiegelman, and Papaioannou, 1992; Wang *et al.*, 1992; Grigoriadis *et al.*, 1994) suggest that Fos/Jun heterodimers may play an important role in mediating the effects of osteogenic stimuli. Knocking down of c-fos expression antagonized the PTH- (or PTHrP-) induced inhibition of proliferation in osteoblastic cells, as well as induction of osteoclast differentiation in the presence of these cells (Kano *et al.*, 1994). However, deletion of the c-*fos* gene in mice does not have any apparent effects on osteoblasts, perhaps because its osteoblastic function is substituted by a related c-fos family member, but, dramatically inhibits osteoclastogenesis (Li *et al.*, 2007a). Thus, c-*fos* is required for osteoclast differentiation, but the role of c-fos in osteoblasts is still not completely determined. c-fos has also been implicated in the process of programmed cell death in other cells, and the PTH-induced increases in c-fos may be relevant to the inhibition of apoptosis in bone cells.

Wnt Signaling and Sclerostin

There is plenty of evidence to suggest that intermittent administration of the PTH can induce osteoblastic lineage commitment and differentiation by stimulating Wnt and BMP-2 signaling in osteoblast progenitors. PTH mediated early stem cell recruitment during fracture repair, in part, by upregulating the expression of Wnt4 and Wnt5b. In an expression profiling of intermittent versus continuous PTH to rats, Wnt4 and the Wnt receptor antagonist secreted frizzled related protein 4 (SFRP4) were upregulated whereas SFRP2 was downregulated by both regiments in bone (Kulkarni *et al.*, 2005). Continuous exposure to PTH (1–38) both *in vivo* and *in vitro* upregulated the expression of LRP6 and the Wnt receptor Frizzled 1 (FZD-1): and decreased LRP5 and Dkk-1. These effects in UMR 106 cells were associated with an increase in β-catenin protein levels and resulted in functional activation (three- to six-fold) of a downstream Wnt responsive TBE6-luciferase (TCF/LEF-binding element) reporter gene. The effect of PTH on the canonical Wnt signaling pathway occurred, at least in part, via the cAMP-PKA pathway through the differential regulation of the receptor complex proteins (FZD-1/LRP5 or LRP6) and the antagonist Dkk-1 (Tobimatsu *et al.*, 2006). PTH stimulated osteoblast β-catenin levels via Smad3 in mouse MC3T3-E1 osteoblastic cells, and both the PKA and PKC pathways were involved (Kakar *et al.*, 2007). Smad3-dependent upregulation of β-catenin levels by PTH may be involved in the anti-apoptotic actions of the hormone in osteoblasts. In osteoblast progenitors derived from the periosteal surface, cyclic PTH treatment to simulate an intermittent mode of administration, upregulated the expression of the Wnt target Axin2; and this effect correlated with a pro-differentiating action of the hormone on periosteal osteoblast progenitors (Ogita *et al.*, 2007).

In vivo, the ability of PTH to enhance the earliest stages of endochondral bone repair correlated with increased level of Wnt-signaling at multiple time points across the time course of fracture repair, indicating that such responses are at least in part mediated through Wnt signaling (Bodine *et al.*, 2007). PTH suppressed the expression of the LRP5 and LRP6 antagonist Dkk1 but activated Wnt signaling in transgenic mice overexpressing Dkk1 in osteoblasts under the control of the α1(I)collagen promoter, indicating that LRP5-independent Wnt signaling may be required for the anabolic actions of PTH. In support of this idea, intermittent PTH administration for four or six weeks increased osteoblast number and cancellous and cortical bone formation in an LRP5-deficient female mice (Sawakami *et al.*, 2006; Iwaniec *et al.*, 2007). In contrast to these findings, a blunting of the anabolic effects of PTH was observed in male LRP5-null mice of the same studies and in the femoral trabecular bone of LRP5 knockout mice receiving up to tenfold higher dose (300 ng/g) of the anabolic dose of daily PTH (Kato *et al.*, 2002). In agreement with the lack of an effect of LRP5 deletion to the anabolic effects of PTH, at least in murine cortical bone of both sexes and in cancellous bone of females, intermittent PTH further increased bone formation in mice transgenic for the G171V LRP5 high bone mass mutation. These observations may not be unexpected because LRP5 and PTH differentially affect the fate of osteoprogenitors. LRP5 increases bone formation by enhancing the proliferation of osteoblast progenitors and without affecting osteoblast differentiation and survival (Bodine *et al.*, 2007). On the contrary, as summarized throughout this chapter, PTH exerts both pro-differentiating and anti-apoptotic effects in murine osteoblast or their osteoprogenitors and exerts mainly an anti-proliferative effect in the same cells. Thus, if Wnt signaling were involved as a mediator of the anabolic effects of PTH, it would be the osteoblastogenic or prosurvival actions of the hormone that it would mediate. In support of this contention, the anabolic effects of intermittent PTH were blunted by deletion of SFRP1 in mice (Poole *et al.*, 2005). Thus, overlapping mechanisms of action may mediate the increased bone mass resulting from administration of the hormone or the activation of endogenous Wnt signaling that follows deletion of the Wnt antagonist SFRP1.

The influence of, specifically, continuous PTH on the Wnt signaling pathway appears to be related to its actions to inhibit the expression of the osteocyte product and Wnt antagonist, sclerostin (Sost). SOST is strongly expressed in osteocytes within bone (Winkler *et al.*, 2003; Van Bezooijen *et al.*, 2004; Wergedal *et al.*, 2003; Brunkow *et al.*, 2001). SOST loss-of-function mutations in humans are the cause of the autosomal recessive bone dysplasias sclerosteosis and van Buchem disease, which are characterized by massive bone overgrowth throughout life and increased bone strength and serum markers of bone formation (Bellido *et al.*, 2005; Keller and Kneissel, 2005). PTH has been shown to suppress Sost expression in osteocytes by a mechanism that is mainly mediated via the cAMP/PKA pathway (Loots *et al.*, 2005). Sost inhibition by PTH was not blocked by the protein synthesis inhibitor cycloheximide, indicating direct regulation, and PTH did not influence Sost mRNA degradation, implying transcriptional regulation. A distant enhancer within the 52 kb Van Buchem disease deletion downstream of the Sost gene was found to be essential for its expression in adult bone (Leupin *et al.*, 2007). Myocyte enhancer factor 2 (MEF2) transcription factors control this enhancer and mediate inhibition of sclerostin expression by PTH (Schmid *et al.*, 1994). Thus, continuous administration of PTH may also have a beneficiary effect on the induction of osteoblast lineage commitment and differentiation by a sustained decrease in the expression of Sost. In support of this contention, overexpression of a constitutive active PTHR1 specifically in osteocytes, suppressed Sost expression and increased bone mass in transgenic mice (O'Brien *et al.*, 2006).

Growth Hormone and IGF-1

Growth hormone (GH) has been evaluated either as a direct regulator of bone cell biology or as a stimulator of IGF-1, which was known to stimulate osteoblast proliferation and differentiation *in vitro* (Canalis, Hock, and Raisz, 1994; Hock and Fonseca, 1990). A study of young male hypophysectomized rats showed that GH was required for PTH to increase bone mass equivalent to the increment seen in treated, intact controls (Schmidt, Dobnig, and Turner, 1995). As the effects of GH on bone can be mediated by IGF-1, it was inferred that IGF-1 might be required for the anabolic effect of PTH, at least during skeletal development when growth plates are still active. The addition of PTH to older, mature hypophysectomized female rats resulted in increased osteoblast number and trabecular bone volume, even in the absence of GH (Gunness and Orwoll, 1995; Mosekilde *et al.*, 2000). Other studies of PTH in combination with either GH alone or combined with IGF-1 in intact old female rats showed a substantial additional effect of either GH or IGF-1 (Mosekilde *et al.*, 2000). It has been suggested that GH in older animals may enhance the effect of PTH by stimulating periosteal formation to change the geometry of the bones, making them more resistant to fracture in biomechanical tests (Canalis *et al.*, 1989): data to prove this additive effect of GH are limited. Collectively, these data suggest that GH is required for the anabolic effect of PTH during the "adolescent" phase of skeletal growth in rats, but is not necessary after skeletal maturation in rats.

IGF-1 acts as both a pro-differentiating and a pro-survival factor in osteoblasts (Conover, 2000). One elegant study showed that a neutralizing IGF-1 antibody blocked the effects of PTH on collagen synthesis in cultured fetal rat calvaria and implicated IGF-1 as a key mediator of PTH effects on osteoblasts (McCauley *et al.*, 1997). In calvarial bone cells isolated from two-day rats and treated with hPTH(1–34) for six hours, osteoblast commitment to differentiation was blocked by the IGF-1 antibody (Grey *et al.*, 2003). These data suggested that IGF-1 may be one of the mediators of PTH effects on skeletal growth and maturation. Indeed, long-term exposure to PTH stimulated IGF-1 synthesis in a cAMP-dependent mechanism in osteoblasts in culture (Locklin *et al.*, 2003). Short-term treatment with the hormone had no effect on osteoblastic cells (Watson *et al.*, 1995). However, intermittent PTH administration to ovariectomized rats for four weeks increased IGF-1 transcripts as determined by *in situ* hybridization (Bikle *et al.*, 2002). The increase in IGF-1 levels in this study could be because of accumulative effects of repeated exposure to PTH. Nevertheless, the role of IGF-1 as a mediator of the anabolic actions of PTH has been established in studies in animal models. Daily PTH administration to IGF-1-deficient mice for up to 14 days failed to stimulated periosteal bone formation and cortical thickness (Miyakoshi *et al.*, 2001) or to increase bone mass in the distal femoral metaphysis. Anabolic actions of PTH at all three sites were observed in wild-type animals (Yamaguchi *et al.*, 2005). Deletion of the insulin receptor substrate adapting molecule-1, which transmits IGF-I receptor signaling, in mice blunted the anabolic response to four weeks of intermittent PTH administration (Yakar *et al.*, 2006). Finally, inactivation of IGF-1 in the liver had no effect on the anabolic actions of PTH in cancellous bone, but attenuated its effects on cortical bone (Wang *et al.*, 2007). These studies suggest that local IGF-1 production mediate PTH-induced anabolism in the cancellous site but liver-derived IGF-1 is involved in the PTH-induced increase in cortical thickness.

Consistent with the involvement of IGF-1 in the anabolic effects of PTH, osteoblast-specific deletion of IGF-1 receptor in mice attenuated PTH-stimulated endosteal bone formation but had no effect on the increase in cortical thickness in the proximal tibia (Schmid *et al.*, 1989; LaTour *et al.*, 1990; McCarthy *et al.*, 1994; Moriwake *et al.*, 1992). IGF-1 receptor inactivation affected the ability of PTH to stimulate osteoprogenitor cell proliferation and differentiation as administration of PTH increased the number of alkaline phosphatase expressing colonies and mineralized nodules in bone marrow-derived stromal cells from control mice, but not from IGF-1 receptor-deficient mice.

As skeletal cells secrete the six known IGF-binding proteins (IGFBPs) and two of the four known IGFBP-related proteins (IGFBP-rP): there may be several levels of regulation if IGF-1 mediates the action of PTH *in vivo* (Pereira and Canalis, 1999; Qin *et al.*, 1998). IGFBPs may prolong the half-life of IGF-1 and compete with its receptors for binding. IGF-BP4 may inhibit osteoblast function *in vitro*, whereas IGF-BP5 enhances the stimulatory effects of IGF-1 (Pereira and Canalis, 1999; Pereira, Durant, and Canalis, 2000). PTH induces IGFBP-rP-1 and IGFBP-rP-2 in osteoblasts by transcriptional control (Hurley *et al.*, 1999). A potential role of IGF-1-binding proteins to the actions of PTH has not yet been elucidated.

FGF-2

FGF-2 exerts both proliferative and anti-apoptotic effects in osteoblasts. In cultured mouse cells and bone organ culture, hPTH-(1–34) increased expression of FGF-2 and FGF receptors 1 and 2 within 30 minutes (Hurley *et al.*, 2006). FGF-2 may contribute to the anabolic effect of intermittent PTH as evidenced by the failure of the hormone to increase cancellous bone volume in growing or adult FGF-2 deficient mice (Okada *et al.*, 2003). PTH-stimulated osteoclast formation in bone marrow cultures or cocultures of osteoblast-spleen cells from FGF-2 deficient mice was also significantly impaired (Lane *et al.*, 2003). Daily injections of FGF-2 and PTH both increased trabecular bone mass in osteopenic rats but had different effects on trabecular bone architecture. Whereas intermittent PTH increased trabecular thickness, FGF-2 increased trabecular number and connectivity. Because the effect of intermittent FGF-2 on trabecular architecture did not mimic the effect of intermittent PTH, either the increase in FGF-2 induced by intermittent PTH was not enough to increase trabecular number and connectivity, or the cellular response of osteoblast progenitors was modified by the anti-proliferative effect of PTH.

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Chapter 31

Actions of Parathyroid Hormone on the Vasculature and Cardiovascular System

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INTRODUCTION

Although not originally conceived as a vasoactive substance, it is now generally appreciated that parathyroid hormone has important effects on the regulation of vascular tone. Parathyroid hormone-induced relaxation of vascular smooth muscle (Mok *et al.,* 1989) is a mechanism for the classical hypotensive property that was first demonstrated by Collip and Clark (1925). In spite of this finding, parathyroid hormone (PTH) has been implicated in multiple aspects of coronary heart disease including hypertension.

Hypercalcemia has been associated with cardiovascular diseases including hypertension, arrhythmias, left ventricular hypertrophy, and vascular and valvular calcification. The effect of PTH on the cardiovascular system has also been explored, because PTH has known positive chronotropic and inotropic effects on the heart. This chapter will review possible mechanisms by which PTH may influence the cardiovascular system including affects of blood pressure and left ventricular function, and examine the studies that address the question of cardiovascular involvement in primary hyperparathyroidism (PHPT).

PRIMARY HYPERPARATHYROIDISM AND THE CARDIOVASCULAR SYSTEM

Any review of this literature must be mindful of the evolution in the clinical profile and severity of PHPT over the past 40 years. In the past, PHPT was characterized by marked hypercalcemia and very elevated levels of PTH. This profile remains typical in some areas of the world. However, in the United States and other developed countries today, PHPT is more commonly characterized by very mild hypercalcemia and PTH levels that are often only minimally elevated. If the cardiovascular manifestations of PHPT are dependent upon the extent to which these biochemical hallmarks are abnormal, one might anticipate a spectrum rather than a single profile of involvement.

Cardiovascular mortality has been assessed in several studies in patients with PHPT (Lunghall *et al.,* 1991; Hedback and *et al.*, 1991; Palmer *et al.,* 1987). In some studies, the increase in the cardiovascular death rates persisted even after curative surgery of the PHPT, although other studies suggest that the risk of cardiovascular death returns to normal after parathyroidectomy. One study demonstrated a decrease in cancer and coronary heart disease (CHD)-related deaths in patients with PHPT. Wermers *et al.* reported mortality figures in patients with PHPT who were diagnosed between 1965 and 1992 (Wermers *et al.,* 1998). Despite the overall finding that overall survival was not adversely affected among patients with PHPT, higher maximal serum calcium levels were shown to be an independent predictor of mortality. This was particularly the case in subjects who were in the highest quartile of serum calcium levels (11.2 to 16.0 mg/dL). It should be noted that the mean serum calcium levels in the cohort of Wermers *et al.* were lower than in the studies that reported increased mortality.

After parathyroid surgery, data are available in the context of subjects whose PHPT was cured and those in whom elevated calcium and PTH levels persisted. In the latter setting, a retrospective review of medical records was performed in 23 of 124 patients who after parathyroid surgery had persistent elevations of serum calcium and PTH levels (Vestergaard and Kristensen, 2002). Follow-up information was available for comparison after 12 months in 14 of these 23 subjects with persistent PHPT and in 38 subjects whose PHPT was cured. In patients with persistently

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elevated levels of PTH, the frequency of ischemic heart disease and hypertension was higher as compared to those whose PHPT was cured. Another case-control study of Danish individuals gleaned from that country's national hospital registry from 1977–1993 found that the diagnosis of PHPT carried with it an increased risk of premature death (Ogard *et al.,* 2004). The overall cardiovascular mortality in the PHPT cohort was elevated compared to the background population. The standard mortality ratios due to ischemic heart disease for women and men with PHPT were 1.9 (95% CI, 1.4–2.4) and 1.5 (95% CI, 0.9–2.3), respectively. It is notable that this study did not address subjects with mild PHPT because it is unlikely that they would have been hospitalized and thus captured in the national registry.

Nilsson *et al.* (2004) suggested that PHPT due to multiglandular disease had an increased risk of death. The patients in this study were identified through a national registry of hospitalized patients in Sweden from 1964–1999, and appear to be restricted to subjects with multiglandular parathyroid disease. There was an increase in standard mortality ratio (1.44; 95% CI, 1.37–1.52) beyond the first postoperative year. Cardiovascular disorders were the predominate cause of death in both genders.

There are limited data on the effect of hyperparathyroidism and hypercalcemia, the hallmarks of PHPT, on cardiovascular disease. Serum PTH levels predicted the presence of coronary heart disease in a study of 3,570 subjects in Tromso (Kamycheva *et al.,* 2004). Linear regression was used to determine associations and a logistic regression model was utilized to find independent predictors of coronary heart disease. Data were obtained by measuring PTH levels and relating them to information obtained from a questionnaire about angina pectoris and myocardial infarction. When stratified for age, the rate of CHD was higher in subjects with elevated serum PTH than in those with normal or low serum PTH levels (relative risk 1.67; 95% CI, 1.26–2.23 in men and 1.78; 95% CI, 1.22–2.57 in women). A positive association was evident after adjustment for body mass index, systolic blood pressure, and total serum calcium and serum cholesterol levels. Other parameters of bone metabolism, such as 25-hydroxyvitamin D levels were not captured. These subjects had serum calcium levels within the reference range, implicating PTH as an associated factor for CHD. However, it is not clear whether the subjects with highest PTH values had primary or secondary hyperparathyroidism. Because the serum calcium levels were normal, it is likely that at least some of these patients had secondary hyperparathyroidism. In those individuals with renal impairment causing hyperparathyroidism there may well be alternative explanations for the increased finding of CHD.

There are also data supporting an association of hypercalcemia itself with cardiovascular disease. In a large prospective, epidemiologic study of Swedish men, hypercalcemia was found to be a risk factor for myocardial infarction (Lind *et al.,* 1997; Jorde *et al.,* 1999). Furthermore, higher serum calcium levels within the normal range are reported to be associated with increased cardiovascular mortality (Leifsson and Ahren, 1996). More recently, a study from Rubin *et al.* documented an association between serum calcium levels and carotid plaque, which is a strong predictor of clinical coronary and cerebrovascular events (Rubin *et al.,* 2007). In this study of 1,194 multi-ethnic subjects from New York City, the interaction of age and serum calcium was the most significant predictor of carotid plaque thickness when traditional cardiovascular risk factors were considered. Unfortunately, PTH levels were not available in this cohort, so it is impossible to determine the relative associations of this finding with calcium and calciotropic hormones.

The mechanism by which coronary heart disease might be associated with PHPT is also not well understood. Several studies have evaluated specific biomarkers of CHD, but a comprehensive assessment of CHD and these biomarkers have not been performed in the same cohort. Markers of inflammation and N-terminal pro-B-type natriuretic peptide that are associated with CHD were elevated in a study of hypertensive PHPT patients (Ogard *et al.,* 2005). In this study, 45 patients with PHPT and 40 matched controls were evaluated. Plasma N-terminal pro-B-type natriuretic peptide (NT-proBNP), high-sensitivity C-reactive protein, and tumor necrosis factor alpha, but not interleukin-6, were higher in patients with PHPT than in controls. NT-proBNP levels were correlated with systolic blood pressure, left-ventricular end-diastolic volume, and peak oxygen uptake (determined on a bicycle exercise test). No decrease in NT-proBNP, markers of inflammation or blood pressure was observed after parathyroidectomy with follow-up evaluations performed at 7 and 18 months. The investigators suggested that hypertension could explain the increased risk of inflammatory markers and NT-proBNP levels that could, in turn, increase cardiovascular mortality rates in this group of patients. Other studies have implicated increased levels of adipokines that are associated with the development of atherosclerosis and cardiovascular disease such as leptin in patients with PHPT and these levels correlated with body mass index (Delfini *et al.,* 2007). Although the connection between leptin and PHPT is not clear, leptin possess direct and indirect effects on bone cells, and the changes in bone remodeling that occur with PHPT may be responsible for the increased leptin levels.

One issue that is difficult to assess is whether patients with mild PHPT without hypertension would be at risk for cardiovascular disease. In a small study, selected patients with PHPT without any evidence of renal or cardiovascular complications were evaluated for heart disease (Barletta *et al.,* 2000). In these subjects, echocardiography of the heart revealed no structural abnormalities. In comparison to normal subjects, the left ventricular isovolumetric relaxation time was normal, but significantly shorter than for control subjects suggesting an increased sympathetic stimulation. The authors suggested that in mild PHPT, cardiovascular function is essentially normal in those patients who are normotensive. These data have not been confirmed in a larger cohort of patients with PHPT.

There is a paucity of data regarding mortality of subjects with normocalcemic PHPT, but one study evaluated lipid levels in a small number of subjects with this disorder (Hagstrom *et al.*, 2006). In this study, individuals with normal serum calcium and an inappropriately high serum PTH were followed over a five-year period. At study entry, these subjects had higher serum levels of calcium, PTH, glucose, low-density lipoprotein/high density lipoprotein-cholesterol ratio (due to a lower HDL-cholesterol level), very low-density lipoprotein-cholesterol, and total triglycerides and a higher body mass index compared to controls. Subjects who underwent parathyroidectomy had a return of their serum calcium and proatherosclerotic lipoprotein levels to more favorable concentrations that were similar to levels of control subjects.

Genetic causes for an increase in cardiovascular risk have been explored for a specific polymorphism of the calcium-sensing receptor. The calcium-sensing receptor is a G-protein-coupled receptor that senses extracellular calcium levels and alters PTH release from the parathyroid gland. One of the more common polymorphisms in the calcium-sensing receptor is an alanine to serine change of amino acid 986. In healthy women, carriers of the S-allele results in asymptomatic, mild increases in calcium concentrations in (Cole *et al.,* 2001). Marz and colleagues evaluated subjects with this polymorphism for coronary heart disease, myocardial infarction, and cardiovascular mortality (Marz, 2007). Compared to AA homozygotes, the prevalence of CHD (odds ratio 1.25; 95% CI, 1.02–1.54) and myocardial infarction (odds ratio 1.33; 95% CI, 1.06–1.68) was increased in carriers of at least one S-allele. The prevalence of CHD and myocardial infarction increased with each S-allele. These associations were independent of cardiovascular risk factors, and calcium and phosphate levels. The S-allele impairs the sensitivity of the renal tubule to ambient calcium, reduces renal calcium elimination, and enhances the excretion of phosphate. The S-allele is likely to increase the set point of the parathyroid gland for calcium ions and increase PTH concentrations. These data indicate that a common genetic polymorphism of the calcium-sensing receptor was associated with angiographic CHD, previous myocardial infarction, and cardiovascular mortality, and that these associations were independent of well-established cardiovascular risk factors.

EVIDENCE FOR HYPERTENSION IN PATIENTS WITH HYPERPARATHYROIDISM

Several studies have addressed the clinical relevance of the association between parathyroid hormone levels and blood pressure measurements, despite a higher prevalence of hypertension in patients with PHPT than in the general population. In a population health survey, serum PTH levels were measured in a large number of subjects ages 39 to 79. In a follow-up study in 1998, 72 subjects had elevated levels of PTH and 100 subjects had normal serum PTH levels. After excluding patients with PHPT, measurements of serum calcium, serum vitamin D, bone mineral density, and systolic and diastolic blood pressure were obtained. Subjects with elevated PTH levels had significantly lower serum calcium levels and intakes of dietary calcium than those with normal PTH levels. Differences in vitamin D intake or serum levels did not differ between the two groups. Subjects with elevated levels of parathyroid hormone had significantly lower bone mineral density in the lumbar spine than those with normal PTH levels. Females with elevated serum PTH levels had significantly higher systolic and diastolic blood pressures, but this was not true for male subjects (Jorde *et al.,* 2000). A second study looked at the association between serum parathyroid hormone levels in normotensive elderly subjects undergoing 24-hour ambulatory blood pressure monitoring. In this group of 123 subjects ages 63 to 88, serum PTH levels correlated to nocturnal systolic blood pressure, nocturnal diastolic blood pressure, daytime systolic blood pressure, and mean 24-hour systolic blood pressure on univariate and multivariate analyses. Nocturnal, daytime, and mean 24-hour blood pressures were not correlated to serum calcium levels, 25(OH) concentrations, age, body mass index, or alcohol consumption. In this study, gender differences were also assessed and men were found to have higher diastolic blood pressure than women. The authors concluded that serum PTH levels were strongly related to blood pressure, particularly nocturnal blood pressure in elderly subjects (Morfis *et al.,* 1997).

To further explore the relationship between blood pressure and levels of PTH, interventional studies have been performed in human subjects. The acute administration of parathyroid hormone was utilized to mimic the role of secondary hyperparathyroidism in the pathogenesis of hypertension in patients with renal failure. Because uremia is characterized by insulin resistance and hyperinsulinemia, administration of physiologic doses of hPTH (1–34) was performed under conditions of a euglycemic clamp technique in 10 healthy male subjects. The study was a double-blind, crossover design using a sham infusion or 200 units of hPTH (1–34). The infusion of hPTH (1–34) resulted in a significant increase in mean arterial pressure compared to sham infusions. Changes in intracellular calcium concentration determined in platelets were significantly correlated with changes in mean arterial pressure, but insulin sensitivity was not affected by PTH infusion. Thus, subacute administration of physiologic doses of parathyroid hormone under hyperinsulinemic conditions alters intracellular calcium and blood pressure in healthy subjects (Fliser *et al.,* 1997).

Several hypotheses have been proposed for the mechanism of hypertension in patients with PHPT and include increased serum calcium levels, increased PTH levels (with attendant changes in cytokines and hormones), raised renin activity and hypomagnesemia. Hypercalcemia created by infusion of calcium to normal volunteers resulted in an increase in systolic and diastolic blood pressure. This may be mediated by a dose-related impairment of endothelial vasodilation (Nilsson, *et al.,* 2001). PTH infusion results in hypercalcemia and hypertension in normal subjects (Hulter *et al.,* 1986). Additional studies have suggested that PTH causes an increase in renin levels (Gennari *et al.,* 1995). In a cohort of subjects with PHPT, there were no differences in calcium or PTH levels between those with hypertension and normotensive individuals. However, plasma renin activity and plasma aldosterone levels were higher in the hypertensive patients. After surgery, blood pressure, renin and aldosterone levels became normal in 8 of the 10 hypertensive patients. Another study supports the hypothesis that hypomagnesemia and hypertension are related (Sangal *et al.,* 1989). In this retrospective analysis, magnesium levels were significantly lower in hypertensive PHPT patients compared to normotensive subjects. The mechanism by which magnesium may cause a change in blood pressure is thought to be due to the role of magnesium in altering vascular tone.

The effects of parathyroid hormone on the vasculature extends to potential actions on vascular reactivity as well as on the blood pressure per se. Nilsson *et al.* (1999) have shown that in primary hyperparathyroidism, there is an abnormal vasodilatory response to the local infusion of metacholine and nitroprusside. Parathyroid hormone modulates the secretion of endothelin-1, whereas endothelin-1, in turn, may influence parathyroid hormone secretion. Endothelin levels have been shown to be elevated in the plasma of patients with primary and secondary hyperparathyroidism (Lakatos *et al.,* 1996).

The most clinically relevant question is whether improvement of blood pressure will occur in patients following normalization of elevated levels of PTH. Several studies have evaluated blood pressure measurements before and after parathyroidectomy (Schleiffer, 1992). In most cases, hypertension persists after removal of the abnormal parathyroid gland(s) and restoration of PTH to normal levels (Sancho *et al.,* 1992). In one study of hypertensive patients on maintenance dialysis, 19 patients were evaluated one month before total parathyroidectomy, the first month after surgery, and also after 16 months. There was neither a clinically relevant nor a statistically significant change in either systolic or diastolic blood pressure over time (Ifudu *et al.,* 1998). Although most studies do not support this, there are limited data suggesting that hypertension may be reversible after surgery for PHPT (Nilsson *et al.,* 2005).

A recent study by Hedback and Oden compared the risk of death after surgery for PHPT in patients with and without hypertension. A series of 845 patients with PHPT was followed for a mean of 10.2 years after surgery. The risk of death was 50% higher in PHPT patients with hypertension than in those who were normotensive. However, the decline in risk of death following surgical cure was twice as great in those with hypertension as compared with those who were normotensive (Hedback and Oden, 2002).

OTHER CARDIOVASCULAR PROPERTIES IN PATIENTS WITH HYPERPARATHYROIDISM

In addition to the effects on blood pressure and cardiovascular mortality, parathyroid hormone has major effects in influencing cardiac function (Dipette *et al.,* 1992; Schluter and Piper, 1998). These actions include increases in heart rate, coronary blood flow, and contractility. PTH is active in the absence of changes in autonomic reflexes (Bogin *et al.,* 1981; Dipette *et al.,* 1992; Roca-Cusachs *et al.,* 1991; Tenner *et al.,* 1983). In isolated perfused hearts, parathyroid hormone is a positive inotropic agent (Nickols *et al.,* 1989).

To determine whether parathyroid hormone and parathyroid hormone related peptide (PTHrP) can directly stimulate cardiac contractility, the isolated, perfused rat heart was studied under conditions where the individual contributions of heart rate, coronary blood flow, and contractility could be assessed independently (Ogino *et al.,* 1995). In this model, both parathyroid hormone and PTHrP stimulated heart rate, coronary blood flow, and contractility in a dose-dependent manner. When heart rate was fixed by pacing, the effect on coronary blood flow and contractility was still appreciated. However, when heart rate and coronary blood flow were rendered constant pacing and by maximal dilatation maintained by treatment with nitroprusside, neither parathyroid hormone nor PTHrP could directly increase inotropy. These studies provide evidence that the cardiac actions of parathyroid hormone and PTHrP are mediated by effects on heart rate and coronary blood flow as compared with direct actions on contractility per se. More recent studies have suggested that the actions of parathyroid hormone and PTHrP on heart rate may be due to increases in the pacemaker current, I_f, of the sinoatrial node (Hara *et al.,* 1995; Hara *et al.,* 1997). These observations provide an electrophysiological basis (Shimoyama *et al.,* 1998a) and, more recently, a biochemical basis (Shimoyama *et al.,* 1998b) for the actions of parathyroid hormone and PTHrP to directly alter automaticity of the heart.

On the clinical side, Smith *et al.* (2000) have shown that in mild PHPT, certain indices of vascular stiffness were higher than in control subjects. The augmentation index is a measure of arterial stiffness that is a strong independent marker for premature coronary artery disease. Rubin *et al.* (2005) evaluated this index in mild PHPT and adjusted for confounding factors such as age, gender, heart rate, height, blood pressure, diabetes mellitus, smoking, and hyperlipidemia. Thirty-nine patients with mild PHPT were compared to 134 healthy controls. The patients with mild HPT had an increased augmentation index, an early measure of arterial stiffness. Another measure of cardiovascular function, flow-mediated dilation, is an early marker of atherosclerotic degeneration and correlates with coronary endothelial dysfunction. In a prospective study, endothelium-dependent, flow-mediated dilation was impaired in patients with PTH compared to controls (Baykan *et al.,* 2007). Other authors have found that successful parathyroidectomy will improve this parameter of endothelial dysfunction (Kosch *et al.,* 2000).

Other parameters of cardiovascular function that have been evaluated in PHPT include measures of the autonomic nervous system. In PHPT, the diurnal variation in parathyroid hormone secretion is attenuated, which might result in an increased risk for CHD. Nilsson *et al.* (2003) evaluated heart rate variability and circadian rhythm in patients with PHPT compared to control subjects. PHPT patients had lower maximal heart rates than controls, but the finding was not statistically significant. Other findings were consistent with an imbalance in cardiac autonomic regulation in hyperparathyroidism with a diminished responsiveness to autonomic input. Another study found no differences in blood pressure profiles, heart rate, or left ventricular mass in a small number of patients and controls, but noted a statistically significant greater increase in carotid intimal-medial thickness, with a trend to more frequent atherosclerotic plaques (Nuzzo *et al.,* 2002). Because this study was limited to patients with marked hypercalcemia, one cannot know what the results would have been if those with mild PHPT had also been studied.

Other markers of endothelium dysfunction have been explored in a small study by Fallo and colleagues (Fallo *et al.,* 2006). Twenty patients with PHPT had measurements of endothelial activation: plasma thrombomodulin, soluble E-selectin, and von Willebrand factor, before and one year after parathyroidectomy. The patients were divided into two groups depending on the absence ($n = 8$) or presence ($n = 12$) of one or more cardiovascular risk factors. Baseline thrombomodulin levels were similar in the two groups and similar to a group of control, normocalcemic subjects. Soluble E-selectin and von Willebrand factors were higher in patients with PHPT with risk factors compared to PHPT patients without cardiovascular risk factors and controls. After surgery, neither thrombomodulin nor soluble E-selectin changed, but plasma von Willebrand factor decreased in patients without risk factors and persisted at elevated levels in those patients with cardiovascular risk factors.

Left Ventricular Dysfunction

Some studies have implicated that patients with a history of PHPT have an increase in congestive heart failure. The reasons for this effect on the cardiovascular system are likely to be multifactorial. Evaluation of left ventricular mass index in patients with PHPT was performed in a group of patients that underwent surgery and compared to a second group that was observed for one year. Left ventricular mass index was larger in the group of patients with PHPT that were observed for one year and the measure correlated with serum concentrations of PTH, but not serum calcium levels (Almqvist *et al.,* 2002). Subclinical changes included a reduction in left ventricular ejection fraction. Several studies have suggested that intervention can reverse these findings, although in some studies, reversible changes were documented only in hypertensive patients (Stefenelli *et al.,* 1997; Piovesan *et al.,* 1999; Nilsson *et al.,* 2000, Nilsson *et al.,* 2005). Nilsson and colleagues, reporting on a cohort with more severe hypercalcemia than is common in the United States, suggested that these findings were coupled with evidence for higher systolic blood pressure, an increase in the number of extrasystolic beats and an increase in ST-segment depression during exercise, all of which were reversible by parathyroidectomy (Nilsson *et al.,* 2000). In another study, myocardial perfusion defects and left ventricular ejection fracture was evaluated in a small sample of patients with PHPT (Ogard *et al.,* 2005). Only patients without a history of myocardial infarction, angina pectoris, or diabetes participated in the study. Using ^{99m}Tc-sestamibi scanning, 5 of 22 subjects had myocardial perfusion defects above 15% at rest. The patients with the perfusion defects had significantly higher levels of PTH than those without a perfusion defect. There were no differences in plasma ionized calcium levels. Patients with the perfusion defects also had a decreased left ventricular ejection fraction. This was a small, uncontrolled study, which suggests that larger evaluation of these findings is warranted. The reversibility of left ventricular dysfunction was assessed by Piovesan *et al.* (1999). Evaluation of left ventricular hypertrophy in patients with PHPT who underwent surgery indicated that the abnormal findings on echocardiography were reversible six months after surgery.

Reversibility of cardiovascular dysfunction after parathyroidectomy was evaluated in 30 consecutive patients with PHPT (mean baseline serum calcium 11.8 mg/dl) who were followed for one and five years postsurgery. Thirty matched controls and the PHPT patients underwent echocardiography and a bicycle exercise test (Nilsson *et al.,* 2005). Serum calcium levels were normal in all patients and normal subjects at five years. Although the maximal blood pressure during exercise was higher before parathyroidectomy, it was not different five years after surgery. ST-segment depression diminished after surgery and the number of ventricular extrasystolic beats at exercise testing before parathyroidectomy was higher in the patient group than in the control group. The authors concluded that successful surgery for PHPT can induce improvements in the regulation of blood pressure, left ventricular diastolic function, and cardiac irritability, which may have implications for cardiac mortality for patients with PHPT.

SUMMARY

Cardiovascular disease and mortality have been associated with PHPT. Various mechanisms have been implicated such as endothelial dysfunction, decreased vascular compliance, autonomic dysfunction, left ventricular hypertrophy, and hypertension. Most studies have had a relatively small number of subjects, have been retrospective in design, and have measured a limited number of variables. It is possible that the seemingly contradictory results of some studies on the cardiovascular manifestations of PHPT may be explained by the evolution of the clinical picture of PHPT. What was once a disease characterized by marked hypercalcemia, and PTH levels that were also very elevated, is now, more commonly a disorder in which hypercalcemia is not extreme (indeed, serum calcium levels are often within 1 mg/dl of the upper end of the normal range) and PTH elevations are similarly moderate. Thus the cardiovascular manifestations of PHPT may well present along a continuum, related to the extent of elevation in PTH, calcium, or both. Longer and larger prospective studies of well-defined populations are needed to understand these relationships more completely.

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Chapter 32

PTH and PTHrP Actions on Kidney and Bone

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INTRODUCTION

This chapter reviews accepted concepts and considers controversial or unsettled elements of parathyroid hormone (PTH) and PTH-related protein (PTHrP) actions. Recent advances in our understanding of the mechanisms and regulatory actions of PTH and PTHrP on kidney and bone are introduced. Mineral ion homeostasis is regulated primarily by the kidneys, largely through the actions of PTH. PTHrP exerts actions on the kidney that are associated with controlling renal vascular tone, though its physiological role is unsettled (Mundy and Edwards, 2008). In bone, PTHrP is a major hormonal regulator of bone formation, whereas PTH contributes to postnatal skeletal integrity. Bone and kidney form a storage and regulatory axis that ensures normal growth, development, and multiple tissue and cellular functions that intimately depend on stringent control of intra- and extracellular mineral ion concentrations. This regulation is accomplished principally in the kidneys. Therefore, the renal effects of PTH and PTHrP action will be discussed first to provide a framework for understanding their actions on bone.

PTH ACTIONS ON KIDNEY

By regulating renal tubular absorption of calcium and phosphate and the synthesis of 1,25$(OH)_2D_3$, PTH controls urinary excretion and intestinal absorption of these mineral ions. Renal tubular responses to PTH deficiency, PTH or PTHrP excess, or defects in function of the type 1 PTH/PTHrP receptor (PTH1R) lead to alterations in blood calcium, phosphate, or dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] that are the hallmarks of numerous clinical disorders, described later in this volume. This chapter reviews the mechanisms of PTH and PTHrP control of renal tubular calcium and phosphate absorption. The discussion focuses principally on PTH because distinct or unique PTHrP actions on tubular ion transport are not well delineated. Because the amino termini of both ligands are similarly recognized by the PTH1R, it is likely that PTHrP shares the effects of PTH. Expression and specific renal actions of PTHrP are associated with regulation of renovascular hemodynamics and may have important effects on hypertension (Massfelder *et al.*, 1998; Massfelder and Helwig, 1999).

Although magnesium is an important mineral ion, its serum levels do not appear to be significantly regulated by PTH and, therefore, will not be discussed further here. Reviews of renal magnesium transport can be found elsewhere (Konrad *et al.*, 2004) and in previous editions of this chapter (Bringhurst and Strewler, 2002).

CALCIUM AND PHOSPHATE HOMEOSTASIS

Serum Calcium

Plasma calcium concentrations in adults average 2.5 mmol/L (or, 5mEq/L, 10 mg/dL[1]). Calcium in plasma is present to varying extents in protein-bound, complexed, and ionized forms. Approximately 45% of the serum calcium is bound to plasma proteins, mostly to albumin. Smaller amounts are bound to globulins. Only a negligible amount of calcium is bound to fibrin. Therefore, serum and plasma calcium concentrations are generally indistinguishable (Miles *et al.*, 2004). Another 45% of serum calcium is ionized (or "free"). The remaining 10% of serum calcium is associated with small polyvalent anions such as bicarbonate, phosphate, and sulfate. Such ion pairs, e.g., calcium bicarbonate, which form by electrostatic forces, are called to as "calcium complexes." Together, ionized and complexed calcium are referred to as "diffusible" because only

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[1] Concentrations of calcium and phosphate are expressed here in SI units (mmol/L). Although more traditional units remain in use, they are confusing for multivalent ions and, in the case of P_i, are ambiguous because the concentration of the different forms is dependent upon pH.

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diffusible calcium is filtered at the glomerulus and is able to cross cell membranes.

Ultrafilterable and ionized fractions of calcium are affected by changes in the total serum calcium concentration, blood pH, plasma protein concentration, and the concentration of complexing anions. Increases in the total serum calcium concentrations are usually accompanied by concomitant elevations of ultrafilterable calcium to a total concentration of about 4 mM (Edwards *et al.*, 1974; Le Grimellec *et al.*, 1974). This upper limit in calcium ultrafiltration with hypercalcemia has been postulated to result from the formation of insoluble $Ca_3(PO_4)$–protein complexes. This idea is supported by the finding that the ultrafilterable phosphate concentration also declines (Cuche *et al.*, 1976). Conversely, hypocalcemia is generally associated with a decline of calcium ultrafiltration (Terepka *et al.*, 1957).

Changes in the concentration of serum proteins are usually accompanied by parallel alterations of total serum calcium concentration such that the ultrafilterable fraction remains constant (Loeb, 1926; Marshall, 1976; Peterson *et al.*, 1961). In severe hypoproteinemia, however, the ultrafilterable fraction increases (Hopkins *et al.*, 1952; Terepka *et al.*, 1957).

Ionized Ca^{2+} varies inversely with blood pH. Acidemia increases ionized Ca^{2+}, whereas alkalosis decreases Ca^{2+} (Hopkins *et al.*, 1952; Loeb, 1926; Peterson *et al.*, 1961). Ionized Ca^{2+} concentrations also change inversely with variations of serum anions. For instance, elevation of phosphate, citrate, sulfate, or bicarbonate increases the serum-free Ca^{2+} secondary to augmented formation of calcium complexes (Walser, 1973).

Symptoms of hypocalcemia vary in relation to the ionized serum calcium concentration. Mild reductions of plasma calcium are associated with paresthesia and muscle cramps; more severe decreases of calcium may induce seizures. Hypercalcemia, on the other hand, has been implicated in the attenuation of the renal effects of PTH, the antidiuretic action of vasopressin, and reduced renal concentrating capacity (Gill and Bartter, 1961; Takaichi and Kurokawa, 1986). The calcium concentration in the extracellular fluid represents a dynamic balance between intestinal absorption, renal reabsorption, and osseous resorption. The parameters affecting calcium balance for an adult are schematically shown in Figure 1. Bidirectional calcium movement from intestine, kidney, and bone are shown and representative magnitudes are provided. Assuming a daily dietary calcium intake of 1000 mg, net intestinal absorption amounts to about 200 mg, with the remaining 800 mg excreted in the feces. When in balance, net intestinal absorption is matched by urinary excretion, whereas calcium accretion and loss from bone are equal. Thus, approximately 200 mg of calcium are excreted daily. In adults, net calcium balance is effectively zero, suggesting that, in the absence of a calcium challenge such as lactation, the kidneys represent the dominant regulatory site of calcium metabolism (Peacock *et al.*, 1969).

Phosphate Chemistry

Terrestrial mammals are characterized by their avidity for calcium and equally keen mechanisms to eliminate phosphorous. Elemental phosphorous (P) exists in both organic and inorganic (P_i) forms. Organic forms include phospholipids and various organic esters. The bulk of phosphate in extracellular fluid exists as the inorganic forms, NaH_2PO_4 and Na_2HPO_4. The Henderson relation determines the ratio of the two:

The dissociation constant, pKa, for phosphate is 6.8. Thus, at pH 7.4, the ratio is essentially 4:1 and the plasma phosphate has an intermediate valence of 1.8.[2]

Serum Phosphate

As in the case of calcium, not all serum P_i is ultrafilterable (see later). Small amounts are bound to plasma proteins. However, some 90%, whether ionized or complexed with Ca, Mg, or Na, is filtered at the glomerulus. Ultrafilterable P_i decreases with elevation of Ca or P_i, presumably because of formation of high-molecular-weight protein complexes.

Although it is commonly stated that P_i is freely and completely filtered at the glomerulus, this is incorrect. Ten percent of the plasma P_i is bound to protein and is not filtered. However, the reduction in ultrafilterable P_i is counterbalanced by an opposite Gibbs–Donnan effect that raises

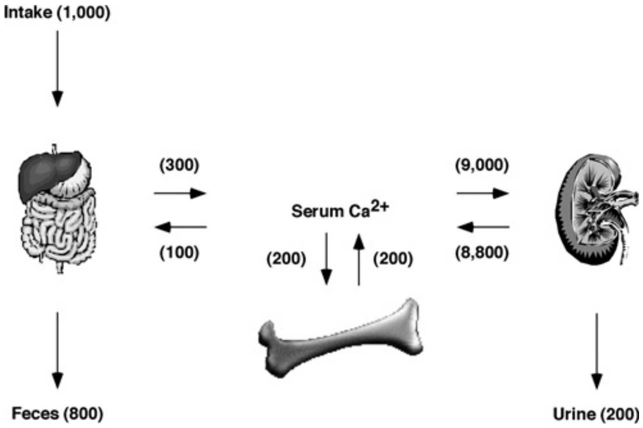

FIGURE 1 Extracellular calcium balance in the adult human. Numeric values for calcium intake, excretion, and fluxes are in mg/day for a 70-kg individual. Original data are from Wilkinson (1976). The recommended adult American daily diet includes 1000 mg of calcium though actual diets generally contain less elemental calcium (Heaney *et al.*, 1975; Mundy, 1990). Using an assumed intake of 1000 mg, 800 mg are excreted in feces and 200 mg in urine.

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[2] The net valence is calculated from the total number of negative charges, 2 × 4 + 1, divided by the number of molecules, 4 + 1, as determined by the Henderson relation, viz., 9/5 = 1.8. Thus, at pH 7.4, 1 mmol of phosphorous = 1.8 mEq.

the P_i concentration in the ultrafiltrate. Correcting the volume occupied by plasma proteins (7%) raises the ultrafiltrate P_i concentration by an additional 7.5% mitigating the reduction of protein-bound P_i. Indeed, both *in vivo* and *in vitro* measurements show that the ratio of ultrafilterable P_i to total plasma P_i is close to unity (Harris *et al.*, 1974).

PTH Actions on Mineral Ion Homeostasis

By regulating renal tubular absorption of calcium and phosphate and the synthesis of $1{,}25(OH)_2D_3$, PTH controls urinary excretion and intestinal absorption and of these mineral ions. Renal tubular responses to PTH deficiency, PTH or PTHrP excess, or defects in function of the PTH1R, lead to alterations in blood calcium, phosphate, or $1{,}25(OH)_2D_3$ that are the hallmarks of numerous clinical disorders, described later in this volume. This chapter reviews current understanding of the mechanisms whereby PTH (and PTHrP) controls renal tubular epithelial function. Because PTH and PTHrP actions in kidney and bone are mediated by the PTH1R, its expression, signaling, and trafficking are presented first as a foundation for understanding hormone actions. Although PTH or PTHrP receptors distinct from the PTH1R have been described (see Chapter 24), their role, if any, in normal renal physiology is unknown. Although not unequivocally proven in each case, it is likely that the effects of PTH and PTHrP described here are mediated by the PTH1R.

PTH1R EXPRESSION, SIGNALING, AND REGULATION IN THE KIDNEY

The PTH1R is expressed both on renal tubular epithelial cells and vascular endothelial cells. The response to PTH1R activation observed in individual renal cells depends on the number and location of PTH1Rs; the expression of cell-specific adapter proteins that modify PTH1R signaling; the array of PTH1R-inducible genes; effector proteins including enzymes, ion channels, and transporters; the local concentration of PTH or PTHrP; exposure to other agents that heterologously regulate PTH1R function; and the history of recent exposure to PTH or PTHrP, which affect the state of receptor desensitization.

PTH1R Expression within the Kidney

Renal localization of the PTH1R was deduced initially from the physiological effects of infused PTH extracts on phosphate and calcium absorption (Lambert *et al.*, 1964; Widrow and Levinsky, 1962). These and similar studies pointed to prominent actions on proximal and distal tubules, respectively. Subsequent investigation amply confirmed the actions of PTH to block P_i absorption by proximal tubules (Agus *et al.*, 1971) and to stimulate calcium absorption by distal nephron segments (Sutton and Dirks, 1975). Later examination of the nephron sites at which PTH promoted cAMP formation (Table I) was generally

TABLE I Stimulation of Adenylyl Cyclase Activity by PTH in Nephron Segments

Nephron Segment	Mouse	Rabbit	Rat	Human
PCT	++++	+++	++	++
PST	+++	+++	+	++
MAL	0	0	0	++
CAL	++++	++	++++	++
DCT	++++	0	++++	++++
CNT	0	+++	++	+
CCT	0	0	0	0
MCT	0	0	0	ND
	Brunette *et al.* Chabardes *et al.*	Chabardès *et al.* Chabardès *et al.* Morel *et al.*	Morel *et al.* Morel and Doucet	Chabardès *et al.*

Results are compiled from references as indicated. Adenylyl cyclase activity is scaled as follows: 0, no effect; +, <100; ++, 100–300; +++, 300-500; ++++, >500 *fmol* mm^{-1} 30 *min*$^{-1}$. (+) *denotes a statistically significant, but minimal effect.* ND, *not determined;* MAL, *medullary thick ascending limb of Henle's loop;* CAL, *cortical thick ascending limb of Henle's loop;* DCT, *distal convoluted tubule;* CNT, *connecting tubule;* CCT, *cortical collecting tubule;* MCT, *medullary collecting tubule. In the case of* DCT *and* CNT, *the structures involved are designated according to current morphological definitions* (*Kriz and Bankir*). *The anatomical origin of the actual structures as they were microdissected is described in the original articles.*

consistent with the pattern of PTH effects. Some distinct species differences also emerge from this analysis. After the PTH1R was cloned, RT-PCR and *in situ* hybridization techniques were used to define the nephron sites of mRNA expression (Lee *et al.*, 1996; Riccardi *et al.*, 1996; Yang *et al.*, 1997). General consensus pointed to conspicuous PTH1R expression in the glomerulus, proximal convoluted and proximal straight tubules, cortical ascending limbs and distal convoluted tubules. Some differences may also be noteworthy. Riccardi and Hebert additionally reported PTH1R expression in rat cortical collecting ducts (Riccardi *et al.*, 1996), a finding not confirmed by Yang *et al.* (1997). Functional examination of PTH actions on Ca absorption by rabbit cortical collecting duct failed to disclose detectable effects (Shareghi and Stoner, 1978; Shimizu *et al.*, 1990b), a nephron site where evidence for cellular calcium transport could not be found (Bourdeau and Hellstrom-Stein, 1982). Of course, PTH1Rs in cortical collecting duct could mediate other regulatory events such as modulating proton secretion and acid–base balance.

More precise PTH1R localization was undertaken by immunoelectron microscopy and immunofluorescence localization. As expected, PTH1R protein was detected on basolateral membranes of proximal convoluted tubule cells (Amizuka *et al.*, 1997). Unexpectedly, and, to a lesser extent, immunoreactivity was found also on the luminal surface. Endothelial cells of the cortical capillaries expressed at high levels of PTH1R. Vascular staining was detected on the surface of glomerular podocytes but not in glomerular vascular endothelial cells. Moderate immunoreactivity was detected in medullary collecting ducts. Other studies employing immunofluorescence detection likewise found both luminal and basolateral PTH1R expression in proximal tubules (Ba *et al.*, 2003). Considerably stronger staining was present in thick ascending limbs of Henle's loop, where both apical and basolateral receptors were detected. By contrast, PTH1R expression in distal convoluted tubules is distributed only on basolateral surfaces. Possible roles of apical membrane PTH1Rs are discussed later.

Considerable evidence points to the bilateral presence of PTH1Rs on apical and basolateral surfaces of proximal tubules. However, PTH actions are asymmetrical (Quamme *et al.*, 1989; Reshkin *et al.*, 1990, 1991). This suggests differential PTH1R coupling to G proteins on apical and basolateral membranes, the presence of adapter proteins such as NHERF1 or AKAPs that modify or specify second messenger signaling, or that the receptor has different functions when present on apical cell membranes. The former possibility seems unlikely because G_s is amply expressed in brush-border membrane vesicles (Brunskill *et al.*, 1991; Stow *et al.*, 1991; Zhou *et al.*, 1990). Interestingly, inhibitory G_i isoforms are found only on apical and not basolateral proximal tubule cells (Stow *et al.*, 1991).

Radioligand-binding studies indicate that PTH1Rs are expressed on glomeruli, apical and basolateral membranes of proximal tubules, cortical thick ascending limbs of Henle's loop, and distal convoluted tubules (Kaufmann *et al.*, 1994; Rouleau *et al.*, 1986). Although the apical expression of PTH1Rs was unexpected, it can be reconciled with the fact that circulating PTH peptides are presumably filtered at the glomerulus and appear in the urine (Bethune and Turpin, 1968). However, apical membrane receptors appear not to be coupled tightly, if at all, to adenylyl cyclase (Kaufmann *et al.*, 1994; Shlatz *et al.*, 1975). Moreover, a high-capacity apical peptide-uptake mechanism, mediated by the multifunctional endocytic clearance receptor megalin (Hilpert *et al.*, 1999) limits the access of filtered bioactive PTH peptides to these receptors. Downstream cortical ascending limbs and distal convoluted tubule sites would be unlikely to encounter biologically meaningful amounts of PTH.

An entirely different function for apical PTH receptors can be envisioned, whereby they serve as clearance receptors. Such a view is supported by studies that examined the disposition of radioiodinated PTH by isolated perfused kidneys. In these studies, the accumulation, extraction, and catabolism of PTH was primarily accomplished from the luminal surface (Kau and Maack, 1977). Such a mechanism, especially when complemented by megalin, would explain the virtual absence of PTH from urine (Hilpert *et al.*, 1999).

As described in more detail in Chapter 24, the PTH1R gene incorporates multiple promoters and 5′-untranslated exons and therefore can generate various transcripts by alternative promoter usage and different patterns of RNA splicing (Amizuka *et al.*, 1997; Bettoun *et al.*, 1998; Jobert *et al.*, 1996; Joun *et al.*, 1997; McCuaig *et al.*, 1995). P1 and P3 promoters in mouse and human, respectively, seem to be used exclusively in kidney cells, whereas the P2 promoter is employed to generate PTH1R mRNAs that are widely expressed in extrarenal tissues and organs (Amizuka *et al.*, 1997; Bettoun *et al.*, 1998; Joun *et al.*, 1997). It is presently unknown whether these differences simply reflect opportunities for tissue-specific gene regulation or lead to expression of structurally different forms of the PTH1R (Jobert *et al.*, 1996; Joun *et al.*, 1997).

PTH1R Signal Transduction in Renal Cells

The PTH1R exerts its biological actions by activating various signaling pathways. Activation is mediated by G_s, $G_{q/11}$, G_i, and G_{12}/G_{13} heterotrimeric G proteins (Abou-Samra *et al.*, 1992; Offermanns *et al.*, 1996; Schwindinger *et al.*, 1998; Singh *et al.*, 2005) (see also Chapters 27 and 67). In most cells and tissues, the PTH1R signals by activating G_s or $G_{q/11}$ (Abou-Samra *et al.*, 1992; Bringhurst *et al.*, 1993). In some instances the PTH1R couples with both G_s and $G_{q/11}$ resulting in activation of adenylyl cyclase and phospholipase C (PLC). In certain instances, however, the PTH1R couples only to a single pathway and does so in a cell-specific manner. For example, in vascular smooth muscle cells, PTH stimulates adenylyl cyclase but not PLC (Maeda *et al.*, 1996; Wu *et al.*, 1993), whereas in keratinocytes (Orloff *et al.*, 1992, 1995; Whitfield *et al.*, 1992), cardiac myocytes (Rampe *et al.*, 1991; Schlüter *et al.*, 1995), and lymphocytes (Atkinson *et al.*, 1987; Klinger *et al.*, 1990; Whitfield *et al.*, 1971), PLC but not adenylyl cyclase is activated. The particular coupling mechanism depends on the cell type in a manner that remains incompletely understood.

PTH administration leads to the rapid excretion of cAMP in the urine, which is referred to as nephrogenous cAMP (Chase and Aurbach, 1967). This effect has been employed as a clinical test to distinguish between primary and secondary hyperparathyroidism (Broadus, 1979). Practically speaking, nephrogenous cAMP is formed exclusively in proximal tubules because of the relative mass of proximal tubules compared with other nephron segments that express PTH1Rs. Further, although PTH-induced nephrogenous cAMP is a robust index of the phosphaturic action of PTH, it fails to disclose the effects of PTH on calcium absorption by distal tubules. Several reasons may account for this including the profusion of proximal tubules compared with distal tubules, receptor abundance on the two cell types, the presence of modifying proteins that alter the coupling of the PTH1R to G proteins, and the signaling array employed.

Considerable evidence indicates that in kidney epithelial or vascular cells PTH can stimulate adenylyl cyclase with attendant formation of cAMP and activation of protein kinase A (PKA); PLC with consequent generation of inositol phosphates + diacylglycerol, release of intracellular calcium, and activation of PKCs, as well as phospholipase A_2 (PLA_2) (Derrickson and Mandel, 1997; Ribeiro *et al.*, 1994; Ribeiro and Mandel, 1992) and phospholipase D (PLD) (Friedman *et al.*, 1996, 1999). Other signaling mechanisms may participate in mediating PTH1R actions in renal cells. For example, PTH-induced activation of mitogen-activated protein kinases in renal epithelial cells may proceed by activation of nonreceptor tyrosine kinases, transactivation of EGF receptors, with subsequent assembly of active Ras/Raf-1/MEK complexes (Cole, 1999; Lederer *et al.*, 2000; Sneddon *et al.*, 2000, 2007).

PTH1R signaling appears to differ depending on the nephron segment where it is expressed. For example, PTH provokes rapid and transient elevations ("spikes") of Ca_i^{2+} in proximal tubules and cells derived from them (Filburn and Harrison, 1990; Friedman *et al.*, 1999; Hruska *et al.*, 1986, 1987; Tanaka *et al.*, 1995). This response is characteristic of PLC activation, as opposed to calcium influx, and results from the formation of inositol trisphosphate and its action to provoke calcium release from endoplasmic reticulum. Distal tubule cells, in contrast, exhibit a delayed and sustained Ca_i^{2+} response to PTH. This effect is because of apical membrane Ca^{2+} entry (Bacskai and Friedman, 1990) and is inhibitable by dihydropyridine-type calcium channel blockers (Gesek and Friedman, 1992b). Furthermore, although PTH-stimulated elevation of Ca_i^{2+} in distal tubule cells involves PLC-independent protein kinase C (PKC) activation (Friedman *et al.*, 1996), PTH-induced PKC activation in distal tubule cells is mediated by PLD (Friedman *et al.*, 1999).

PTHrP, though not as exhaustively characterized, exerts effects on Ca_i^{2+} that are similar to those seen with PTH (Abou-Samra *et al.*, 1992; Bringhurst *et al.*, 1993; Yamamoto *et al.*, 1989). Recent clinical studies revealing a larger calcemic action of PTH compared with PTHrP owing to greater renal $1,25(OH)_2$ vitamin D production (Horwitz *et al.*, 2005) will surely provoke further examination of the mechanistic details by which PTH and PTHrP bind and activate the PTH1R (Dean *et al.*, 2008).

Regulation of PTH1R Signaling in Renal Cells

The magnitude of physiological responses mediated by the PTH1R is tightly linked to the balance between signal generation and signal termination. Rapid termination of PTH1R signaling is mediated by receptor desensitization and internalization, whereas prolonged reduction in responsiveness is caused by downregulation and diminished receptor biosynthesis. Desensitization and internalization of the PTH1R, as with other G protein-coupled receptors is generally thought to be regulated by intracellular proteins, termed arrestins, and by G protein-coupled receptor kinases (GRKs). Hormone-induced receptor phosphorylation facilitates binding between the receptor and arrestin. Phosphorylation may be mediated by both second messenger-dependent protein kinases, e.g., PKA and PKC, and by GRKs. Just as receptor desensitization provides a mechanism to protect cells against excessive stimulation, resensitization guards cells against prolonged inactivity and hormone resistance. As in other PTH/PTHrP target cells, the responsiveness of renal epithelial cells to PTH or PTHrP may be regulated both by previous or chronic exposure to the homologous ligand and by other agonists that do not interact directly with the PTH1R. Desensitization of renal cellular responsiveness during continuous exposure to high concentrations of PTH or PTHrP has been well documented and studied extensively. Chronic hyperparathyroidism, either primary or secondary to calcium or vitamin D deficiency, as well as acute infusion of PTH, leads to PTH resistance in humans or animals, which is manifested by impaired cAMP and phosphaturic responses (Bellorin-Font *et al.*, 1995; Carnes *et al.*, 1980; Forte *et al.*, 1976; Tomlinson *et al.*, 1976; Tucci *et al.*, 1979). The nephrogenous cAMP reaction to PTH in humans desensitizes more readily than the phosphaturic response at low doses of hormone (Law and Heath, 1983).

Similar desensitization is observed in cultured renal epithelial cells (Fujimori *et al.*, 1993; Guo *et al.*, 1997; Henry *et al.*, 1983; Pernalete *et al.*, 1990; Ureña *et al.*, 1994).

GPCR activation is generally followed by a loss of responsiveness, termed desensitization. Receptor desensitization protects cells against excessive stimulation and may be rapid or develop only slowly. Although receptor desensitization is caused by phosphorylation of the PTH1R by GRKs and arrestin-mediating uncoupling with the cognate G proteins that mediate effector activation, hormone resistance may also arise by receptor downregulation, i.e., reduced expression of cell surface PTH1Rs. Receptor downregulation can arise from diminished receptor synthesis, increased receptor degradation, or a combination of the two. The relative roles of receptor desensitization or downregulation in causing PTH1R refractoriness appear to vary with the specific situation and experimental system (Carnes *et al.*, 1978; Forte *et al.*, 1976; Mahoney and Nissenson, 1983; Mitchell *et al.*, 1988; Tamayo *et al.*, 1982; Turner *et al.*, 1995).

GPCR desensitization occurs as a consequence of uncoupling the receptor from its cognate G protein in response to phosphorylation of Ser or Thr residues within the intracellular tail. Upon ligand binding to the PTH1R, the receptor is phosphorylated both by G protein receptor kinases (GRKs) and by second messenger-dependent protein kinases, PKA and PKC (Blind *et al.*, 1996; Dicker *et al.*, 1999; Flannery and Spurney, 2001). Phosphorylation of Ser residues in the PTH1R is mediated by G protein-coupled receptor kinase-2 (GRK2) (Dicker *et al.*, 1999; Malecz *et al.*, 1998), and to a lesser extent by PKC (Castro *et al.*, 2002). GRK2 preferentially phosphorylates the distal sites of the intracellular PTH1R tail, whereas PKC phosphorylates more upstream residues (Blind *et al.*, 1996). Nonetheless, PTH1R phosphorylation is not required for the interaction of the PTH1R with β-arrestin and receptor internalization (Dicker *et al.*, 1999; Ferrari *et al.*, 1999; Malecz *et al.*, 1998; Sneddon *et al.*, 2002).

The particular signals mediating PTH1R desensitization in renal epithelial cells is complex and may be cell-type specific. It was suggested that homologous desensitization of the PTH1R in opossum kidney (OK) proximal tubule-like cells is mediated by PKC (Pernalete *et al.*, 1990). However, further and more detailed analysis revealed that neither PKC nor PKA mediated PTH-induced PTH1R desensitization because blockade of PKA with H-89 or of PKC with bisindolylmaleimide-1 did not inhibit phosphorylation (Blind *et al.*, 1995). These results suggest that agonist-stimulated PTH1R phosphorylation and desensitization involves GRKs. Indeed, GRK2 was shown specifically to mediate PTH(1–34)-induced receptor phosphorylation (Malecz *et al.*, 1998). Mutagenesis of serine residues 483, 485, 486, 489, 495, and 498 within the carboxy-terminal tail of the PTH1R abolished GRK2-mediated phosphorylation. However, the rate and extent of PTH-stimulated internalization of the phospho-deficient PTH1R was indistinguishable from that of the wild-type PTH receptor. Nonetheless, inhibition of PTH1R desensitization by GRKs was seen in the absence of receptor phosphorylation, suggesting that inhibition is effected prior to phosphorylation and that GRK binding to the PTH1R may by itself impair receptor signaling (Dicker *et al.*, 1999). Subsequent β-arrestin binding may stabilize this inhibition and initiate receptor internalization.

As noted earlier, the PTH1R signals facultatively through adenylyl cyclase and PLC. In LLC-PK$_1$ kidney cells stably overexpressing the PTH1R, PTH increases cAMP and inositol phosphate accumulation (Guo *et al.*, 1995). Continuous exposure to PTH causes desensitization of both adenylyl cyclase and PLC (Guo *et al.*, 1997). Desensitization may be pathway specific. Treatment with forskolin reduced subsequent cAMP formation but not inositol phosphate formation in response to a challenge with PTH. Conversely, exogenous PLC activation with a phorbol ester produced desensitization, albeit modest, of inositol phosphate formation but not of cAMP accumulation (Guo *et al.*, 1997). Remarkably, receptor desensitization proceeded without concomitant downregulation of cell surface receptor expression, an exception to the normal pattern of coupled desensitization and PTH1R downregulation (Mitchell and Goltzman, 1990; Teitelbaum *et al.*, 1986).

PTH1R abundance may modulate the signaling pathway employed, as well as the relative and absolute intensities of signaling. Guo and Bringhurst generated LLC-PK$_1$ cells stably expressing PTH1R at densities of 20,000 to 400,000 receptors per cell (Guo *et al.*, 1995). Receptor density had a negligible effect on PTH-stimulated adenylyl cyclase insofar as cAMP accumulation was essentially maximal even at the lowest levels of receptor number. Half-maximal stimulation occurred at a PTH(1–34) concentration of 2 n*M*. In contrast, activation of PLC by PTH(1–34) was strongly dependent on PTH1R density, increasing exponentially as a function of receptor copy. Maximal stimulation was achieved only at receptor numbers of 400,000 per cell. The EC$_{50}$ for stimulation was 25 n*M*. These findings are consistent with the view that the PTH1R couples more efficiently with G$_s$ than with G$_{q/11}$. If these observations can be extended to other cells and to intact tissue, they suggest that receptor density has little effect on the potency of adenylyl cyclase stimulation. The results also provide a mechanism for the differential modulation of PTH1R signaling responses in a given renal cell.

Rapid internalization of PTH1Rs following ligand occupancy also contributes to abrogating further activation and signaling by reducing cell surface receptor expression (Bisello *et al.*, 2002; Malecz *et al.*, 1998; Sneddon *et al.*, 2003; Wang *et al.*, 2007). Recent work disclosed that PTH1R internalization, like activation, occurs in a ligand- and renal cell-specific manner. It was well established that full PTH1R agonists such as PTH(1–34) or PTHrP elicited prompt receptor internalization (Bounoutas *et al.*, 2006; Huang *et al.*, 1995, 1999; Sneddon and Friedman, 2007; Sneddon *et al.*, 2003; Tawfeek *et al.*, 2001; Teitelbaum *et al.*, 1986; Wheeler and Sneddon, 2006). Although the PTH1R lacks a canonical NPXXY internalization motif, other endocytotic signals have been identified within the carboxy terminus. Detailed analysis of the intracellular tail of the PTH1R revealed bipartite sequences that negatively or positively regulate receptor endocytosis (Huang *et al.*, 1995). An endocytic signal was detected within residues 475 to 494 of the opossum kidney PTH1R (corresponding to D482 to S501 of the human PTH1R). Mutations or deletions within this region result in diminished PTH-induced receptor endocytosis (Huang *et al.*, 1995; Sugai *et al.*, 2003). A smaller sequence (EVQ), which is present in other Family B receptors, is a negative regulator of endocytosis and, when removed, results in enhanced receptor internalization (Huang *et al.*, 1995; Vazquez *et al.*, 2005).

Expression of PTH1Rs on the surface of kidney cells is also controlled by the rate of PTH1R gene transcription, although current understanding of this process is incomplete. Hypoparathyroidism, induced by either parathyroidectomy or dietary phosphate depletion, upregulates PTH1R mRNA levels in rat renal cortex (Kilav *et al.*, 1995). However, high concentrations of PTH have no detectable effect on PTH1R mRNA (Kilav *et al.*, 1995; Ureña *et al.*, 1994). Renal PTH1R mRNA expression is reduced in rats with renal failure, but this apparently is caused by some aspect of uremia or renal disease (Disthabanchong *et al.*, 2004) other than secondary hyperparathyroidism *per se*, because it is not prevented by a parathyroidectomy (Largo *et al.*, 1999; Urena *et al.*, 1994, 1995). In rats with secondary hyperparathyroidism owing to vitamin D deficiency, renal cortical PTH1R mRNA levels were found to be twice as high as normal and could not be corrected by normalizing serum calcium (Turner *et al.*, 1995). These results suggest that vitamin D impairs PTH1R gene transcription in proximal tubules. However, in immortalized distal convoluted tubule cells PTH1R expression was upregulated severalfold by $1,25(OH)_2D_3$ (Sneddon *et al.*, 1998). In OK cells, TGFβ1 diminished PTH1R mRNA expression (Law *et al.*, 1994). The physiological significance of this effect has not been clarified. PTH1R mRNA expression was not affected by the mild secondary hypoparathyroidism induced by ovariectomy in rats nor by subsequent estrogen treatment (Cros *et al.*, 1998).

A critical insight to PTH1R regulation was achieved with the discovery that the receptor interacts with the cytoplasmic Na/H Exchanger Regulatory Factors NHERF1 and NHERF2 (NHERF1/2), which govern certain aspects of the signaling and trafficking (Mahon *et al.*, 2002). NHERF1/2 are cytoplasmic proteins consisting of two tandem PSD95/Discs Large/ZO-1 (PDZ) domains and a merlin/ezrin/radixin/moesin (MERM). The PTH1R binds to these domains through a recognition sequence at its carboxy terminus. This Glu-Thr-Val-Met (ETVM) sequence corresponds to a canonical PDZ domain of D/E-S/T-X-Φ, where X is any amino acid and Φ is a hydrophobic residue, generally L/I/V but it can also be M (Songyang *et al.*, 1997; Wang *et al.*, 1998). Mutation of any residue of the PDZ sequence of the PTH1R, other than the permissive position, abrogates interaction with NHERF1/2 (Mahon *et al.*, 2002; Sneddon *et al.*, 2003). In seminal work, Mahon and Segre found that NHERF1/2 switched PTH1R signaling from adenylyl cyclase to PLC. NHERF1 also regulates mitogen-activated Erk signaling (Wang *et al.*, 2008). NHERF1 is abundantly expressed on proximal tubule apical membranes (Wade *et al.*, 2001). Mouse proximal tubules express both NHERF1 and NHERF2 (Wade *et al.*, 2003). NHERF1 is strongly expressed in microvilli, whereas NHERF2 is detected only weakly in microvilli. However, it is expressed predominantly at the base of the microvilli in the vesicle-rich domain. Notably, neither NHERF1 nor NHERF2 is found is distal tubules. Human and mouse kidneys exhibit comparable NHERF1 localization (Shenolikar *et al.*, 2002; Wade *et al.*, 2003; Wade *et al.*, 2001; Weinman *et al.*, 2002). As will be discussed in greater detail later, NHERF1 is expressed in the proximal nephron, where it importantly regulates PTH-dependent phosphate absorption.

In addition to governing PTH1R signaling, NHERF1 also is significantly involved in determining receptor endocytosis. Disrupting the interaction of NHERF1 with the PTH1R by mutating the PDZ-binding domain, by mutating NHERF1, or by depolymerizing the actin cytoskeleton cause important alterations in PTH1R endocytosis. Notably, in the absence of NHERF1, both PTH(1–34) and PTH(7–34) (along with their corresponding full-length peptides) promoted efficient PTH1R sequestration. In the presence of NHERF1, PTH(1–34)-induced receptor internalization was unaffected, whereas PTH(7–34)-initiated endocytosis was largely inhibited (Sneddon *et al.*, 2003). PDZ core-binding domains and the NHERF1 MERM domain are required for inhibition of endocytosis (Wang *et al.*, 2007). NHERF1 does not alter PTH1R recycling and in this regard differs from β_2-adrenergic and κ-opioid receptors, where NHERF1 promotes rapid recycling to the plasma membrane (Cao *et al.*, 1999; Li *et al.*, 2002).

The PTH1R interacts with other adapter proteins including Tctex and 4.1G. Tctex1, which forms part of the dynein motor complex, interacts with the carboxy terminus of the receptor (Sugai *et al.*, 2003). Tctex1 binds to G protein-coupled receptors through an R/K-R/K-X-X-R/K motif (Mok *et al.*, 2001). A KRKAR sequence is found at positions Lys^{484}–Arg^{488} of the hPTH1R. This region was previously defined by Nissenson to contain a positive receptor endocytic signal (Huang *et al.*, 1995).

4.1G is a multifunctional cytoskeletal protein involved in cell morphology and membrane stability. It contains a 4.1/ezrin/radixin/moesin domain in common with NHERF1/2. 4.1G binds to the intracellular tail of the PTH1R within the Gln^{467}–Gly^{490} region. This portion of the PTH1R is involved in β-arrestin and AP-2 binding. When heterologously expressed in COS-7 cells, 4.1G increased membrane targeting of the PTH1R (Saito *et al.*, 2005). Although 4.1G is not expressed in kidney, 4.1R is expressed in thick ascending limbs; 4.1 N is found in thin limbs of Henle's loop, distal convoluted tubules, and all regions of the collecting ducts; and 4.1B is restricted to Bowman's capsule and proximal convoluted tubules (Ramez *et al.*, 2003). Homologous recombination of 4.1R resulted in mild hemolytic anemia but no conspicuous mineral ion phenotype (Shi *et al.*, 1999). The colocalization of certain 4.1 splice variants with the PTH1R opens the possibility for a role in governing receptor trafficking, signaling, and function.

CALCIUM ABSORPTION AND EXCRETION

The kidneys are responsible for controlling extracellular calcium balance. Renal calcium regulation occurs by a series of sequential events as the urine passes through the nephron. As described later in some detail, the bulk of the filtered calcium is absorbed by proximal tubules, with progressively smaller fractions recovered as the insipient urine passes through downstream tubule segments. The majority of the calcium absorption is not subject to hormone regulation. Hormonal, and pharmacological, regulation of calcium absorption is achieved by its action in modifying the fine control of calcium transport that occurs in distal segments.

Participation of PTH in the maintenance of the extracellular calcium concentration was described soon after the discovery of the parathyroid glands. Early observations in animals or in patients with hypo- or hyperparathyroidism clearly implicated renal calcium handling in PTH abnormalities (Carney, 1996; Gley, 1893; Hackett and Kauffman, 2004; Sandström, 1879–1880).

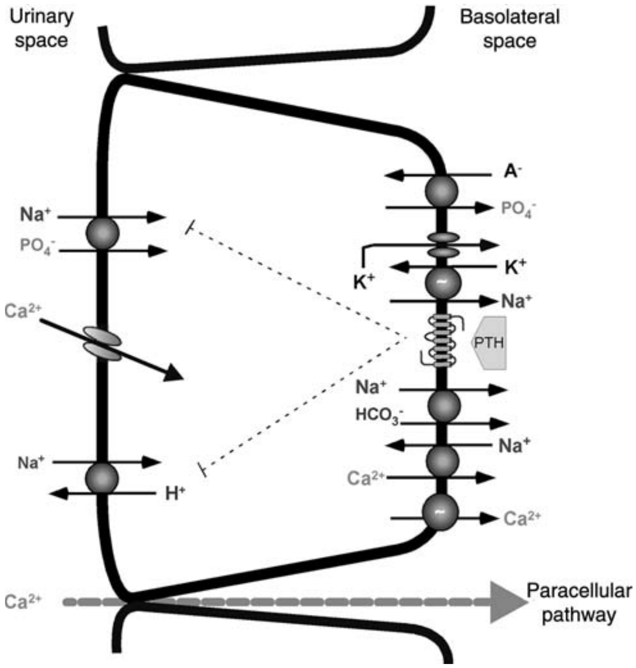

FIGURE 2 Ion transport in proximal tubules. Proximal tubule Na-P$_i$ cotransport is mediated principally by apical membrane Npt2a. Apical brush-border membranes also express the NHE3 Na^+/H^+ exchanger. Both transporters are inhibited by PTH as described in the accompanying text. The mechanism of basolateral PO_4 extrusion is uncertain and is illustrated here as proceeding by a nonspecified anion (A^-) exchange. Notably, calcium absorption by proximal tubules is largely, if not entirely, a passive process that proceeds through the paracellular pathway of the lateral intercellular spaces (dashed line). Evidence for apical membrane Ca^{2+} entry and basolateral efflux has been described, but whether these single processes contribute to transcellular calcium absorption is controversial. For simplicity, the PTH1R is shown only on basolateral cell membranes, though it is present also on luminal, brush-border membranes and signals differentially from basolateral PTH1R to inhibit Na-Pi cotransport.

Renal Calcium Absorption

PTH reduces renal calcium excretion. Although calcium is absorbed throughout the nephron, the calcium-sparing action of PTH occurs primarily in distal tubules (Friedman, 2007; Sutton and Dirks, 1975). The sites of renal calcium absorption and mechanism of PTH action are discussed in the following paragraphs.

Approximately 60% of filtered calcium is reabsorbed by the proximal tubules (Frick *et al.*, 1965; Lassiter *et al.*, 1963). Absorption is mostly passive, driven by the favorable electrochemical gradient generated by the progressive absorption of chloride and water (Berry and Rector, 1978; Bomsztyk *et al.*, 1984; Ng *et al.*, 1984), and proceeding through the lateral intercellular spaces that define the paracellular pathway (Fig. 2). A small fraction of active calcium absorption could be detected in euparathyroid rats under conditions where passive calcium movement was eliminated (Ullrich *et al.*, 1976). It should be borne in mind that, although small by comparison with paracellular calcium absorption, active cellular absorption by proximal tubules amounts to some 20 μmol/min (Ullrich *et al.*, 1976), which, in fact, is approximately twice that of the distal nephron, where calcium absorption is entirely cellular. Thus, even a small fraction of active calcium absorption would be substantial compared with that occurring in more distal nephron segments.

Conflicting evidence precludes a definitive statement regarding PTH effects on apical membrane calcium uptake by proximal tubules. Calcium entry has been reported to increase (Khalifa *et al.*, 1983), decrease (Agus *et al.*, 1973; Dolson *et al.*, 1985), or not to change (Frick *et al.*, 1965; Lajeunesse *et al.*, 1994) in response to PTH. Neither PTH, the calcium channel blockers nitrendipine or nisoldipine, nor the calcium channel agonist Bay K 8644 affect Ca^{2+} entry in apical membrane vesicles prepared from proximal tubules (Lajeunesse *et al.*, 1994) or ^{45}Ca efflux from perfused rat tubules (Kauker *et al.*, 1997). PTH has been reported to stimulate Na^+/Ca^{2+} exchange-mediated calcium efflux by basolateral membrane vesicles obtained from the cortex of rat and canine kidneys (Jayakumar *et al.*, 1984; Scoble *et al.*, 1985). It is not known whether this effect occurs directly or it involves an electrogenic action of PTH. PTH stimulates Ca^{2+}-ATPase activity in basolateral membranes from proximal tubules of the rat and the dog (Levy *et al.*, 1986; Tsukamoto *et al.*, 1992) but not in the rabbit (Bouhtiauy *et al.*, 1991). It should be borne in mind that because virtually all cells require calcium for various regulatory activities, the presence of calcium uptake and calcium efflux mechanisms, *per se*, even if regulated by PTH, should not be construed as evidence for the presence of transcellular or transepithelial calcium absorption. Indeed, PTH exerts an inhibitory effect on proximal tubule calcium absorption (Agus *et al.*, 1973, 1971; Amiel *et al.*, 1970). This modest inhibitory action of PTH is attributable to reduced Na$^+$ absorption secondary to inhibition by PTH of Na-P$_i$ cotransport, Na$^+$/H$^+$ exchange, and Na$^+$-K$^+$-ATPase activity. However, it should be underscored that the overall effect of PTH on the kidney is to increase calcium absorption. Indeed, because calcium absorption by distal nephron segments is load dependent (Costanzo and Windhager, 1978; Greger *et al.*, 1978), it is likely that calcium rejected by proximal tubules in response to PTH is recovered in a compensatory manner by distal nephron segments.

Thin descending and thin ascending limbs of Henle's loop exhibit low calcium permeability (Rocha *et al.*, 1977; Rouse *et al.*, 1980), consistent with limited calcium transport. Moreover, neither segment expresses the PTH1R and, thus, are not sites where PTH stimulates or regulates calcium absorption.

Thick ascending limbs of Henle's loop, in contrast, are sites of appreciable calcium absorption. Approximately 20% to 25% of the filtered calcium is absorbed by thick ascending limbs, which consist of medullary and cortical portions. Calcium is absorbed by both medullary and cortical portions of thick ascending limbs, though to differing degrees (Friedman, 2007). In contrast to proximal tubules, where calcium absorption is virtually all passive and proceeds through the paracellular pathway, calcium movement in thick ascending limbs is characterized by parallel active transcellular movement and passive calcium transport (Fig. 3). Passive calcium absorption is driven by the favorable lumen-positive transepithelial voltage, which provides the driving force for movement through the calcium-permeable tight junctions. In this setting, the rate and magnitude of calcium transport are parallel and proportional to those of sodium. Physiological responses or interventions that enhance sodium absorption increase the voltage and thereby augment calcium absorption. Conversely, maneuvers that decrease sodium absorption also reduce calcium absorption. Such maneuvers commonly involve the use of diuretics such as furosemide or bumetanide that inhibit sodium absorption by thick ascending limbs and increase calcium excretion. Less commonly, mutations of the Na-K-2Cl cotransporter (Slc12a1), or the ROMK apical K$^+$ channel (*KCNJ1*) or basolateral ClC-K2 basolateral Cl$^-$ channel (*CLCNKB*) associated with the different forms of Bartter's Syndrome, are accompanied by hypercalciuria, underscoring the parallel nature of sodium and calcium absorption by thick ascending limbs (Hebert, 2003).

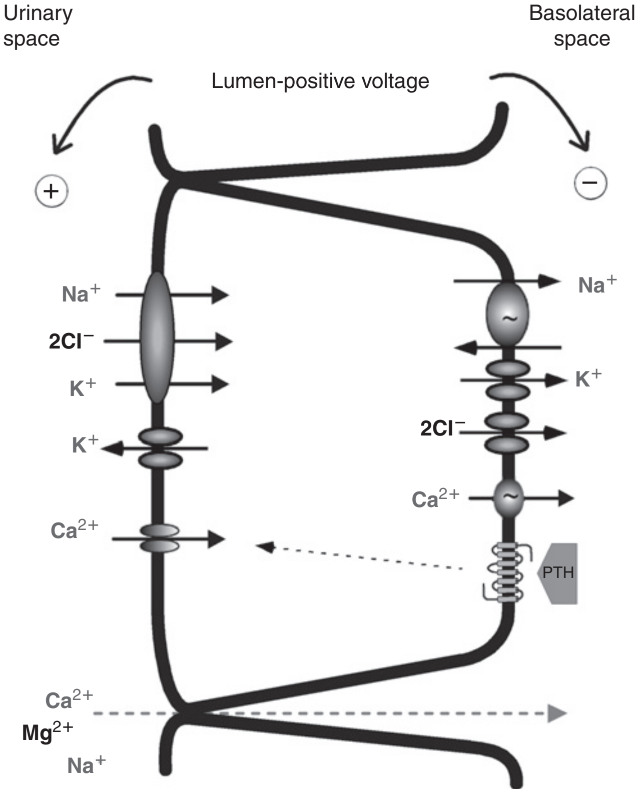

FIGURE 3 Parallel calcium transport pathways in thick ascending limbs. Calcium absorption in cortical and medullary thick ascending limbs of Henle's loop proceeds through an active, transcellular pathway and a parallel passive mechanism that traverses the paracellular pathway. Basal calcium absorption is passive and is driven by the ambient electrochemical gradient. PTH stimulates active, cellar calcium absorption by cortical thick ascending limbs as detailed in the accompanying text. Calcitonin exerts a similar effect on medullary thick limbs (Suki and Rouse, 1981) (data not shown). Other hormones, such as vasopressin, which stimulate sodium absorption by the Na-K-2Cl cotransporter, cause parallel elevations of calcium absorption by increasing the transepithelial voltage and the driving force for passive calcium absorption. Conversely, loop diuretics such as bumetanide and furosemide, by inhibiting Na-K-2Cl cotransport, decrease the transepithelial voltage and diminish passive calcium absorption with an attendant increase in calcium excretion.

Experimental evidence exists for active, transcellular calcium absorption by medullary and cortical thick ascending limbs (Rocha *et al.*, 1977; Suki *et al.*, 1980). Calcitonin stimulates active calcium absorption by medullary thick limbs, whereas PTH promotes active calcium absorption by cortical thick limbs (Suki and Rouse, 1981). Active calcium absorption is regulated by PTH as discussed later.

Initial reports failed to identify an effect of PTH on calcium transport by thick ascending limbs (Shareghi and Stoner, 1978). However, utilizing protocols to abolish passive calcium flux revealed that PTH stimulated transepithelial calcium transport (Bourdeau and Burg, 1980; Di Stefano *et al.*, 1990; Friedman, 1988; Suki and Rouse, 1981; Suki *et al.*, 1980). The stimulatory action of PTH on calcium absorption by thick ascending limbs is limited to the cortical segment; PTH does not affect calcium transport by medullary thick ascending limbs (Di Stefano *et al.*, 1990; Friedman, 1988; Suki and Rouse, 1981; Suki *et al.*, 1980). PTH augments net calcium absorption by cortical thick ascending limbs in the absence of changes of the transepithelial voltage (Bourdeau and Burg, 1980). Bourdeau and Burg interpreted these findings as being compatible with either active calcium absorption or with single file diffusion. When the effects of PTH were analyzed in mouse cortical thick ascending limbs in the absence of transepithelial electrochemical driving forces for calcium (Friedman, 1988), PTH clearly stimulated active, transcellular calcium absorption. These findings notwithstanding, the extent to which such hormone-dependent calcium transport contributes to overall renal calcium economy is uncertain. The reason for this is that substantial passive calcium movement is present under virtually all conditions and this fraction likely dominates net calcium absorption by thick ascending limbs.

The calcium-sensing receptor (CaSR) is expressed on basolateral membranes of thick ascending limbs of Henle's loop, especially of cortical thick ascending limbs (Riccardi *et al.*, 1998). CaSR activation selectively inhibits passive calcium but not sodium transport, and also inhibits PTH-dependent active calcium absorption (see later) (Motoyama and Friedman, 2002).

Distal convoluted tubules reabsorb 5% to 10% of the filtered calcium. Here, calcium absorption is entirely transcellular, proceeding against a steep adverse electrochemical gradient (Fig. 4) (Costanzo and Windhager, 1978; Lau and Bourdeau, 1995; Shareghi and Stoner, 1978). Transcellular calcium movement is a two-step process, wherein calcium enters the cell across apical membranes and, following diffusion across the cell, is extruded across basolateral membranes. Calcium entry is mediated by calcium ion channels (Lau *et al.*, 1991; Matsunaga *et al.*, 1994; Poncet *et al.*, 1992; Saunders and Isaacson, 1990) and extrusion is accomplished by the plasma membrane Ca^{2+}-ATPase and Na^{+}/Ca^{2+} exchange (Bacskai and Friedman, 1990; Matsunaga *et al.*, 1994; Poncet *et al.*, 1992).

Calcium entry is now thought to be mediated by TrpV5 (ECaC1, CAT2) (Hoenderop *et al.*, 1999, Vennekens *et al.*, 2000). TrpV5-null mice exhibit renal calcium wasting, thus establishing its participation in some aspects of calcium homeostasis in rodents (Hoenderop *et al.*, 2003). TrpV5 is a homotetramer that is constitutively active, which is difficult to reconcile with a regulated, negative feedback process (Nilius *et al.*, 2000). Indeed, TrpV5 is insensitive to PTH or to thiazide diuretics, two well characterized agents that stimulate calcium absorption by distal convolutedtubules. In contrast, an oligomeric calcium channel that is activated by PTH and by thiazide diuretics has been described (Bacskai and Friedman, 1990; Barry *et al.*, 1998; Matsunaga *et al.*, 1994), though its molecular identity is unknown.

Notably, whereas calcium and sodium transport proceed in parallel in proximal tubules and in thick ascending limbs, their movement is inversely related in distal convoluted tubules and this is a hallmark of calcium absorption by this nephron segment. Evidence for the inverse relationship comes from the pharmacological effects of thiazide diuretics, which increase sodium excretion but diminish that of calcium, as well as from patients with Gitelman's syndrome. This inherited disorder is caused by inactivating

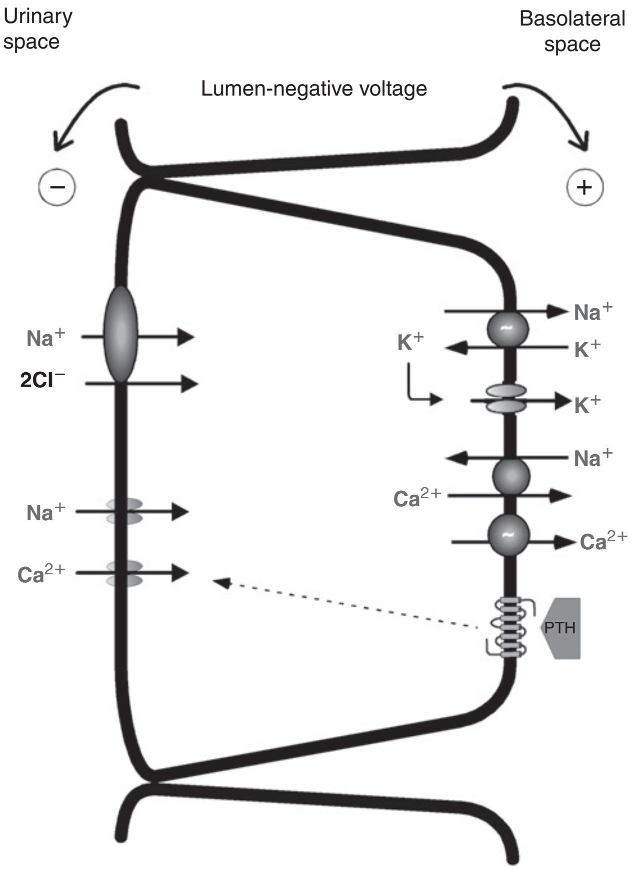

FIGURE 4 Calcium absorption by distal convoluted tubules. Two features distinguish distal tubule calcium absorption. First, calcium transport proceeds exclusively through a cellular pathway. Because of the substantial adverse electrochemical barrier, calcium movement is negligible in the absence of PTH (Costanzo and Windhager, 1980). Second, calcium and sodium movement in distal convoluted tubules are inversely related. Thus, thiazide diuretics, which block sodium transport, augment calcium absorption (Gesek and Friedman, 1992a). The cell model shows two apical membrane sodium entry mechanisms, the thiazide-diuretic-inhibitable Na-Cl cotransporter and the amiloride-blockable, epithelial Na channel, ENaC. Amiloride also stimulates calcium transport by distal tubule cells (Friedman and Gesek, 1995). Calcium enters the cell across apical membranes through dihydropyridine-sensitive calcium channels (Bacskai and Friedman, 1990). Basolateral calcium efflux is mediated by the plasma membrane Ca^{2+}–ATPase (PMCA) and the Na^{+}/Ca^{2+} exchanger as discussed in detail in the text and elsewhere (Friedman, 1998).

mutations of the Na-Cl cotransporter (NCC; *Slc12a3*) that mediates sodium absorption in distal convoluted tubules. Patients (Bettinelli *et al.*, 1992; Gitelman *et al.*, 1966) and experimental animals (Schultheis *et al.*, 1998) with Na-Cl cotransporter mutations exhibit characteristic hypocalciuria.

PTH stimulation of calcium absorption by distal tubules involves hyperpolarization of the membrane voltage (Gesek and Friedman, 1992b). Shimizu *et al.* (1990a) found that PTH induced biphasic changes of transepithelial voltage in single rabbit connecting tubules, the functional analogue of distal tubules in the rabbit. In other studies, however, alterations of voltage in response to PTH were not found (Imai, 1981; Suki *et al.*, 1980). Membrane hyperpolarization increases calcium entry in mouse distal convoluted tubule cells (Bacskai and Friedman, 1990; Matsunaga *et al.*, 1994) and in rabbit connecting tubules (Tan and Lau, 1993). This effect is caused by increases in the open probability of apical membrane Ca^{2+} channels and by an elevation of the driving force for calcium entry (Matsunaga *et al.*, 1994). PTH-induced hyperpolarization also enhances the driving force for basolateral calcium extrusion mediated by Na^+/Ca^{2+} exchange (Bouhtiauy *et al.*, 1991; Friedman, 1998; White *et al.*, 1996, 1998).

It has been proposed recently that α-klotho modulates renal calcium homeostasis by acting on distal tubules (Imura *et al.*, 2007). Homologous recombination of α-klotho results in a phenotype that includes osteoporosis and renal calcium wasting. (Kuro-o *et al.*, 1997; Tsuruoka *et al.*, 2006). The proposed mechanism for this effect involves enhanced Ca^{2+} entry secondary to augmentation of the Na^+ gradient following recruitment by α-klotho of the Na^+,K^+-ATPase to basolateral cell membranes. This action, in cooperation with TRPV5, calbindin$_{28k}$, and the NCX1 Na^+/Ca^{2+} calcium exchanger, it was suggested, enhances calcium absorption. Although there is little doubt that α-klotho participates in renal calcium economy, we believe it unlikely that the mechanism proposed by Imura accounts for this action because it would result in parallel increases of Ca^{2+} and Na^+ transport, whereas the signature of distal tubule calcium absorption is the well-established inverse relation between Ca^{2+} and Na^+ flux. Alternatively, the finding that α-klotho activates TRPV5 by hydrolyzing extracellular sugar residues provides a more compelling mechanism (Chang *et al.*, 2005). α-Klotho effects on PTH-dependent renal calcium absorption in intact animals may involve indirect actions on PTH secretion (Imura *et al.*, 2007) in combination with rather than direct effects on distal nephron calcium movement. A comprehensive picture of the mechanism of PTH action on renal calcium homeostasis requires further examination.

The contribution of cortical collecting tubules to renal calcium conservation is modest at best and uncertain. A small absorptive flux of calcium has been noted as has an equally small secretory transport (Bourdeau and Hellstrom-Stein, 1982; Imai, 1981; Shareghi and Stoner, 1978; Shimizu *et al.*, 1990b). Net calcium transport in isolated perfused rabbit cortical collecting ducts varied with the magnitude and direction of the transepithelial voltage (Bourdeau and Hellstrom-Stein, 1982). A small net secretory calcium flux was observed under ambient conditions. The Ca^{2+} permeability was very low compared with that of thick ascending limbs and proximal tubules. It is conceivable that secretory calcium transport could play a role in regulating calcium excretion when long-term changes of transepithelial voltage occur, for instance, as an adaptive response to changes in mineralocorticoid status (O'Neil, 1990). A model of calcium transport by collecting tubules is shown in Fig. 5. PTH exerts no detectable action on calcium movement by collecting ducts (Shimizu *et al.*, 1990b), and, as described earlier, the presence of the PTH1R at this nephron site is uncertain.

PHOSPHATE EXCRETION

Extracellular phosphate homeostasis is regulated primarily by controlling its transport in the kidneys, where it is

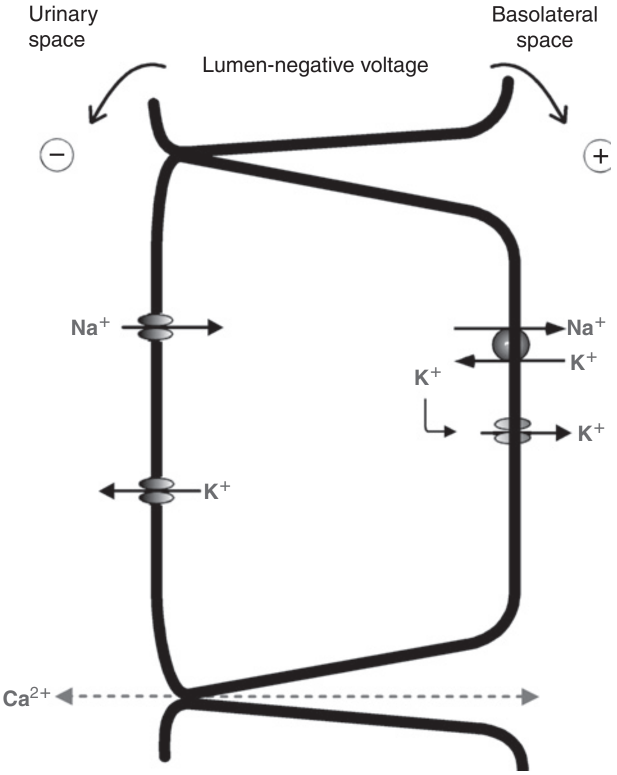

FIGURE 5 Calcium transport by collecting ducts. Collecting ducts are not a primary site of net calcium transport. Studies suggest that calcium movement is small in magnitude and thermodynamically passive (Bourdeau and Hellstrom-Stein, 1982). Transport may be absorptive or secretory, driven by the lumen-negative transepithelial voltage, and proceeds through the paracellular pathway (dashed line). PTH1Rs may or may not be expressed but PTH does not affect the rate of calcium absorption (Shareghi and Stoner, 1978).

absorbed mostly by proximal tubules. The prompt phosphaturia caused by PTH was one of the earliest recognized and best studied actions of PTH (Albright *et al.*, 1929; Greenwald and Gross, 1925). Early confusion about other purported actions can be attributed to the substantial contamination of the primitive extracts employed. With the availability of purified PTH, the direct phosphaturic action of PTH on the kidney was firmly established (Levinsky and Davidson, 1957; Pullman *et al.*, 1960).

Classical renal clearance studies established that more than 90% of the plasma phosphate is freely filtered at the glomerulus and 80% is actively reabsorbed. Ensuing investigations applying micropuncture and microperfusion techniques delineated the sites and cellular mechanisms of renal phosphate transport and its hormonal regulation. Based on those findings it is now well accepted that 60% to 70% of the filtered P_i is absorbed by late proximal convoluted tubules (Amiel *et al.*, 1970; Brunette *et al.*, 1973; Le Grimellec *et al.*, 1974; Staum *et al.*, 1972; Strickler *et al.*, 1964; Wen, 1974). P_i absorption was nearly three times greater in isolated perfused rabbit proximal convoluted tubules than in proximal straight tubules (Dennis *et al.*, 1976). The existence of distal P_i absorption is uncertain. Evidence from micropuncture experiments suggests that distal tubules absorb up to 10% of the filtered P_i (Pastoriza-Munoz *et al.*, 1978). Other studies failed to uncover evidence for distal P_i transport (Lang *et al.*, 1977). P_i transport by terminal nephron segments has also been suggested by studies showing a higher fraction of filtered P_i remaining in late distal tubules than that appearing in the final urine (de Rouffignac *et al.*, 1973; Lang *et al.*, 1977; Pastoriza-Munoz *et al.*, 1978). Collecting tubules may absorb 2% to 3% of the filtered P_i (Dennis *et al.*, 1977; Peraino and Suki, 1980). Phosphate transport by inner medullary collecting ducts was absent in control rats but was stimulated after acute thyroparathyroidectomy and was reversed by PTH administration (Bengele *et al.*, 1979). However, insofar as PTH receptors are not expressed in terminal nephron segments, the origin of this effect and its interpretation are uncertain. In summary, proximal convoluted and straight tubules are the major sites of P_i absorption. The extent of P_i absorption by distal and terminal nephrons is uncertain.

Addition of calcium to the lumen of perfused proximal tubules increased P_i absorption (Rouse and Suki, 1985). It was postulated that calcium modulates the anion affinity of the Na-P_i cotransporter. However, the identification of CaSRs at the luminal surface of proximal tubules (Riccardi *et al.*, 1998) suggests that the stimulatory effect of calcium might arise from a regulatory action exerted by the CaSR. The role of the CaSR in modulating basal and PTH-dependent proximal P_i transport was directly examined (Ba *et al.*, 2003). Activation of the CaSR by addition of luminal Ca^{2+} or Ba^{3+} specifically reduced PTH-inhibitable P_i transport without affecting basal P_i absorption.

Mechanisms of Proximal Tubular Phosphate Absorption

Clarification of the cellular mechanisms of proximal tubular phosphate absorption was largely derived from experiments utilizing renal membranes and membrane vesicles (Murer *et al.*, 2008, 2000). Apical P_i entry is rate-limiting for cellular P_i absorption and is the major site of its regulation. P_i enters the cell across apical brush-border membranes against a steep electrochemical gradient established primarily by the strongly negative intracellular voltage. This electrochemical barrier is overcome by Na^+/P_i cotransporters that couple P_i influx to the favorable dissipative entry of Na^+ (see Fig. 2).

Three classes (types I, II, and III) of Na-P_i ("NaP_i") cotransporters have been identified by molecular cloning techniques. The nomenclature, general features, and GenBank accession numbers for the human forms are summarized in Table II. The presence of three types of Na-P_i cotransporters underscores the complexity of the cell and molecular mechanisms and regulation of renal P_i absorption. Comprehensive reviews of the background, biochemistry, and expression patterns of NaP_i cotransporters can be found in previous editions of this chapter (Bringhurst and Strewler, 2002) and elsewhere (Murer *et al.*, 2001, 2003, 2008; Tenenhouse, 2007; Virkki *et al.*, 2007). Our attention here focuses on the present understanding of the mechanisms of renal P_i homeostasis and its regulation by PTH. Other hormonal or cytokine control of P_i handling by FGF23 and klotho has been reviewed recently (Kuro-o, 2006; Prie *et al.*, 2005; Stubbs *et al.*, 2007; Torres *et al.*, 2007).

Type I and type IIa NaP_i cotransporters are localized on apical brush-border membranes of proximal convoluted tubule cells. Type II cotransporters are structurally and genetically distinct from type I transporters, sharing only 20% identity. Type IIb transporters, an isoform of type IIa, are expressed exclusively in intestine and not in kidney (Hilfiker *et al.*, 1998). Type III NaP_i cotransporters, originally identified as cell surface virus receptors Glvr-1 and Ram-1 are widely expressed, both within and outside the kidney (Kavanaugh and Kabat, 1996). Type III cotransporters, which are expressed on basolateral cell membranes, are regulated by extracellular P_i deprivation and PTH (Ohkido *et al.*, 2003; Segawa *et al.*, 2007). Type II cotransporters are also expressed by distal convoluted tubule cells and may thus be involved in phosphate absorption by both proximal and distal nephrons (Collins *et al.*, 2004; Tenenhouse *et al.*, 1998). Targeted mutagenesis of Npt2 suggests that type IIa cotransporters account for 70% of renal P_i absorption (Beck *et al.*, 1998). Interestingly, despite an earlier account to the contrary (Hoag *et al.*, 1999), Npt2c protein abundance increased in Npt2a-null mutant mice suggesting that it may provide some compensatory response for Npt2a deficiency (Tenenhouse *et al.*,

TABLE II Sodium Phosphate Transporter Family

Type	Family Name	Common Name	Genbank	Function	References
NaPi-I	NaPi-1	NPT1	SLC17A1	Modulator of organic and inorganic anion transport	Busch *et al.* (1996)
NaPi-II	NaPi-2: rat NaPi-3: human NaPi-4: opossum NaPi-5: xenopus NaPi-6: rabbit NaPi-7: mouse	NPT2a NPT2b NPT2c	SLC34A1 SLC34A2 SLC34A3	3:1 electrogenic BBM proximal tubule P_i uptake Intestinal P_i transport 2:1 electroneutral BBM proximal tubule P_i uptake	Murer *et al.* (2000) Hilfiker *et al.* (1998) Bacconi *et al.* (2005) Bergwitz *et al.* (2006) Madjdpour *et al.* (2004) Segawa *et al.* (2002) Tenenhouse *et al.* (2003)
NaPi-III		NPT3	SLC20A1, SLC20A2	P_i transporter in bone. Broadly expressed	Suzuki *et al.*(2006) Suzuki *et al.* (2001)

2003). Type II cotransporters are 80- to 90-kDa glycoproteins that are predicted to span the membrane eight times, with both their amino and carboxyl termini in the cytoplasm (Murer and Biber, 1997). Recent work shows two variant transcripts of the mouse Npt2a gene, Npt2a-v1 and Npt2a-v2 (Yamamoto *et al.*, 2005). These are characterized by the presence of alternative first exons (either exon 1 A or exon 1B). Npt2a-v2 is important for 1,25(OH)$_2$D$_3$-dependent renal cell-specific activation. Type IIa cotransporters are electrogenic and transport Na$^+$ and H$_2$PO$_4$$^-$ at a molar ratio of 3:1 (Murer and Biber, 1997). Expression and activity of type IIa NaP$_i$ cotransporters ("NaP$_i$2," in rat; "NaP$_i$3" or "NPT2" in human) are strongly regulated by both parathyroid status and dietary phosphate (Keusch *et al.*, 1998; Lotscher *et al.*, 1999; Murer *et al.*, 1996; Pfister *et al.*, 1997; Ritthaler *et al.*, 1999; Takahashi *et al.*, 1998). PTH regulation of serum P$_i$ and tubular NaP$_i$ absorption is lost in mice lacking type IIa cotransporters (Zhao and Tenenhouse, 2000). Thus, regulation of Npt2 activity is the principal mechanism whereby PTH controls phosphate absorption in proximal convoluted tubules.

The type IIc transporter (Npt2c) has been identified in rat and human kidneys (Ohkido *et al.*, 2003). Npt2c accounts for approximately 30% of Na/P$_i$ cotransport in kidneys of P$_i$-deprived adult mice (Ohkido *et al.*, 2003). The contribution of Npt2c to overall renal P$_i$ reabsorption is uncertain. Recent work showed upregulation of renal Npt2c but not Npt1 expression in Npt2a-null mice (Tenenhouse *et al.*, 2003). This compensatory response occurred in the absence of a corresponding upregulation of mRNA transcription or stability. Further, Npt2c protein abundance was not further increased by dietary P$_i$ restriction. These findings are consistent with maximum upregulation of Npt2c protein in Npt2a-null mice fed P$_i$-replete diets. These findings were interpreted to suggest that hypophosphatemia is insufficient for upregulation of Npt2c gene expression and that Npt2c is responsible for residual Na-P$_i$ cotransport in Npt2a-null mice. The precise contribution of Npt2c to overall P$_i$ economy and homeostasis in intact animals is, nonetheless, uncertain.

PTH Regulation of Renal Phosphate Absorption

PTH exerts a prompt phosphaturic action that early biochemical studies demonstrated involved reduction of the maximal rate of Na-P$_i$ cotransport and that recovery from this effect required microtubules and new protein synthesis (Dousa *et al.*, 1976; Malmström and Murer, 1987). Based on classical transport mechanisms and their regulation, it had been widely assumed that PTH inhibited Na-P$_i$ cotransport through phosphorylation or other actions that would control the kinetics or driving force. In fact, functional and immunohistochemical analyses of Npt2 in intact kidneys or in cultured cells revealed that PTH induces rapid (15 min) removal of Npt2 from the apical membrane, into the subapical endocytic apparatus, followed by microtubule-dependent delivery to lysosomes and proteolytic degradation (Fig. 6) (Bacic *et al.*, 2006; Kempson *et al.*, 1995; Keusch *et al.*, 1998; Lötscher *et al.*, 1999; Pfister *et al.*, 1997; Zhang *et al.*, 1999). The inhibitory action of PTH on proximal tubular Pi absorption correlates with the abundance of brush-border membrane Npt2 cotransporters (Lötscher *et al.*, 1996, 1999). PTH-induced retrieval of Npt2 from apical membranes requires the presence of a specific Lys^{503}–Arg^{504} dibasic amino acid motif in the proximal carboxyl-terminal cytosolic loop of Npt2 (Karim-Jimenez *et al.*, 2000). In chronic kidney disease, elevated phosphaturia is maintained by parallel reductions of Npt2a mRNA and protein abundance. In contrast, decreased phosphate excretion associated with parathyroidectomy or a low-P$_i$ diet is mediated by increased Npt2a protein

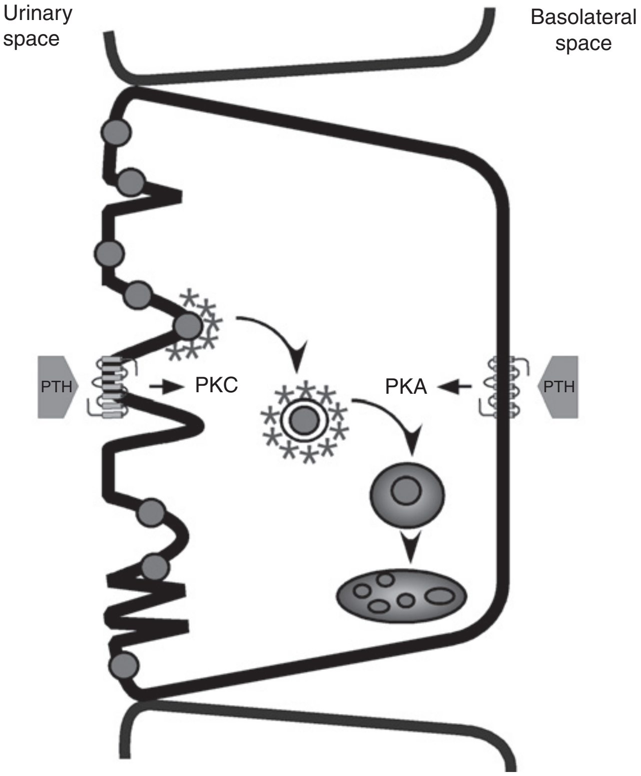

FIGURE 6 Simplified model of proximal tubule Na-Pi cotransport by endocytosis and downregulation by PTH. Stimulation of apical membrane PTH1Rs activate PKA, whereas basolateral membrane PTH1R predominantly activate PKC. PKA and PKC induce rapid retrieval of apical Npt2 transporters. Apical targeting of Npt2 requires the adapter protein NHERF1 (data not shown), as does PKC activation and efficient Npt2 internalization. Npt2 is translocated to clathrin-coated pits, which are pinched off to form vesicles. The clathrin-coated vesicles containing Npt2 is transported to endosomes and then to lysosomes, where it is degraded, causing downregulation.

without a change in mRNA expression, suggesting differential post-transcriptional regulation of Npt2a (Elhalel *et al.*, 2004).

Hypoparathyroidism and hypophosphatemia increase both PTH1R and NaP$_i$ mRNA and protein showing that there is coordinate regulation of the proteins mediating PTH effects on renal P$_i$ transport (Kilav *et al.*, 1995). PTH does not acutely reduce Npt2 mRNA expression, although parathyroidectomy does lead to severalfold increases of both apical Npt2 protein and mRNA (Kilav *et al.*, 1995; Saxena *et al.*, 1995; Takahashi *et al.*, 1998).

PTH1R Signal Transduction in the Regulation of Calcium and Phosphate Excretion

As described earlier, and discussed in Chapter 27, the PTH1R mediates its actions through multiple signaling pathways. Signaling is both ligand- and cell-specific. PTH exerts physiologically distinct and spatially separated effects on the kidney. In proximal tubules, PTH inhibits Na-P$_i$ cotransport and Na/H exchange, increases Na$^+$/Ca^{2+} exchange (see Fig. 2), activates 25-hydroxyvitamin D$_3$-1α-hydroxylase, while suppressing Na$^+$, K$^+$-ATPase activity, and stimulates gluconeogenesis and ammoniagenesis. Complex, but precise, individual control over these multiple physiological activities ensures that all are not simultaneously engaged in response to elevations or reductions of PTH. Just how this is accomplished is far from understood. However, important insight has been gained in to PTH-dependent regulation of renal calcium and phosphate transport by the identification of cytoplasmic scaffolding proteins that specifically modulate individual PTH actions. Moreover, emerging evidence supports a role of these cytoplasmic adapter proteins in accounting for this remarkable specificity(Biber *et al.*, 2005).

PTH Signaling of Renal Calcium Transport

PTH stimulates calcium absorption in distal tubules. The specific subsegment where this occurs differs somewhat between species. In humans, and in the rat, the mouse, and the dog, distal convoluted tubules are the primary site of PTH-dependent calcium absorption. This has been demonstrated directly or is inferred from the localization of the PTH1R or the presence of PTH-stimulated adenylyl cyclase activity (Chabardès *et al.*, 1980; Friedman and Gesek, 1993). Connecting tubules are the site of PTH-stimulated calcium transport in the rabbit (Shimizu *et al.*, 1990b).

The initial entry of Ca^{2+} across apical cell membranes in murine distal convoluted tubule cells requires activation of both PKA and PKC (Friedman *et al.*, 1996, 1999; Hilal *et al.*, 1997). Considerable evidence suggested that PTH action on calcium absorption was mediated exclusively through activation of PKA. That is, stimulation of adenylyl cyclase was thought to be necessary and sufficient for induction of calcium transport. The most compelling evidence for this conclusion was that addition of exogenous cAMP analogues or forskolin, which directly activates adenylyl cyclase, or phosphodiesterase inhibitors stimulated calcium absorption in different tubule preparations (Bindels *et al.*, 1991; Bourdeau and Burg, 1980; Bourdeau *et al.*, 1990; Bourdeau and Lau, 1992; Costanzo and Windhager, 1980; Friedman, 1988; Imai, 1981; Lau and Bourdeau, 1989; Shimizu *et al.*, 1990b; Suki and Rouse, 1981). PTH stimulates cAMP formation and increases calcium uptake by murine distal convoluted tubule cells. However, isoproterenol, vasopressin, and glucagon stimulated cAMP formation to levels comparable to or greater than those evoked by PTH but had no significant effect on calcium uptake (Friedman and Gesek, 1993). These findings were consistent with the idea that activation of both PKA and PKC is required to stimulate calcium uptake. Forskolin, however, induced the greatest cAMP accumulation and

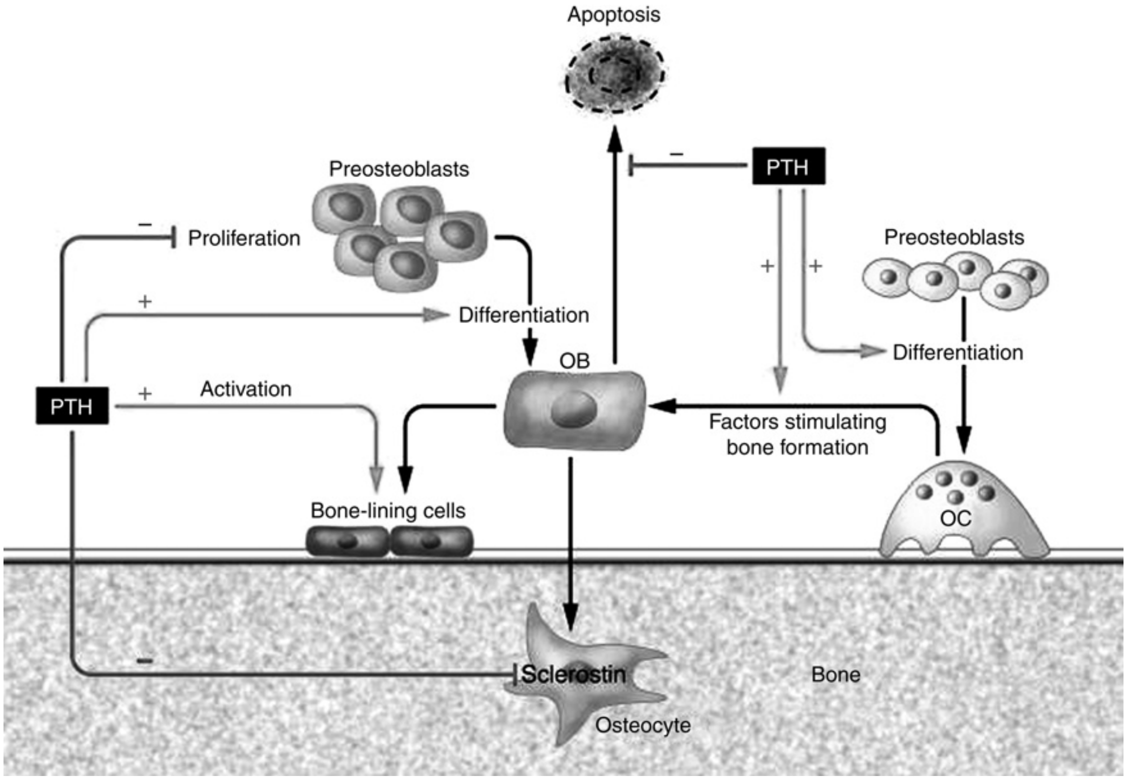

FIGURE 7 Cellular effects of PTH on bone. Bone resorption. PTH stimulates differentiation and activation of osteoclasts by increasing RANKL, and possibly decreasing OPG, production by stromal cells and osteoblasts. Bone formation. PTH decreases proliferation of preosteoblasts and stimulates their differentiation. PTH decreases osteoblast apoptosis and can activate bone lining cells into functioning osteoblasts. These combined actions result increased numbers of osteoblast. Moreover, PTH suppresses the production of the Wnt inhibitor sclerostin by osteocytes, thereby increasing β-catenin signaling in osteoblasts. From Khosla *et al*. (2008).

also augmented calcium uptake, but only to a fraction of that observed with PTH. When the concentration of forskolin was adjusted to one eliciting comparable stimulation of cAMP formation as that maximally induced by PTH, forskolin no longer enhanced calcium uptake. However, simultaneous addition of forskolin and a phorbol ester, which had no effect of its own, to stimulate PKC, resulted in calcium uptake that was similar in magnitude to that elicited by PTH. These results support the view that activation of both PKA and PKC pathways are necessary for stimulation of calcium uptake by mouse distal convoluted tubule cells. The findings also indicate that relatively high concentrations of forskolin (= 1 μM) may induce nonspecific crossover activation of PKC (Anderson and Breckon, 1991; Jiang *et al.*, 1992; MacNicol and Schulman, 1992; Shabb *et al.*, 1991).

PKC is typically stimulated secondary to mobilization of $G_{q/11}$ with subsequent activation of PLC, hydrolysis of phosphatidylinositol to form inositol phosphates and diacylglycerol, which, in the presence of elevated Ca^{2+}, activates PKC. Further evidence implicated a Ca^{2+}-independent ("atypical") PKC as a mediator of PTH effects (Hoenderop *et al.*, 1999). Surprisingly, however, although PTH activates PKC in distal tubule cells, and stimulation of calcium transport requires such activation, stimulation of PLC was undetectable (Friedman *et al.*, 1999). This raised the question as to the mechanism by which PTH activates PKC independent of PLC. Subsequent investigation explored an alternative pathway, in which PTH stimulates PLD. PLD hydrolyzes phosphatidylcholine or phosphatidylethanolamine with attendant formation of phosphatidic acid, conversion to diacylglycerol, and activation of PKC. The PTH1R uses this G_q- and PLC-independent mechanism in a cell-specific manner to activate PKC (Friedman *et al.*, 1999; Singh *et al.*, 1999). Notably, the PTH1R employs this pathway despite the fact that bradykinin and α_2-adrenergic receptors in distal convoluted tubule cells exhibit conventional PKC and calcium signaling through PLC. An important question remaining from these findings is how the PTH1R activates PLD. PTH1R activation of $G\alpha_{12}/G\alpha_{13}$ mediates PTH activation of PLD in UMR-106 cells (Singh *et al.*, 2005). Other pathways involving EPAC (exchange protein activated by cAMP) (Lopez De Jesus *et al.*, 2006) or the sorting motif, NPXXY (Johnson *et al.*, 2006), have been described.

Cellular efflux of calcium is mediated by Na^+/Ca^{2+} exchange and the plasma membrane Ca^{2+}-ATPase, both of which are expressed on distal tubule cell membranes (Magyar *et al.*, 2002; White *et al.*, 1997). Although opinions differ regarding the relative contribution of each process, we think it likely that Ca^{2+}-ATPase mediates basal Ca^{2+} efflux because the electrochemical driving force for Ca^{2+} extrusion by Na^{+}/Ca^{2+} exchange is insufficient (Friedman, 1998). After stimulation by PTH, distal tubule cells hyperpolarize (Gesek and Friedman, 1992b; Shimizu *et al.*, 1990a), thereby augmenting the driving force for Na^{+}/Ca^{2+} exchange. In this setting, Na^{+}/Ca^{2+} exchange likely is responsible for the elevated Ca^{2+} efflux that accompanies transcellular calcium absorption. Regardless of its relative importance, PTH stimulates Na^{+}/Ca^{2+} exchange (Bouhtiauy *et al.*, 1991; Hanai *et al.*, 1986).

Ca^{2+}-ATPase activity is also stimulated by PTH (Itoh *et al.*, 1988; Levy *et al.*, 1986). However, this effect was demonstrated in proximal tubule basolateral membranes, where PTH inhibits transcellular phosphate absorption. The effect on Ca^{2+}-ATPase may be associated with a homocellular action of PTH or represent an epiphenomenon. Indeed, PTH fails to alter Ca^{2+}-ATPase activity in membranes prepared from distal tubule cells (Bouhtiauy *et al.*, 1991).

Considering that PTH may orchestrate a series of coordinated single events including membrane hyperpolarization, insertion or activation of apical membrane Ca^{2+} channels, calbindin-D_{28K} expression, increased Na^{+}/Ca^{2+} exchange, and, perhaps, increased Ca^{2+}-ATPase activity to achieve distal tubular Ca^{2+} absorption, it is not surprising that some uncertainty attends the precise roles of PKA, PKC, or mitogen-activated protein kinases (Sneddon *et al.*, 2000) (or other PTH1R-activated effectors) in controlling distal tubular Ca^{2+} transport. Apparent requirements for multiple effectors may reflect a convergence of several signals on a single mechanism; independent actions of different effectors on one or more of the elemental cellular responses that contribute to the overall Ca^{2+}-reabsorptive response, or both.

PTH Signaling of Renal Phosphate Transport

Early experiments *in vivo* or with isolated renal membranes indicated that regulation of P_i absorption was mediated by cAMP. The evidence marshaled in support of this conclusion was based, *inter alia*, on the observation that cAMP analogues or phosphodiesterase inhibitors mimicked the phosphaturic action of PTH (Agus *et al.*, 1973; Coulson and Scheinman, 1989; Gmaj and Murer, 1986; Hammerman, 1986). Indeed, the excretion of urinary cAMP in response to PTH administration, so-called nephrogenic cAMP, reflects virtually exclusively the action of PTH on proximal tubule P_i absorption (and not distal tubule Ca absorption), and is a direct index of PTH1R sensitivity or refractoriness (Besarab and Swanson, 1984). Although these conclusions are solid, the mechanisms by which PTH inhibits renal P_i transport are complex and incompletely resolved. The intricacy stems from the recognition that the PTH1R activates multiple signaling paths. The possibility that differentially expressed PTH1R types (Pun *et al.*, 1988) or isoforms account for the heterogeneity of action can be excluded. The first studies profiling the effect of PTH on inositol phosphate and Ca^{2+} signaling, in fact, preceded the cloning of the PTH1R (Bidot-López *et al.*, 1981; Meltzer *et al.*, 1982). PTH stimulation of inositol phosphate and diacylglycerol accumulation is independent of cAMP formation (Hruska *et al.*, 1987) and occurs in parallel (Friedman *et al.*, 1996).

The involvement of specific PTH1R-generated signals in the regulation Na-P_i cotransport has been pursued extensively *in vitro* using immortalized opossum kidney (OK) cells, which exhibit a proximal tubule-like phenotype that includes expression of the Npt2a NaP_i cotransporter and the PTH1R (Jüppner *et al.*, 1991; Rabito, 1986). Considerable evidence supports cAMP/PKA-mediated signaling, but other observations point to an important contribution of inositol phosphates/PKC. The extensive body of work on this subject has been reviewed elsewhere (Muff *et al.*, 1992; Murer *et al.*, 2000; Pfister *et al.*, 1999) and described in the previous edition this text (Bringhurst and Strewler, 2002). Recent incisive work and the discovery of cytoplasmic binding partners for the PTH1R and Npt2a have largely reconciled the apparently disparate views. The salient features of PTH1R signaling of proximal tubule P_i transport can be summarized as follows.

PTH1Rs, as noted earlier, are expressed on both apical and basolateral membranes of proximal tubule cells (Amizuka *et al.*, 1997; Ba *et al.*, 2003; Traebert *et al.*, 2000). Studies selectively applying PTH(1–34) or PTH(3–34) to apical or basolateral surfaces of perfused murine proximal tubules indicate that PTH1Rs present at both membrane domains induce rapid retrieval of apical NaP_i proteins (Traebert *et al.*, 2000). Notably, this occurs exclusively by a PKC-dependent mechanism that entails activation of apical PTH1Rs. In contrast, basolateral PTH1Rs induce cotransporter endocytosis by PKA-dependent signaling. More recent studies uncovered the molecular basis for the sidedness of PTH action and the dependence on PKC or PKA. The likely explanation for the asymmetric actions and signaling stem from the localized expression of the adapter protein, NHERF1. NHERF1 is a PDZ protein that binds both to the PTH1R and to Npt2a (Hernando *et al.*, 2002; Mahon *et al.*, 2002; Wade *et al.*, 2003). In the absence of NHERF1, mice exhibit a phosphate-wasting phenotype (Shenolikar *et al.*, 2002). NHERF1 is abundantly expressed at proximal tubule brush-border membranes in the rat, and is also detected in the cytoplasm and on basolateral membranes (Wade *et al.*, 2001). In mice, NHERF1 is found exclusively on proximal tubule brush-border microvilli (Wade *et al.*, 2003). In OK/H cells, which lack NHERF1, PTH fails to inhibit Na-P_i cotransport and this defect can be surmounted by overexpressing NHERF1 (Mahon *et al.*, 2003).

According to the presently accepted understanding, PTH1R signals through PKA in the absence of NHERF1 and by means of PKC in its presence (Mahon *et al.*, 2002). Thus, at basolateral membranes, where there is little NHERF1, PTH would be expected to signal through adenylyl cyclase and PKA. At apical (i.e., luminal) cell membranes, signaling would be predicted to be mediated by PLC and PKC because of the coupling between the PTH1R and PLC (Mahon and Segre, 2004). These predictions are compatible with the observations that PTH(1–34) evoked Npt2a sequestration when added to either cell surface, whereas PTH(3–34), which activates PKC but not PKA, induced Npt2a internalization only when applied to the luminal surface (Traebert *et al.*, 2000). Consistent with this scheme, more recent studies reveal that PTH(3–34) was unable to promote Npt2a internalization from proximal tubule brush-border membranes prepared from NHERF1-null mice (Capuano *et al.*, 2006). Activation of PLC by PTH was impaired. Bypassing this defect by directly activating PKC with a diacylglycerol analogue caused Npt2a internalization in proximal tubules of both wild-type and NHERF1-null mice. Although these are elegant and compelling observations, their relevance to regulation of phosphate transport under physiological or pathophysiological conditions is unsettled. As described earlier, Na-P_i cotransport is regulated by cAMP and PKA. Metabolism of cAMP involves nucleotide degradation to adenosine by brush-border membrane ectoenzymes such as ecto-5′-nucleotidase. PTH(1–34) augmented ecto-5′-nucleotidase activity in apical membranes of OK cells (Siegfried *et al.*, 1995). This effect was mimicked by PTH(3–34) but not by forskolin. Both PTH fragments increased cytoplasmic Ca^{2+} and stimulated PKC activity. Conversely, stimulation of ecto-5′-nucleotidase activity was blocked by inhibitors of PKC. Thus, PKC-mediatedstimulation of 5′-nucleotidase thereby bolster the effects transduced by PKA.

PTH1Rs present on proximal tubule luminal membranes presumably may be activated by PTH peptides that are filtered through the glomerulus and appear in the luminal fluid. It is unlikely that these peptides or smaller fragments escape in the final urine, because they are absorbed (Kau and Maack, 1977), internalized (Hilpert *et al.*, 1999), and metabolized (Brown *et al.*, 1991; Daugaard *et al.*, 1994; Yamaguchi *et al.*, 1994) during their passage through the nephron. However, given the increased generation and accumulation of amino-truncated PTH peptides during kidney disease and other pathological settings, it is likely that significant amounts reach luminal brush-border membranes and would be expected to promote Npt2a/NPT2a internalization with concomitant renal phosphate wasting. Such an action might represent an adaptive response to the elevation of plasma phosphate in chronic kidney disease. By contrast, in distal tubules, which lack NHERF1, no such effect would be anticipated.

Activation by PTH fragments of PKA and PKC may lead to temporally and qualitatively distinct changes in Npt2 expression and activity (Cole *et al.*, 1987; Lederer *et al.*, 1998; Pfister *et al.*, 1999). Each can separately inhibit Npt2 activity by internalizing the cotransporter; only the PKA pathway, however, leads to Npt2 downregulation. Stimulation of both kinases may be necessary for maximal PTH action, and PTH1Rs located on opposite membrane surfaces of proximal tubule cells may be coupled to different effectors of Npt2a regulation.

SODIUM AND HYDROGEN EXCRETION

PTH exerts three principal and direct renal effects on ion homeostasis: increasing P_i excretion, and decreasing calcium and proton (H^+) excretion. Acute (Ellsworth and Nicholson, 1935; Hellman *et al.*, 1965; Kleeman and Cooke, 1951; Nordin, 1960) or chronic (Hulter and Peterson, 1985) PTH exerts an acute alkalotic action attended by elevated bicarbonate excretion. Primary hyperparathyroidism, however, is generally not attended by consistent or remarkable changes of serum pH and is largely asymptomatic (Bilezikian and Silverberg, 2004). This occurs despite marked hyperchloremia because of compensating metabolic acidosis (Wills, 1971). Similar metabolic changes have been described in chronic kidney disease (Liborio *et al.*, 2007).

Elimination of the daily metabolic acid load by the kidneys involves the secretion of H^+ by proximal tubules. This is accomplished by a plasma membrane H^+-ATPase and by Na^+/H^+ exchange. PTH inhibits acidification by suppressing Na^+/H^+ exchange. As described later, this involves activation of adenylyl cyclase, NHERF1, which serves as a scaffold to assemble PKA and NHE3, the Na^+/H^+ exchanger isoform expressed on proximal tubule brush-border membranes, followed by phosphorylation and inhibition of NHE3 (Moe *et al.*, 1995; Weinman *et al.*, 1998, 2005). Notably, the inhibitory effect of PTH on proximal tubule bicarbonate absorption due to blockade of Na^+/H^+ exchange does not necessarily result in increased bicarbonate excretion (Bank and Aynedjian, 1976; Puschett *et al.*, 1976) and is not accompanied by appreciable reductions in serum bicarbonate (Hulter, 1985). These results suggest that the presence of distal compensatory bicarbonate absorption (Bichara *et al.*, 1986) and acidification by downstream tubule segments in response to PTH (Paillard and Bichara, 1989). Secondary actions of PTH to mobilize bicarbonate from bone also contribute to the absence of an effect on systemic pH (Hulter and Peterson, 1985). More important than its modest effects on net bicarbonate balance or pH homeostasis, however, is the permissive action of bicarbonate to facilitate PTH-dependent distal tubule calcium absorption (Marone *et al.*, 1983; Mori *et al.*, 1992; Sutton *et al.*, 1979), a primary function of PTH.

Much of the transcellular sodium absorption in proximal convoluted tubules is mediated by Na^+/H^+ exchange.

As a corollary of the inhibitory action of PTH on Na^+/H^+ exchange, proximal sodium absorption is diminished. However, PTH has a negligible effect on sodium excretion because of compensatory absorption by downstream tubule segments (Harris *et al.*, 1979). Natriuretic actions of PTH, however, have been described in experimental animals (Bichara *et al.*, 1986; Hellman *et al.*, 1965) and in humans (Jespersen *et al.*, 1997).

The fundamental mechanism whereby PTH inhibits Na^+/H^+ exchange differs from its action on $Na\text{-}P_i$ cotransport. As described earlier, PTH decreases $Na\text{-}P_i$ cotransport by retrieving the protein from brush-border membranes. In contrast, PTH acutely regulates Na^+/H^+ exchange by *in situ* phosphorylation of NHE3 that is mediated by PKA (Hamm *et al.*, 2008). The immediate decrease of NHE3 activity is followed several hours later by decreased brush-border membrane abundance of NHE3 protein (Fan *et al.*, 1999). Details of this process and the involvement of NHERF1 are detailed in the next section.

Na^+/H^+ exchange is a dissipative, i.e., secondary active, transport process that depends on the continuous extrusion of Na across basolateral cell membranes. This is accomplished by the ATP-dependent Na^+,K^+-ATPase. Data showing that PTH inhibits the Na^+,K^+-ATPase have been published (Ribeiro and Mandel, 1992; Zhang *et al.*, 1999). An inhibitory action of PTH on Na^+,K^+-ATPase would certainly contribute to, or explain, reduced Na^+/H^+ exchange. However, such an action would be expected to have profound effects on proximal tubule ion transport and all Na-coupled transport process such as those mediating glucose (Na-glucose) and amino acid (Na-amino acid) cotransport, and perhaps $Na\text{-}P_i$ cotransport. Moreover, the inhibitory action of PTH should extend to virtually all cells expressing PTH1R including the distal tubule. In this setting, blockade of the Na^+,K^+-ATPase would be expected to reduce Na^+/Ca^{2+} exchange and decrease net calcium absorption when, in fact, just the opposite occurs. The described actions of PTH seem remarkably specific for the proximal tubule Na^+,K^+-ATPase and for Na^+/H^+ exchange. A rationale explication of these findings awaits further clarification.

PTH Regulation of Proximal Tubular Sodium and Hydrogen Excretion

Efficient bicarbonate absorption by proximal tubules requires NHERF1. Targeted disruption of NHERF1 virtually eliminates PTH- or forskolin-inhibited Na^+/H^+ exchange in proximal tubule cells (Cunningham *et al.*, 2004). This defect could be restored by infecting the cells with an adenovirus containing NHERF1. Interestingly, cAMP generation and PKC activation were equivalent in proximal tubule cells from wild-type mice or NHERF1-null animals. This result seemingly contrasts with the paradigm of the PTH "signaling switch" advanced by Mahon and Segre (Mahon *et al.*, 2002), where PTH1R signaling proceeds through cAMP in the presence of NHERF1 but by PKC in its absence. However, mouse proximal tubules express both NHERF1 and NHERF2, and NHERF2 expression is normal in NHERF1-null mice (Cunningham *et al.*, 2008; Shenolikar *et al.*, 2002). Thus, it is entirely possible that NHERF2 compensates for the absence of NHERF1, binds the PTH receptor, and directs appropriate signaling and cell functions. Notably, OK cells express NHERF1 but not NHERF2 (Wade *et al.*, 2001).[3] Thus, the results of studies conducted with OK cells demonstrating an absolute role of NHERF1 in PTH1R mediated inhibition of $Na\text{-}P_i$ cotransport (Mahon *et al.*, 2003) may have fortuitously benefited from the absence of NHERF2.

The majority of PTH effects appear to be mediated by PKA and, to a lesser extent, by PKC. However, recent evidence uncovered an alternative pathway that involves cAMP but utilizes EPAC (exchange protein directly activated by cAMP) rather than PKA as an effector (Fujita *et al.*, 2002). Studies employing proximal tubule cells derived from wild-type or NHERF1-null mice revealed a role for EPAC in mediating Na^+/H^+ exchange (Murtazina *et al.*, 2007).

Following the secretion of H^+ (in exchange for one Na^+ ion by the apical cell membrane Na^+/H^+ exchanger) OH^- is generated within the cell. This is converted to HCO_3^- and CO_3^-, which are then extruded across basolateral cell membranes. This is accomplished by Na^+-dependent HCO_3^- (NBC) cotransporters (Boron, 2006; Romero *et al.*, 2004; Soleimani and Burnham, 2001). The possibility that PTH inhibits bicarbonate absorption by an action on basolateral HCO_3^- exit was a matter of uncertainty (Pastoriza-Munoz *et al.*, 1992; Sasaki and Marumo, 1991), and this action was attributed by some to an effect on the Na^+,K^+-ATPase as discussed earlier. More recent studies reveal direct effects of cAMP on NBC activity. This action requires NHERF1 (Bernardo *et al.*, 1999; Weinman *et al.*, 2001). PTH presumably exerts comparable actions but this has not been explicitly determined.

PTH1R Signal Transduction in the Regulation of Sodium and Hydrogen Excretion

The mechanisms by which the PTH1R regulates Na^+/H^+ exchange are both complex and incompletely resolved. NHE3 is rapidly phosphorylated and inactivated following *in vivo* PTH exposure, after which it is internalized (Fan *et al.*, 1999; Hensley *et al.*, 1989; Zhang *et al.*, 1999). NHE3 is a target for PKA-mediated phosphorylation (Zhao

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[3] Rats too express only NHERF1 on proximal tubule brush-border membranes. Thus, OK cells are more of a model for the rat proximal tubule than for that of humans or mice, where both NHERF1 and NHERF2 are expressed.

et al., 1999). Considerably greater uncertainty attends the question of whether NHE3 is also phosphorylated by PKC. Indirect evidence derived from the application of PTH fragments that largely, but not entirely, activate signaling-selective protein kinases is consistent with the view that PKC activation is insufficient to promote NHE3 internalization *in vivo* (Zhang *et al.*, 1999). Comparable results were obtained in a cell culture system (Maeda *et al.*, 1998). PTH induces phosphorylation of multiple serine residues within the cytoplasmic tail of NHE3 (Collazo *et al.*, 2000). Evidence developed using kinase inhibitors and signal-selective PTH analogues supports the involvement of both PKA- and PKC-dependent pathways in NHE3 regulation (Azarani *et al.*, 1995, 1996; Helmle-Kolb *et al.*, 1990; Kahn *et al.*, 1985). Phosphorylation is maximal within 5 minutes and is associated with a reduction in NHE3 activity but not surface expression. NHE3 phosphorylation requires NHERF1 (Weinman *et al.*, 1993, 2005). Following the initial inactivation of NHE3, the exchanger is retrieved from the cell membrane by dynamin-dependent endocytosis (Chow *et al.*, 1999; Collazo *et al.*, 2000).

By way of summary, PTH regulates Na^+/H^+ exchange primarily by an immediate inhibitory action that depends on phosphorylation and requires the presence of NHERF1. Phosphorylation is thought generally to be mediated by PKA, though a regulatory influence of PKC can not be excluded. Following the initial inhibition of membrane-associated NHE3, the protein is withdrawn from the membrane. However, it does not appear to be degraded, which would lead to downregulation. Though its fate is uncertain, it seems plausible that after dephosphorylation the transporter is recycled to the plasma membrane, as are the PTH1R and other G protein-coupled receptors.

PTH Regulation of Sodium and Hydrogen Excretion beyond the Proximal Tubule

The effect and physiological significance of downstream PTH actions on sodium and proton transport are poorly defined. Studies, particularly *in vivo* experiments, published before the molecular cloning and tubular localization of the PTH1R should be considered suspect. Actions attributed to PTH evidently must have involved indirect actions insofar as present consideration would argue that no detectable receptors are present at the presumed site of action. Such an interpretation is especially relevant because it is now appreciated that phosphaturia induced by PTH contributes to net acid secretion (Mercier *et al.*, 1986), and PTH can increase net renal acid secretion during metabolic acidosis (Bichara *et al.*, 1990).

VITAMIN D METABOLISM

The biologically active form of vitamin D, $1,25(OH)_2D_3$, is synthesized by a cascade of multiorgan processes, the penultimate step of which is 1-alpha hydroxylation that proceeds in proximal renal tubules and is regulated by PTH and the CaSR (DeLuca, 2004; Dusso *et al.*, 2005; Holick, 2007; Maiti and Beckman, 2007).

Parathyroidectomy reduces $1,25(OH)_2D_3$ synthesis and PTH administration increases renal 1α-hydroxylase activity (Armbrecht *et al.*, 2003; Fraser and Kodicek, 1973; Walker *et al.*, 1990). Increased synthesis of the $25(OH)D_3$ 1α-hydroxylase gene results from regulated expression in proximal tubular cells, where the promoter is rapidly induced in response to PTH (Fukase *et al.*, 1982; Kremer and Goltzman, 1982). This *in vitro* effect of PTH can be overridden *in vivo* by the direct suppressive action of hypercalcemia on 1α-hydroxylase expression (Weisinger *et al.*, 1989). Induction of 1α-hydroxylase mRNA by PTH is transcriptional; additive to that of calcitonin; occurs in the absence of the vitamin D receptor; and is antagonized by coadministration of $1,25(OH)_2D_3$, which directly inhibits its expression when administered alone (Murayama *et al.*, 1999). Positive actions of PTH on 1α-hydroxylase expression and $1,25(OH)_2D_3$ biosynthesis are counter-regulated by the CaSR (Maiti and Beckman, 2007; Maiti *et al.*, 2008).

PTH not only promotes $1,25(OH)_2D_3$ synthesis, but also regulates its degradation. 24-Hydroxylation of $1,25(OH)_2D_3$ is the first step in the catabolic inactivation of $1,25(OH)_2D_3$. It was long held that only proximal renal tubules expressed 24-hydroxylase (Iida *et al.*, 1993; Kawashima *et al.*, 1981). Distal tubules, however, take up $1,25(OH)_2D_3$ (Stumpf *et al.*, 1980), express the vitamin D receptor (VDR) (Kumar *et al.*, 1994), and possess vitamin D-dependent calcium-binding proteins (Christakos *et al.*, 1981). 24-Hydroxylase message, protein, and activity have been demonstrated in murine distal tubule cells (Yang *et al.*, 1999), human distal tubules (Kumar *et al.*, 1994), and collecting duct cells (Bland *et al.*, 2001). Whereas PTH reduces 24-hydroxylase activity in proximal tubule cells (Barletta *et al.*, 2004; Matsumoto *et al.*, 1985), PTH and cAMP augment 24-hydroxylase activity in murine distal tubule cells (Yang *et al.*, 1999). Oppositely oriented actions of PTH on 24-hydroxylase activity suggest differential regulation of 24-hydroxylase expression in proximal and distal tubules.

The signaling pathways employed by the PTH1R to increase $1,25(OH)_2D_3$ synthesis have been examined extensively *in vivo* and *in vitro*. Involvement of cAMP is suggested by the fact that the PTH effect can be mimicked by cAMP analogues, forskolin, or phosphodiesterase inhibitors (Armbrecht *et al.*, 1984; Henry, 1985; Horiuchi *et al.*, 1977; Korkor *et al.*, 1987; Larkins *et al.*, 1974; Rost *et al.*, 1981; Shigematsu *et al.*, 1986). PTH- or forskolin-stimulated transcriptional induction of 1α-hydroxylase occurred and was blocked by the PKA inhibitor H89 (Murayama *et al.*, 1999). A role for PKC in regulating 1α-hydroxylation has been identified from studies of the effects of PTH on isolated proximal tubules (Janulis *et al.*, 1992, 1993). PKC activation produced rapid (30 to 60 minute) increases of 1,25$(OH)_2D_3$ synthesis [PKC and 1,25$(OH)_2D_3$ synthesis correlated with PTH concentrations 100- to 1000-fold lower than required for activation of PKA]. Moreover, inhibition of PKC blocked 1,25$(OH)_2D_3$ synthesis, and amino-truncated PTH fragments that stimulate PKC but not PKA likewise enhanced 1,25$(OH)_2D_3$ formation. Taken together, the results suggest a predominant effect of PKA on transcriptional regulation of 1α-hydroxylase gene expression and a more rapid, post-transcriptional action of PKC on 1α-hydroxylase enzymatic activity.

OTHER RENAL EFFECTS OF PTH

PTH exerts a variety of additional described, but far less well understood, effects on ion transport, such as activation of apical Cl^- channels (Suzuki *et al.*, 1991), and metabolism.

The kidney contributes significantly to systemic gluconeogenesis (Friedman and Torretti, 1978; Schoolwerth *et al.*, 1988), although this is not normally apparent because of balancing by renal medullary glycolysis. PTH-dependent renal gluconeogenesis is attended by segmental and internephron heterogeneity. PTH increases gluconeogenesis (Chobanian and Hammerman, 1988), ammoniagenesis (Wang and Kurokawa, 1984), and phosphoenolpyruvate carboxykinase (PEPCK) (Watford and Mapes, 1990) mRNA expression in proximal tubules. PTH primarily stimulates gluconeogenesis in cortical S1 and S2 proximal tubules (Wang and Kurokawa, 1984). Although juxtamedullary S1 proximal tubules exhibit the highest rate of gluconeogenesis, they are unaffected by PTH.

Gluconeogenesis is linked to ammoniagenesis because both are stimulated by acidosis and by PTH. Moreover, L-glutamine, which is the major gluconeogenic precursor, is also a substrate for ammoniagenesis. Thus, there may be some metabolic interdependence of PTH actions on gluconeogenesis and ammoniagenesis.

PTH also exerts conspicuous morphological effects causing rapid microvillus shortening (Goligorsky *et al.*, 1986). This action appears to be specific for proximal tubule cells. Emerging observations suggest that this cell-specific effect may be caused by the prominent brush-border expression of NHERF1 in proximal tubule cells and its absence from distal tubule cells.

Whether proximal tubule PTH regulation of Na-P_i cotransport, Na^+/H^+ exchange, or Na^+/Ca^{2+} exchange, or its metabolic actions on the 25-hydroxyvitamin D-1α-hydroxylase, gluconeogenesis, or ammoniagenesis involves more complicated second messenger signaling is unknown. It should be mentioned that emerging evidence points to the ability of PTH receptors to activate additional signaling pathways involving phospholipase A_2 (Mandel and Derrickson, 1997; Ribeiro *et al.*, 1994), PLD (Friedman *et al.*, 1996; Somermeyer *et al.*, 1983), and MAP kinase (Quamme *et al.*, 1994; Swarthout *et al.*, 1997; Verheijen and Defize, 1997).

RENAL EXPRESSION AND ACTIONS OF PTHrP

PTHrP is expressed by glomeruli, distal tubules, and collecting ducts of fetal kidneys and by proximal convoluted tubules, distal convoluted tubules, and glomeruli of adult kidneys (Aya *et al.*, 1999; Philbrick *et al.*, 1996). In rat kidneys, PTHrP mRNA is found in glomeruli, proximal convoluted tubules, and macula densa but not in cortical ascending limbs, medullary ascending limbs, distal convoluted tubules, or collecting ducts (Yang *et al.*, 1997), whereas other studies reported PTHrP transcripts in glomerular mesangial cells, proximal and distal tubules (Soifer *et al.*, 1993). Although PTHrP is critical for normal cardiovascular and bone development, the kidneys of PTHrP-null mice appear histologically normal (Karmali *et al.*, 1992) and a physiological role for PTHrP on the kidney is uncertain (Mundy and Edwards, 2008).

Amino-terminal fragments of PTHrP exhibit renal actions, including stimulation of cAMP production, regulation of Na-P_i cotransport, and calcium excretion, that are largely indistinguishable from those of PTH (Everhart-Caye *et al.*, 1996; Horiuchi *et al.*, 1987; Horwitz *et al.*, 2003; Pizurki *et al.*, 1988; Scheinman *et al.*, 1990; Yates *et al.*, 1988). Some differences may be discerned. In distal tubule cells, for instance, PTHrP(1–34) and PTHrP(1–74) were more potent than equimolar concentrations of PTH(1–34) and PTH(1–84) in stimulating adenylyl cyclase (Friedman *et al.*, 1989). In contrast, PTHrP(1–36) has a markedly lower effect to raise serum 1,25$(OH)_2D_3$ levels (a consequence of its action on proximal tubules) in human volunteers than does comparable administration of PTH(1–34) (Horwitz *et al.*, 2003).

PTHrP possesses mid- and carboxy-terminal regions that have distinct biological actions (Clemens *et al.*, 2001; Philbrick *et al.*, 1996). Longer PTHrP fragments may exhibit unique renal properties. For example, excretion in perfused rat kidneys was enhanced equivalently by PTHrP(1–34) and PTH(1–34), whereas PTHrP(1–84), PTHrP(1–108), and PTHrP(1–141) were each less active than PTH(1–34) (Ellis *et al.*, 1990). As discussed in Chapter 25, the PTHrP gene can generate multiple transcripts and protein products, some of which may undergo unique nuclear localization. It is possible, therefore, that locally expressed PTHrP exerts actions in the kidney that are not shared with PTH.

A possible role for locally produced PTHrP in the renal response to ischemia has been suggested by findings that PTHrP expression is induced by ischemia or after recovery from ATP depletion (García-Ocaña *et al.*, 1999; Largo *et al.*, 1999; Soifer *et al.*, 1993). PTHrP is expressed in the intima and media of human renal microvessels and in the macula densa (Massfelder *et al.*, 1996). PTHrP (like PTH) increases renin release from the juxtaglomerular apparatus and also stimulates cAMP in renal afferent and efferent arterioles, leading to vasodilation and enhanced renal blood flow (Endlich *et al.*, 1995; Helwig *et al.*, 1991; Musso *et al.*, 1989; Saussine *et al.*, 1993). Both cAMP and nitric oxide have been implicated in PTHrP-induced *in vitro* vasorelaxation (Massfelder *et al.*, 1996). Thus, enhanced local PTHrP production induced by inadequate renal perfusion or ischemia may be involved in both local and systemic autoregulatory mechanisms. According to this view, direct local vasodilatory actions are supplemented by the systemic activation of angiotensinogen, which increases arterial pressure and further sustains renal blood flow.

PTHrP promotes fibrogenesis and is upregulated in experimental nephropathy (Funk, 2001; Ortega *et al.*, 2006; Ortega *et al.*, 2005). Locally produced PTHrP may contribute to the pro-inflammatory effect by activating NF-κB and extracellular signaling-related kinases (ERK) (Ramila *et al.*, 2008). The incidence of renal hypertrophy is greater in diabetic mice overexpressing PTHrP than in control animals, suggesting that constitutive PTHrP overexpression may elicit adaptive responses such as nitric oxide production to mitigate against renal damage (Izquierdo *et al.*, 2006).

PTHRP AND RECEPTORS FOR PTH AND PTHrP IN BONE

Receptors and Second Messenger Systems for PTH and PTHrP in Bone

Most PTH and PTHrP effects in bone are mediated by the type 1 PTH/PTHrP receptor (PTH1R) (Abou-Samra *et al.*, 1992; Jüppner *et al.*, 1991). As discussed in detail in Chapter 24, this is a G protein-coupled receptor that recognizes both PTH and PTHrP, as well as their biologically active N-terminal peptides PTH(1–34) and (1–36) and PTHrP(1–36). The PTH1R binds PTH and PTHrP with equal affinity (although some differences in the affinity for G protein-coupled and uncoupled states have recently been reported) (Dean *et al.*, 2008) and, in response to these ligands, signals through various cellular effector systems. The best characterized are the adenylyl cyclase/cAMP/PKA pathway via activation of Gs proteins and the PLC/PKC pathway via activation of Gq proteins (see Chapters 24 and 26) (Abou-Samra *et al.*, 1992; Civitelli *et al.*, 1989; Jüppner *et al.*, 1991; Pines *et al.*, 1996; Syme *et al.*, 2005). In addition, important effects of the PTH1R on mitogen-activated protein kinases (MAPK), especially ERK1 and ERK2, have been described (Cole, 1999; Miao *et al.*, 2001; Sneddon0 *et al.* ,2000, 2003; Swarthout *et al.*, 2001; Syme *et al.*, 2005).

The PTH1R is expressed widely in cells of the osteoblast lineage. Receptor expression is greater in more differentiated cells such as mature osteoblasts on the trabecular, endosteal, and periosteal surfaces (Fermor and Skerry, 1995; Lee *et al.*, 1993) and osteocytes (Fermor and Skerry, 1995; van der Plas *et al.*, 1994). PTH1R are also expressed in marrow stromal cells near the bone surface (Amizuka *et al.*, 1996), a putatively preosteoblast cell population that had been shown previously to bind radiolabeled PTH (Rouleau *et al.*, 1988, 1990). However, PTH1R is virtually absent in STRO-1+ positive, alkaline phosphatase negative marrow stromal cells (Gronthos *et al.*, 1999; Stewart *et al.*, 1999), which are thought to represent relatively early osteoblast precursors. Indeed, PTH1R expression can be induced by the differentiation of stromal cells, MC3T3 cells, or C3H10T1/2 cells with dexamethasone, ascorbic acid, or bone morphogenetic proteins (Feuerbach *et al.*, 1997; Hicok *et al.*, 1998; Liang *et al.*, 1999; Stewart *et al.*, 1999; Wang *et al.*, 1999; Yamaguchi *et al.*, 1987). Other data suggest that PTH receptors are limited to a relatively mature population of osteoprogenitor cells that express the osteocalcin gene (Bos *et al.*, 1996).

Whether receptors for PTH or PTHrP are expressed on the osteoclast is controversial. Initial studies using receptor radioautography failed to demonstrate them (Rouleau *et al.*, 1990; Silve *et al.*, 1982), and other studies have not identified PTH1R mRNA or protein on mature osteoclasts (Amizuka *et al.*, 1996; Lee *et al.*, 1993, 1995). However, PTH1R are reportedly present on normal human osteoclasts (Dempster *et al.*, 2005) and from patients with renal failure (Langub *et al.*, 2001) and relatively low-affinity binding of radiolabeled PTH peptides to osteoclasts or preosteoclasts has been reported (Teti *et al.*, 1991). The functional importance of such putative receptors is unclear.

Both PTH and PTHrP have additional receptors besides the PTH1R. The PTH2R is a G protein-coupled receptor closely related to the PTH1R (Usdin *et al.*, 1995), which recognizes the amino-terminal domain of PTH but not of PTHrP (see Chapter 24). The endogenous ligand for the PTH2R is likely to be tuberoinfundibular peptide of 39 amino acids (TIP39). The PTH2R and TIP39 are expressed predominantly in brain, vasculature, and pancreas (Dobolyi *et al.*, 2003; Eichinger *et al.*, 2002; Usdin *et al.*, 1996., 1999) but have yet to be demonstrated in adult bone, and at present no evidence is available for a direct role of PTH2R and TIP39 on bone metabolism.

A large body of evidence exists for the presence of specific receptor(s) for carboxyl-terminal PTH peptides on osteoblasts (Divieti *et al.*, 2005; Inomata *et al.*, 1995; Nguyen-Yamamoto *et al.*, 2001) and osteocytes (Divieti *et al.*, 2001) and evidence for actions of carboxyl-terminal PTH peptides on bone has been presented (Murray *et al.*, 1989, 1991; Nakamoto *et al.*, 1993; Sutherland *et al.*, 1994).

PTHrP is expressed and secreted by osteoblast-like osteosarcoma cells (Rodan *et al.*, 1989; Suda *et al.*, 1996) and by rat long-bone explants *in vitro* (Bergmann *et al.*, 1990). Messenger RNA for PTHrP is detected in periosteal cells of fetal rat bones (Karmali *et al.*, 1992). *In situ* hybridization and immunohistochemistry localized PTHrP mRNA and protein to mature osteoblasts on the bone surface of fetal and adult bones from mice and rats (Amizuka *et al.*, 1996; Lee *et al.*, 1995) and to flattened bone lining cells and some superficial osteocytes (Amizuka *et al.*, 1996) in postnatal mice. In addition, the PTHrP gene is expressed in preosteoblast cells in culture, and in some studies its expression is reduced as preosteoblasts undergo differentiation (Kartsogiannis *et al.*, 1997; Oyajobi *et al.*, 1999; Suda *et al.*, 1996).

As discussed in Chapter 25, PTHrP is cleaved to produce a set of peptides: those that contain the amino terminus [such as PTHrP(1–36)] activate the PTH1R, and additional peptides representing the midregion and carboxyl terminus of PTHrP appear to have distinct biological actions mediated by their own receptors (Philbrick *et al.*, 1996; Wysolmerski and Stewart, 1998). Receptors that are specific for amino-terminal PTHrP and do not recognize PTH have been identified in brain (Yamamoto *et al.*, 1997) and other tissues (Gaich *et al.*, 1993; Orloff *et al.*, 1992; Valin *et al.*, 2001), and midregion peptides of PTHrP have actions on placental calcium transport that imply a distinct receptor (Care *et al.*, 1990; Kovacs *et al.*, 1996), but there is presently no evidence for either receptor in bone. Carboxyl-terminal PTHrP fragments [e.g., PTHrP(107–139)] are reported to inhibit bone resorption (Cornish *et al.*,1997; Fenton *et al.*, 1991a, 1991b, 1993) and stimulate (Goltzman and Mitchell, 1985) or inhibit (Martinez *et al.*, 1997) the growth of osteoblasts and their function (Esbrit *et al.*, 2000; Grey *et al.*, 1999), and it is thus likely that a specific receptor for this peptide is present on osteoblasts, and conceivably also on osteoclasts.

EFFECTS OF PTH AND PTHrP ON BONE CELLS

Molecular mechanisms of action in osteoblasts

Transcription Factors

A major effect of PTH and PTHrP in osteoblasts is directed to modulating the expression and/or function of a number of transcription factors known to be important in bone metabolism. Among these, the most prominent are the cAMP response element-binding protein CREB (Brindle and Montminy, 1992; Papavassiliou, 1994), the immediate early gene of the activator protein-1 (AP-1) family *c-fos* (c-fos, fra-1, fra-2) and *c-jun* (c-jun, junD) (Clohisy *et al.*, 1992; Lee *et al.*, 1994; McCauley *et al.*, 1997., 2001; Stanislaus *et al.*, 2000), and Runx2 (Komori, 2002).

PTH induces *c-fos* transcription in a fashion that does not require protein synthesis and is mediated by the transcription factor CREB. Binding and phosphorylation of CREB to cAMP responsive elements (CRE) within the c-fos promoter (Evans *et al.*, 1996; Pearman *et al.*, 1996; Tyson *et al.*, 1999) are required to activate transcription. These events are stimulated by PTH through its ability to activate PKA, whereas the PKC signaling pathway is not involved in this response (Evans *et al.*, 1996; McCauley *et al.*, 1997). Activation of AP-1 transcription factors is important for osteoblast function because the *collagenase-3* (MMP-13) promoter contains AP-1 binding sites (Pendas *et al.*, 1997; Selvamurugan *et al.*, 1998).

One member of the runt-domain transcription factor family, namely Runx2 (also called cbfa1 and OSF2), is a specific osteoblast transcriptional activator and is required for determination of the osteoblast phenotype (Ducy *et al.*, 1997; Komori, 2002). Runx2 stimulates transcription of a number of key osteoblastic genes such as osteocalcin, osteopontin, collagenase-3, and collagen α1 and α2 (Banerjee *et al.*,2001; Porte *et al.*, 1999; Selvamurugan *et al.*, 1998). The importance of Runx2 for osteoblast formation and bone metabolism has been established in a number of *in vitro* and *in vivo* models (Ducy *et al.*, 1997, 1999; Komori *et al.*, 1997; Otto *et al.*, 1997). Short PTH treatment stimulates rapid and transient increases in Runx2 mRNA and protein both in osteoblastic cell cultures and in mice (Krishnan *et al.*,2003). In addition, PTH, likely via cAMP/PKA activation, stimulates Runx2 activity (Selvamurugan *et al.*, 2000; Winchester *et al.*, 2000). In contrast, long-term treatment with PTH reduces Runx2 levels (Bellido *et al.*, 2003). The dual effect of PTH on Runx2 levels and activity is particularly interesting. As will be discussed later, PTH promotes osteoblast survival in part by increasing Runx2 activity and, consequently, inducing expression of the survival gene *Bcl2*. However, PTH also induces proteasomal degradation of Runx2 though a mechanisms involving Smurf2. Therefore, the ability of PTH either to increase or decrease Runx2 levels in osteoblasts in a fashion dependent on the administration schedule may provide one of the possible (and likely many) mechanisms to explain the complex effect of PTH on bone formation and resorption.

Osterix is a zinc finger domain transcription factor expressed specifically in osteoblasts (Nakashima *et al.*, 2002). Its function is necessary for full osteoblastic differentiation and maturation (Nakashima *et al.*, 2002), and studies in cells (Nishio *et al.*, 2006) and genetically modified mice (Nakashima *et al.*, 2002) established that osterix lies downstream of Runx2. Similar to Runx2, PTH rapidly stimulates expression of osterix both in cell cultures (Wang *et al.*, 2006) and *in vivo* (Tanaka *et al.*, 2004).

Finally, PTH injection stimulates rapid and transient increases in the expression of at least three members of the nerve growth factor-inducible factor B (NR4A; NGFI-B) family of orphan nuclear receptors: Nurr1, Nur77, and NOR-1 (Pirih *et al.*, 2005). Although the functional significance of these molecules is largely undetermined, Nurr1 mediates PTH-stimulated activation of the *osteopontin* (Lammi *et al.*, 2004) and *osteocalcin* (Nervina *et al.*, 2006; Pirih *et al.*, 2004) promoters.

Growth Factors and Cytokines

Insulin-like Growth Factors

PTH stimulates synthesis and secretion of insulin-like growth factors (IGF), IGF-I and IGF-II, in bone. Both IGF-I and IGF-II are expressed in rat (McCarthy *et al.*, 1989) and mice (Linkhart and Mohan, 1989; Watson *et al.*, 1995). The PTH-dependent increase of IGF requires activation of the cAMP/PKA pathway because its effects are mimicked by cAMP analogues or agents that increase cAMP, but not by stimulation of calcium signals or PKC (McCarthy et al., 1990).

IGF-I exerts both proliferative and antiapoptotic actions in osteoblasts (Grey *et al.*, 2003) and a number of observations support the requirement of IGF-I as a mediator of the anabolic action of PTH. First, treatment of intact rats with PTH under conditions where it has an anabolic effect on bone leads to an increase in IGF-I mRNA (Watson *et al.*, 1995) and the bone matrix content of IGF-I. Second, daily PTH injection failed to stimulate bone formation in mice lacking IGF-I (Bikle *et al.*, 2002). Third, a similar absence of an anabolic effect by PTH was observed in mice null for the insulin receptor substrate-1 (IRS-1) (Yamaguchi *et al.*, 2005), the key intracellular mediator of IGF-I receptor signaling.

Additionally, both PTH and PTHrP affect the secretion of IGF-binding proteins (IGFBP), in particular, IGFBP-4 (LaTour *et al.*, 1990) and IGFBP-5 (Conover *et al.*, 1993) in bone. The role of IGFBP on IGF function is complex, because it can exert both inhibitory (IGFBP-4) and stimulatory (IGFBP-5) effects on IGF action in a cell- and context-specific manner. Moreover, some IGFBP have been proposed to have effects independent of IGF (Conover, 2008). Thus, the role of IGFBP on PTH action remains mostly to be defined.

Fibroblast Growth Factor-2 (FGF-2)

Like IGF-I, FGF-2 is a potent mitogen (Ling *et al.*, 2006), induces differentiation (Woei Ng et al., 2007) and exerts antiapoptotic effects on osteoblasts (Chaudhary and Hruska, 2001; Debiais *et al.*, 2004). PTH rapidly increases FGF-2 and FGF receptors in both primary and clonal osteoblastic cells (Hurley *et al.*, 1999). Stimulation of bone formation by PTH is impaired in mice null for FGF-2 (Hurley *et al.*, 2006) and bone marrow cultures from these mice produce fewer osteoclasts in response to PTH (Okada *et al.*, 2003). Collectively, these observations provide evidence that FGF-2 participates in the skeletal actions of PTH in vivo.

Amphiregulin

Recent studies demonstrated an important role for amphiregulin (AR), a member of the epidermal growth factor (EGF) family, as a mediator of PTH action. PTH stimulates AR expression in osteoblastic cells in a cAMP/PKA-dependent manner (Qin and Partridge, 2005). The increase in AR mRNA is mediated by phosphorylation of CREB and binding to a conserved CRE site in the AR promoter. AR appears to be important for bone metabolism because mice lacking AR have decreased trabecular bone (Qin *et al.*, 2005). In addition, AR stimulates rapid increases in *c-fos* and *c-jun* expression (Qin *et al.*, 2005). Treatment of primary calvarial cultures in vitro with AR stimulated cell proliferation and concomitant inhibition of osteoblastic differentiation, suggesting a role for AR on preosteoblastic mesenchymal stem cells (Qin and Partridge, 2005; Qin *et al.*, 2005). It is also notable that PTH stimulates the release heparin-bound EGF, which belongs to the same family of membrane bound and releasable EGF receptor agonists as AR, by activation of ADAM proteins in osteoblasts (Ahmed *et al.*, 2003). This process leads to the activation of EGF receptor in an auto- and paracrine fashion. It is therefore likely that PTH may not only induce expression of AR in osteoblasts but also its release via a similar mechanism.

Transforming Growth Factor-β

All three TGF-β isoforms are detected in bone, with TGF-β1 being the most abundant (Hering *et al.*, 2001; Seyedin *et al.*, 1985). TGF-β1 is secreted in by bone cells and is stored in the extracellular matrix. TGF-β is a potent mitogen for osteoprogenitors (Hock *et al.*, 1990) and has varied effects on differentiation and mineralization. Intermittent PTH treatment of rats increases the bone matrix content of TGF-β1 (Pfeilschifter *et al.*, 1995; Watson *et al.*, 1995) and the expression of TGF-β in osteoblasts (Oursler *et al.*, 1991; Pfeilschifter and Mundy, 1987; Wu and Kumar, 2000). Moreover, PTH and PTHrP increase the secretion of TGF-β by osteoblast-like bone cells (Wu and Kumar, 2000), TGF-β1 activity (Pfeilschifter and Mundy, 1987; Sowa *et al.*, 2003), and the release of TGF-β from bone matrix. Collectively, these observations suggest the possibility that TGF-β may contribute to the anabolic effects of intermittent PTH administration.

RANK Ligand and Osteoprotegerin

The regulation of these molecules by PTH and PTHrP is now recognized as the most prominent mechanism linking PTH1R-mediated actions in osteoblasts (and possibly stromal cells) and osteoclastogenesis. RANK ligand (RANKL)[4] is a member of the TNF family of cytokines that is expressed on the osteoblast and stromal cell surface. Its receptor, called receptor activator of NFkB (RANK), is expressed in osteoclast precursors and mature osteoclasts, and its activation by RANKL stimulates osteoclast formation, activity, and survival (Burgess *et al.*, 1999; Fuller *et al.*, 1998; Hofbauer *et al.*, 2000; Lacey *et al.*, 1998). A second component of this system, expressed and secreted by osteoblasts, is osteoprotegerin (OPG) (Simonet *et al.*, 1997; Yasuda *et al.*, 1998), a released protein that binds RANKL and prevents its actions on RANK. Therefore, it is the balance between RANKL and OPG (in other words, the ratio RANKL:OPG) that ultimately determines the extent of osteoclast formation and function.

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[4] It is also called osteoprotegerin ligand (OPGL), osteoclast differentiation factor (ODF), and TNF-related activation-induced cytokine (TRANCE).

Both PTH and PTHrP regulate the expression of RANKL and OPG. It is interesting to note that the frequency of administration of PTH affects the ratio RANKL: OPG differently. Thus, prolonged exposure to PTH in rats causes sustained stimulation of RANKL expression while inhibiting OPG expression (Huang *et al.*, 2004; Kondo *et al.*, 2002; Lee and Lorenzo, 1999; Ma *et al.*, 2001)—with the final effect being an overall stimulation of osteoclast number and resorptive capacity. In contrast, intermittent PTH treatment affected RANKL and OPG only transiently (Ma *et al.*, 2001). Exposure to PTH increases the expression of RANKL in murine bone marrow cultures, cultured osteoblasts, and mouse calvariae (Hofbauer *et al.*, 2000; Lee and Lorenzo, 1999; Tsukii *et al.*, 1998), and simultaneously decreases the expression of OPG (Lee and Lorenzo, 1999). In addition, studies using primary cultures indicate that the effect of PTH on RANKL expression is more pronounced in differentiated osteoblasts, whereas the inhibition of OPG occurs at all differentiation stages (Huang *et al.*, 2004). Notably, the stimulation of osteoclastic differentiation by RANKL requires the presence of macrophage-colony-stimulating factor (M-CSF), which is also upregulated by PTH (Horowitz *et al.*, 1989; Weir *et al.*, 1989).

Strong evidence supports the key role of the RANK/RANKL/OPG system in the activation of bone resorption upon prolonged exposure to PTH and PTHrP. Stimulation of osteoclastogenesis by PTH is blocked by antibodies to RANKL (Tsukii *et al.*, 1998) or by OPG (Lacey *et al.*, 1998; Yasuda *et al.*, 1998) and OPG inhibits the hypercalcemic response to PTH or PTHrP (Morony *et al.*, 1999; Oyajobi *et al.*, 2001).

Wnt/β-catenin/sclerostin

The Wnt/β-catenin signaling pathway is recognized as an important regulator of bone mass. This is a complex signaling system that comprises a number of members. Canonical Wnt signaling is mediated by a receptor complex formed by a Frizzled (a seven transmembrane domain receptor) and a coreceptor lipoprotein-receptor-related proteins 5 or 6 (LRP5/6). Upon engagement by Wnt, this complex activates the cytoplasmic protein dishevelled (Dsh), followed by the accumulation of unphosphorylated β-catenin. As a result, β-catenin translocates to the nucleus and regulates gene transcription. Moreover, cells secrete proteins (such as DKK-1) that interact with the coreceptors LRP5/6 and inhibit Wnt signaling (Baron and Rawadi, 2007). All the key elements of this pathway are expressed in bone cells and in osteoblastic cultures and are regulated by PTH (Kulkarni *et al.*, 2005). Treatment of rats with PTH increased Frizzled-1 and β-catenin levels and decreased DKK-1, with a resultant activation of Wnt responses (Kulkarni *et al.*, 2005). Sclerostin, the product of the Sost gene, is also a secreted Wnt inhibitor and binds LRP5 and LRP6 (Li *et al.*, 2005; Semenov *et al.*, 2005), and its level in osteocytes is dramatically reduced by continuous PTH treatment (Bellido, 2006; Bellido *et al.*, 2005). It is interesting that sclerostin appears to inhibit osteoblast differentiation (van Bezooijen *et al.*, 2004, 2007), thereby providing a functional link between osteocytes and osteoblasts. The profound effects of the Wnt signaling complex on bone metabolism are well described by a number of genetic studies, both in human and in animal models (Baron and Rawadi, 2007), and the observation that PTH engages this pathway in bone cells is indeed of great interest.

Adaptor Proteins

G protein-Coupled Receptor Kinase 2 and β-Arrestins

These two ubiquitously expressed proteins play important roles in the regulation of several G protein-coupled receptors, including the PTH1R. It is not surprising that alterations in their expression or activity impact the function of osteoblasts and their responsiveness to PTH. G protein-coupled receptor kinase 2 (GRK2) phosphorylates agonist-occupied PTH1R and promotes the binding of β-arrestins (Dicker *et al.*, 1999; Ferrari *et al.*, 1999; Flannery and Spurney, 2001; Vilardaga *et al.*, 2001). These combined actions result in decreased signaling and receptor desensitization (Bisello *et al.*, 2002; Dicker *et al.*, 1999; Ferrari and Bisello, 2001). Targeted overexpression of GRK-2 in osteoblasts promotes bone loss (Wang *et al.*, 2005). In contrast, inhibition of GRK2 activity by expression of a dominant negative mutant increases PTH-stimulated cAMP, and mice overexpressing this molecule have increased bone remodeling with a net gain in bone content (Spurney *et al.*, 2002). Similarly, intermittent PTH increased the number of osteoblasts in mice null for β-arrestin2 (Ferrari *et al.*, 2005). However, no net increase in bone mass was observed, likely because of the intense stimulation of osteoclastogenesis. Another regulator of G protein-coupled receptor signaling, the regulator of G protein signaling-2 (RGS2), which increases the rate of hydrolysis of GTP bound to G proteins, thereby terminating signaling, has also been implicated in PTH1R actions in bone cells. RGS2 mRNA is rapidly and transiently increased by PTH in rat bones, as well as in osteoblast cultures (Miles *et al.*, 2000) and its expression in bone cells decreased PTH-stimulated cAMP production (Thirunavukkarasu *et al.*, 2002). Interestingly, RGS2 upregulation was also observed in cells overexpressing

Runx2 (Thirunavukkarasu *et al.*, 2002), suggesting the possibility that mechanisms limiting PTH1R signaling by G proteins may be activated upon differentiation of cells along the osteoblastic lineage.

Effects on Gap Junctions

PTH increases intercellular communication of bone cells by increasing connexin-43 gene expression (Schiller *et al.*, 1992) and opening gap junctions (Donahue *et al.*, 1995; Schiller *et al.*, 1992). The reduction of connexin-43 levels by transfection of antisense cDNA markedly inhibited the cAMP response to PTH (Vander Molen *et al.*, 1996) and blocked the effect of PTH on mineralization by osteoblast-like cells (Schiller *et al.*, 2001). These effects appear to be significant to the anabolic action of PTH because increases in bone mineral content in response to intermittent PTH administration were significantly decreased in mice with targeted deletion of connexin-43 in osteoblasts (Chung *et al.*, 2006).

Effects on Bone Matrix Proteins and Alkaline Phosphatase

PTH regulates the expression of a number of bone matrix proteins, including type I collagen, osteocalcin, osteopontin, bone sialoprotein, osteonectin, and alkaline phosphatase. In most cases, these genes (with the exception of osteocalcin) are downregulated by continuous exposure to PTH (Wang *et al.*, 2005), whereas intermittent administration of PTH has inhibitory or stimulatory effects in different genes.

Type I collagen is the most abundant bone matrix protein. PTH and PTHrP acutely inhibit collagen synthesis *in vitro* (Kream *et al.*, 1980, 1986; Partridge *et al.*, 1989; Pines *et al.*, 1990). Similar inhibition is observed upon infusion of PTH in humans (Simon *et al.*, 1988). However, anabolic PTH treatment can stimulate type I collagen expression (Canalis *et al.*, 1990; Opas *et al.*, 2000), an effect that is likely mediated by increases in IGF-I (Canalis *et al.*, 1989). Intermittent treatment of mouse bone marrow cells with PTH modestly increased collagen expression (Locklin *et al.*, 2003). Similarly, PTH treatment inhibits osteopontin (Noda *et al.*, 1988) and osteonectin (Termine *et al.*, 1981) expression.

The osteocalcin gene is also importantly regulated by PTH (Noda *et al.*, 1988; Towler and Rodan, 1995; Yu and Chandrasekhar, 1997). However, in contrast to the effect on type I collagen, expression of osteocalcin is stimulated by chronic administration of PTH or PTHrP, whereas the acute effect of these hormones is inhibitory (Gundberg *et al.*, 1995).

Finally, the effect of PTH on the expression of bone sialoprotein can be either stimulatory (Ogata *et al.*, 2000; Yang and Gerstenfeld, 1997) or inhibitory (Ma *et al.*, 2001; Wang *et al.*, 2000).

The reported effects of PTH on the expression of alkaline phosphatase are inconsistent. PTH can either stimulate or inhibit secretion of alkaline phosphatase from bone cells (Jongen *et al.*, 1993; Kano *et al.*, 1994; Majeska and Rodan, 1982; McPartlin *et al.*, 1978; Yee, 1985) and may not be particularly indicative of PTH-specific actions. Indeed, although anabolic therapy with PTH generally increases the circulating levels of alkaline phosphatase (Finkelstein *et al.*, 1998), this effect is likely caused by an increase in osteoblast number rather than an increase in protein expression.

Effects on Bone Proteases

PTH stimulates the secretion of a number of matrix metalloproteases (MMPs) in bone cells (see Chapter 16) that are involved in bone remodeling. These include collagenase-3 (MMP-13) (Partridge *et al.*, 1987; Quinn *et al.*, 1990; Scott *et al.*, 1992; Winchester *et al.*, 1999 , 2000), stromelysin-1 (MMP-3) (Meikle *et al.*, 1992), gelatinase B (Meikle *et al.*, 1992), and the disintegrin and metalloprotease with thrombospondin repeats ADAMTS-1 (Miles *et al.*, 2000). Bone proteases, in particular collagenase-3 and gelatinase B, partially mediate the stimulation of bone resorption by PTH (Witty *et al.*, 1996). As described earlier, stimulation of the collagenase-3 promoter by PTH requires the combined action of AP-1 transcription factors and Runx2, effects that are mediated by cAMP-dependent activation of CREB (Porte *et al.*, 1999; Selvamurugan *et al.*, 1998, 2000). All of these events are stimulated by PTH. PTH treatment also increases secretion of the tissue inhibitor of matrix metalloproteins TIMP-1 by osteoblasts (Meikle *et al.*, 1992). This is relevant to the action of PTH because mice overexpressing TIMP-1 in osteoblasts responded to intermittent PTH with increases in bone mineral density higher than those in normal mice (Merciris *et al.*, 2007). This was also accompanied by decreased osteoclastic differentiation (Geoffroy *et al.*, 2004; Merciris *et al.*, 2007).

Effects of PTH and PTHrP on Bone Cell Proliferation

Continuous exposure to relatively high concentrations of PTH(1–34) or PTHrP(1–34) inhibits proliferation of virtually every osteoblastic cell line, including UMR 106-01, MC3T3-E1, SaOS-2, and calvarial primary cultures (Civitelli *et al.*, 1990; Kano *et al.*, 1991; Onishi and Hruska, 1997; Qin *et al.*, 2005). This effect is mediated by changes in the expression levels of several components of the cell cycle, ultimately resulting in arresting cells in the G_1 phase. PTH treatment decreases expression of cyclin D1, while increasing the levels of $p21^{Cip1}$ and $p27^{Kipl}$ (Onishi and Hruska, 1997; Qin *et al.*, 2005), with evident cell cycle arrest.

In contrast, some *in vitro* studies demonstrated that in certain circumstances PTH stimulates the proliferation of osteoblastic cells (Finkelman *et al.*, 1992; Onishi *et al.*, 1997; Somjen *et al.*, 1990). In particular, very low concentrations of PTH (Swarthout *et al.*, 2001) and brief exposure to PTH (Scutt *et al.*, 1994) resulted in increased cell proliferation. In the preosteoblast cell line TE-85, PTH stimulated proliferation by increasing expression of the cyclin-dependent kinase cdc2 (Onishi *et al.*, 1997).

The effects of PTH and PTHrP on the cell cycle in osteoblastic cells have important consequences *in vivo*. Several studies indicate that intermittent injections of PTH increase the number of osteoblasts (Kostenuik *et al.*, 1999; Nishida *et al.*, 1994) and this contributes to the stimulation of bone formation. However, this effect does not appear to be related to a direct stimulation of osteoblast mitogenesis. Indeed, although intermittent PTH administration in rats greatly increased osteoblast number and function, osteoblast proliferation was not detected (Dobnig and Turner, 1995; Onyia *et al.*, 1995, 1997). It is possible that the increase in osteoblast number produced by intermittent treatment with PTH is caused by the activation of bone lining cells to osteoblasts (Dobnig and Turner, 1995), a process that does not require mitosis. These data are compatible with the conclusion that PTH inhibits cell cycle progression of committed osteoprogenitors, thereby permitting their maturation.

Effects of PTH and PTHrP on Bone Cell Differentiation

PTH and PTHrP have a profound influence on the differentiation program of bone marrow cells to form osteoblasts. Several studies indicate that anabolic administration of PTH stimulates rapid changes in histomorphometry and gene expression, which have been interpreted as resulting from cell differentiation (Hodsman and Steer, 1993; Onyia *et al.*, 1995). The effects of PTH and PTHrP on cell differentiation in culture are also well documented and appear to depend on both the duration and frequency of PTH exposure. Early, transient PTH treatment enhances the commitment of progenitor cells and increases osteoblast differentiation (Wang *et al.*, 2007). *In vitro*, primary osteoblasts briefly (1 hour every 48 hours) exposed to PTH showed inhibited expression of alkaline phosphatase activity and bone nodule formation (Ishizuya *et al.*, 1997). In contrast, intermittent PTH treatment for 6 hours every 2 days stimulated osteoblastic differentiation and formation of mineralized nodules (Ishizuya *et al.*, 1997). Similarly, transient PTH treatment of calvarial osteoblasts inhibited the initial osteoblast differentiation but ultimately resulted in increased mineralized nodules and osteoblastic differentiation (Wang *et al.*, 2007). In cultured murine marrow cells, intermittent PTH treatment increases the expression of osteoblast differentiation markers (such as Runx2, alkaline phosphatase, and type I collagen) (Locklin *et al.*, 2003). In all cases, however, continuous exposure to PTH strongly inhibits osteoblast differentiation (Ishizuya *et al.*, 1997; Wang *et al.*, 2007). In this respect, it is interesting to note that in humoral hypercalcemia of malignancy (HHM), PTHrP is continuously secreted by tumors and results in the virtual absence of mature osteoblasts (Stewart *et al.*, 1982). In contrast, elevated PTH levels observed in hyperparathyroidism do not have the same effect: indeed, hyperparathyroidism is characterized by increased bone remodeling (i.e., higher formation and resorption) with complex effects on bone (Stewart *et al.*, 1982). Although the mechanisms of this difference remain to be fully elucidated, one possible underlying basis is the pulsatile secretion of PTH by the parathyroids in hyperparathyroidism versus the continuous, unregulated secretion of PTHrP in HHM.

Effects of PTH and PTHrP on Bone Cells: Survival

Cell culture studies show that PTH rapidly stimulates transcription of the prosurvival gene *Bcl-2*, while increasing the inactivation of the apoptotic protein Bad (Bellido *et al.*, 2003; Jilka *et al.*, 1998), suggesting that activation of the PTH1R in osteoblasts may inhibit apoptosis. Indeed, various studies showed that PTH reduces the apoptotic effects of etoposide, dexamethasone, and serum deprivation in osteoblastic cultures (Bellido *et al.*, 2003; Chen *et al.*, 2002; Jilka *et al.*, 1998). The effect of PTH on the expression of survival proteins appears to require the action of CREB and Runx2 (Bellido *et al.*, 2003). Considering the disparate effects of PTH on Runx2 stability discussed previously, it is therefore possible that the timing and frequency of PTH stimulation may be a key determinant for its prosurvival action. Thus, although some studies in mice demonstrated a significant decrease in apoptotic osteoblasts following daily PTH administration (Bellido *et al.*, 2003; Jilka *et al.*, 1999), a study in rats reported an actual increase in apoptotic osteoblasts by intermittent PTH (Stanislaus *et al.*, 2000). An additional complexity results from the observation that daily administration of PTH in postmenopausal women resulted in increased osteoblast apoptosis (Lindsay *et al.*, 2007). Finally, it should be noted that differences have been observed in the effect of PTH on osteoblast apoptosis at different skeletal sites (cortical and trabecular) and possibly between primary and secondary spongiosa (Jilka *et al.*, 1999; Stanislaus *et al.*, 2000).

EFFECTS OF PTH AND PTHrP ON BONE

Bone Resorption

Cellular Basis of PTH Action

PTH and PTHrP increase bone resorption by stimulating both osteoclastogenesis and activating the mature osteoclast. These effects require the participation of classical PTH target cells, including stromal cells and osteoblasts (Akatsu *et al.*, 1989; McSheehy and Chambers, 1986). Moreover, as described earlier, osteoclasts do not express high-affinity PTH1R (Amizuka *et al.*, 1996; Rouleau *et al.*, 1990; Silve *et al.*, 1982). The identification of the central role of the RANK/RANKL system in osteoclast formation and function, and the appreciation that PTH and PTHrP affect the expression of RANKL and OPG in stromal cells and osteoblasts, provides a molecular basis for understanding PTH-stimulated bone resorption. The precise target cell in the osteoblast lineage responsible for mediating the bone-resorbing effects of PTH and PTHrP, if any, has not been identified, but various marrow stromal cell lines suffice *in vitro* (Aubin and Bonnelye, 2000) and bone resorption is still active when mature osteoblasts have been ablated (Corral *et al.*, 1998). By binding to its cognate receptor, RANK, on osteoclast precursors and mature osteoclasts, RANKL stimulates both osteoclastogenesis and the activity of mature osteoclasts. Therefore, the increased expression of RANKL mediated both the acute and chronic actions of PTH on bone resorption. The major difference in the two effects is likely related to the stronger suppression of the decoy receptor OPG upon prolonged exposure to PTH (Huang *et al.*, 2004). Osteoclast activation by RANKL is apparently responsible for both bone resorption at the cellular level and for hypercalcemia, because both are blocked by the decoy receptor OPG (Morony *et al.*, 1999; Yamamoto *et al.*, 1998).

The bone-resorbing effects of amino-terminal PTH and PTHrP are essentially indistinguishable when studied using isolated osteoclasts (Evely *et al.*, 1991; Murrills *et al.*, 1990), bone explant systems (Raisz *et al.*, 1990; Yates *et al.*, 1988), or infusion into the intact animal (Kitazawa *et al.*, 1991; Thompson *et al.*, 1988). In contrast, PTHrP is considerably less potent than PTH in inducing hypercalcemia in humans (Horwitz *et al.*, 2006). This difference is most likely because of lower induction of renal $1,25(OH)_2D_3$ by PTHrP compared with PTH (Horwitz *et al.*, 2003). Notably, studies in humans showed that intermittent administration of PTHrP(1–36) over 3 months stimulated bone formation without attendant increases in markers of bone resorption (Horwitz *et al.*, 2003).

As discussed in Chapter 3, PTHrP is a polyhormone, the precursor of multiple biologically active peptides. Carboxy-terminal peptides that are predicted to arise from cleavage of PTHrP in the polybasic region PTHrP(102–106) have been synthesized and shown to inhibit bone resorption in several explant systems (Fenton *et al.*, 1991b, 1993) and *in vivo* (Cornish *et al.*, 1997). On this basis, the minimal peptide that inhibits bone resorption, PTHrP(107–111), has been identified and called osteostatin.

EFFECTS OF PTH AND PTHrP ON BONE

Bone Formation

The mechanisms by which PTH increases bone formation are complex. As described previously, PTH and PTH exert a variety of effects on osteoblasts. The increase in bone formation in response to intermittent administration of PTH and PTHrP correlates with marked increases in the number of active osteoblasts (Boyce *et al.*, 1996; Dempster *et al.*, 1999; Shen *et al.*, 1993).

It is evident from the preceding discussion that every aspect of the osteoblast existence is affected by PTH, and all of these cellular actions may contribute to the increase in osteoblast number observed in the stimulation of bone formation. First, activation of the PTH1R produces various actions on the cell cycle of both the osteoblasts and their precursors. It is clear that in most circumstances PTH and PTHrP cause cell cycle arrest in osteoblasts and pre-osteoblasts and this may be a prerequisite to induce further differentiation and activation. Most *in vivo* evidence does not support a direct proliferative effect of PTH on mature osteoblasts (Dobnig and Turner, 1995; Onyia *et al.*, 1995, 1997). However, PTH and PTHrP increase expression and release of a number of potent mitogens (such as IGF-I, TGFβ, and amphiregulin), which may act in a paracrine fashion to expand the pool of osteoprogenitors (Grey *et al.*, 2003; Hock *et al.*, 1990; Qin *et al.*, 2005). Second, intermittent PTH administration stimulates transcription factors, such as Runx2 and osterix, which in turn stimulate differentiation. This effect is accompanied by increases in osteoblastic differentiation markers (Hodsman and Steer, 1993; Onyia *et al.*, 1995; Wang *et al.*, 2007). Third, some of the rapid hystomorphometric changes observed upon anabolic administration of PTH may derive from activation of bone lining cells to become active osteoblasts (Dobnig and Turner, 1995). Fourth, intermittent PTH administration exerts antiapoptotic actions in osteoblasts and osteocytes (Bellido *et al.*, 2003; Jilka *et al.*, 1999). Obviously, prolonging the life span of osteoblasts would contribute to increasing the number of mature osteoblasts. It is interesting to observe that Runx2, a key molecule mediating PTH effects on osteoblast survival, is also involved in the stimulation of osteoblastic differentiation.

The relative contribution of each of these mechanisms to the anabolic action of PTH has not been completely established. It seems likely that the remarkable increases in bone formation in response to intermittent administration of PTH and PTHrP arise from a combination of these effects. Moreover, it is possible that PTH and PTHrP may not have the same effect under all circumstances and in the presence of other treatments affecting bone metabolism.

Finally, it has long been thought that the anabolic effects of PTH and PTHrP are substantially equivalent because most of their cellular effects *in vitro* and activities in animal models are quite similar. However, it has recently become apparent that some basic differences exist in the action of these two hormones in humans. Intermittent administration of PTHrP(1–36) for 2 weeks (Plotkin *et al.*, 1998) and 3 months (Horwitz *et al.*, 2003), for instance, leads to increases in biochemical markers of bone formation without changing markers of bone resorption, suggesting that PTHrP(1–36) may uncouple bone formation and resorption.

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Chapter 33

Physiological Actions of Parathyroid Hormone (PTH) and PTH-Related Protein

Epidermal, Mammary, Reproductive, Pancreatic Tissues

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INTRODUCTION

Documentation of the skeletal abnormalities in mice that either overexpressed parathyroid-related protein (PTHrP) in their skeletons or had the genes for PTHrP and the PTH receptor ablated by the techniques of homologous recombination provided an exciting impetus for the rapid accumulation of knowledge regarding the mechanisms by which PTHrP regulates bone and cartilage development and physiology. These findings are reviewed in Chapters 13 and 15. Since the mid-1990s, increasing evidence has accumulated that PTHrP and the PTH/PTHrP receptor family also contribute to the development and functioning of several nonskeletal organs. Data regarding the action of PTHrP in the vascular system and the central nervous system are reviewed in Chapter 16. In this, the last of the series of chapters on the physiological actions of PTHrP, we review data regarding the function(s) of PTHrP in several other nonskeletal sites. We first consider the functions of PTHrP in skin. Next, we review its functions in the mammary gland, placenta, and other reproductive tissues. Finally, we examine its role in the endocrine pancreas.

SKIN

PTHrP and PTHrP Receptor Expression

Normal human keratinocytes were the first nonmalignant cells shown to produce PTHrP (Merendino *et al.,* 1986), and multiple studies have confirmed that rodent and human keratinocytes in tissue culture express the PTHrP gene and secrete bioactive PTHrP (reviewed in Phlbrick *et al.,* 1996). PTHrP expression has also been examined in skin *in vivo* by using both immunohistochemistry and *in situ* hybridization. During fetal development in rats and mice, PTHrP is expressed principally within the epithelial cells of developing hair follicles (Karmali *et al.,* 1992; Lee *et al.,* 1995). In mature skin, the PTHrP gene is also expressed most prominently within hair follicles, and mRNA levels appear to vary with the hair cycle. Cho and colleagues (2003) found that PTHrP expression increased within the outer root sheath and isthmus of late anagen hair follicles in mice. During catagen and telogen, PTHrP transcripts were abundant within the isthmus, but expression was downregulated during early anagen (Cho *et al.,* 2003). In addition to hair follicles, low levels of PTHrP expression may also be found throughout the interfollicular epidermis from the basal layer to the granular layer. Some studies have suggested that PTHrP is more highly expressed in the superbasal keratinocytes (Danks *et al.,* 1989; Hayman *et al.,* 1989), although not all studies have reported this pattern (Atillasoy *et al.,* 1991; Grone *et al.,* 1994). A variety of factors have been reported to regulate PTHrP production by cultured keratinocytes (see Philbrick *et al.,* 1996, for review). For example, glucocorticoids and 1,25-$(OH)_2D$ have been shown to suppress PTHrP production, whereas fetal bovine serum, matrigel, and an as-yet-unidentified factor(s) secreted from cultured fibroblasts have been shown to enhance PTHrP production. The upregulation of PTHrP production by fibroblast-conditioned media is particularly interesting, as PTHrP, in turn, acts back on dermal fibroblasts, suggesting that it may function in a short regulatory loop

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between keratinocytes and dermal fibroblasts (Shin *et al.,* 1997; Blomme *et al.,* 1999a). Finally, *in vivo,* PTHrP expression has been shown to be upregulated at the margins of healing wounds in guinea pigs (Blomme *et al.,* 1999b). Interestingly, in this study, PTHrP was also detected in myofibroblasts and macrophages, suggesting that keratinocytes may not be the only source of PTHrP in skin.

The general consensus has been that keratinocytes do not express the type I PTH/PTHrP receptor (PTH1R), but dermal fibroblasts do (Hanafin *et al.,* 1995; Orloff *et al.,* 1995). PTHrP has been shown to bind to skin fibroblasts and to elicit biochemical and biological responses in these cells (Shin *et al.,* 1997; Blomme *et al.,* 1999a; Wu *et al.,* 1987). In addition, studies utilizing *in situ* hybridization in fetal skin have demonstrated that PTH1R mRNA is absent from the epidermis, yet abundant in the dermis, especially in those cells adjacent to the keratinocytes (Karmali *et al.,* 1992; Lee *et al.,* 1995; Dunbar *et al.,* 1999a). There are fewer data concerning the expression patterns of the PTH1R in more mature skin, but, in mice, it appears that the relative amount of PTH1R mRNA in dermal fibroblasts is reduced in adult compared with fetal skin (Cho *et al*., 2003). As with PTHrP, there also appears to be a hair-cycle-dependent variation in expression of the PTH1R gene in the connective tissue adjacent to the isthmus of the hair follicles. However, unlike PTHrP mRNA, PTH1R mRNA appears to be most plentiful during early anagen (Cho *et al*., 2003). In addition to fibroblasts, recent studies using sensitive PCR-based detection methods have also reported expression of the PTH1R in keratinocytes, although these studies have used cultured cells (Errazahi *et al.,* 2003, 2004). Furthermore, studies have shown that cultured keratinocytes bind and respond to PTHrP by inducing calcium transients, suggesting the presence of PTHrP receptors, either the classical PTH1R or nonclassical, alternative PTHrP receptors (Orloff *et al.,* 1992, 1995). However, to date, no such alternative receptors have been isolated, so their existence remains uncertain. Furthermore, it is not clear whether the PTH1R is expressed on keratinocytes *in vivo*. Therefore, although the possibility of both paracrine and autocrine signaling exists, no functional studies address the relative importance of either pathway to the biology of PTHrP in skin.

Biochemistry of PTHrP

As described in Chapters 3 and 4, during transcription, the PTHrP gene undergoes alternative splicing to generate multiple mRNAs, which in human cells give rise to three main protein isoforms. In addition, each of these isoforms is subject to post-translational processing to generate a variety of peptides of varying length. Human keratinocytes have been shown to contain mRNA encoding for each of the three main isoforms, although, as in other systems, no clearly defined or unique role has yet emerged for any of the three individual isoforms (Philbrick *et al.,* 1996). Keratinocytes have also been shown to process full-length PTHrP into a variety of smaller peptides, including PTHrP(1–36) and a midregion fragment beginning at amino acid 38 (Soifer *et al.,* 1992). These cells have also been shown to secrete a large (≈10 kDa) amino-terminal form that is glycosylated (Wu *et al.,* 1991). There is currently no specific information regarding the secretion of COOH-terminal peptides of PTHrP in skin, but keratinocytes are also likely to produce these peptides.

Function of PTHrP

Several studies suggest that PTHrP is involved in the regulation of hair growth. As noted earlier, the PTHrP gene in embryonic skin is expressed most prominently in developing hair follicles, and overexpression of PTHrP in the basal keratinocytes of skin in transgenic mice leads to a severe inhibition of ventral hair follicle morphogenesis during fetal development (Wysolmerski *et al.,* 1994). This appears to be the result of an interaction between PTHrP and BMP signaling that normally is involved in the regulation of Msx2 expression, the patterning of the mammary mesenchyme and the lateral inhibition of hair follicle development around the nipple (Hens *et al*., 2007) (see "Mammary Gland"). However, it is unlikely that PTHrP is critical to hair follicle morphogenesis elsewhere because disruption of the PTHrP or PTH1R genes does not seem to affect hair follicle formation or patterning in mice except for the vicinity of the nipple (Karaplis *et al.,* 1994; Lansk *et al.,* 1996; Foley *et al.,* 1998).

It has also been suggested that PTHrP may participate in the regulation of the hair cycle. Systemic or topical administration of PTH1R antagonists to mice appears to perturb the hair cycle by prematurely terminating telogen, prolonging anagen growth, and inhibiting catagen (Schilli *et al.,* 1997; Safer *et al.,* 2007). In a reciprocal fashion, mice overexpressing PTHrP in their skin (K14-PTHrP mice) demonstrate a delayed emergence of dorsal hair and have shorter hairs, and their hair follicles enter catagen approximately 2 days early (Cho *et al.,* 2003; Diamond *et al.,* 2006). These findings were associated with a lower proliferation rate in the hair matrix and a less well-developed perifollicular vasculature during anagen. Together, these studies imply that PTHrP may regulate the anagen to catagen transition, acting to inhibit hair follicle growth by pushing growing hair follicles into the growth-arrested or catagen/telogen phase of the hair cycle. If this hypothesis were correct, one would expect PTHrP knockout mice to exhibit findings similar to PTH1R antagonist-treated mice. However, this does not appear to be the case. In mice that lack PTHrP in their skin, the hair cycle appears to be normal (Foley *et al.,* 1998). In fact, rather than a promotion of hair growth, these mice demonstrate a thinning of their coat over time. These conflicting results are difficult to rationalize at this point, but suggest that although PTHrP may contribute to the regulation of the hair cycle, it is unlikely to be necessary for this process to unfold normally.

PTHrP has also been implicated in the regulation of keratinocyte proliferation and/or differentiation. Data from studies *in vitro* have suggested that PTHrP promotes the differentiation of keratinocytes (reviewed in Philbrick *et al.,* 1996), but studies *in vivo* have suggested that PTHrP inhibits keratinocyte differentiation (Foley *et al.,* 1998). A careful comparison of the histology of PTHrP-null and PTHrP-overexpressing skin demonstrated reciprocal changes. In the absence of PTHrP, it appeared that keratinocyte differentiation was accelerated, whereas in skin exposed to PTHrP overexpression, keratinocyte differentiation appeared to be retarded (Foley *et al.,* 1998). Therefore, in a physiological context, PTHrP appears to slow the rate of keratinocyte differentiation and to preserve the proliferative, basal compartment. Remarkably, these changes in the rate of keratinocyte differentiation are exactly analogous to those noted for chondrocyte differentiation in the growth plates of mice overexpressing PTHrP compared with PTHrP- and PTH1R-null mice (Philbrick *et al.,* 1996) (see Chapter 15). Again, at present, it is difficult to rationalize conflicting data regarding the effects of PTHrP on keratinocyte differentiation, but studies in genetically altered mice indicate that PTHrP participates in the complex regulation of these processes *in vivo*. Further research will be needed to understand its exact role.

As alluded to previously, an important but still unresolved question is whether the effects of PTHrP on keratinocyte proliferation, differentiation, and hair follicle growth are the result of its effects on keratinocytes directly or via its effects on dermal fibroblasts. At present there are more data to support the paracrine possibility. PTH1R is expressed on dermal fibroblasts *in vivo* and in culture (Lee *et al.,* 1995; Hanafin *et al.,* 1995). Dermal fibroblasts have been demonstrated to show biochemical and biological responses to PTHrP (Shin *et al.,* 1997; Blomme *et al.,* 1999a; Wu *et al.,* 1987, Thomson *et al.*, 2003). Furthermore, PTHrP has been shown to induce changes in growth factor and extracellular matrix production that could, in turn, lead to changes in keratinocyte proliferation and/or differentiation and hair follicle growth (Shin *et al.,* 1997; Blomme *et al.,* 1999a; Insogna *et al.,* 1989). Of course, the autocrine and paracrine signaling pathways are not mutually exclusive, but any direct autocrine effects of PTHrP on keratinocytes would require the presence of PTHrP receptors. An alternative possibility by which PTHrP might have cell autonomous effects on keratinocytes is via an intracrine pathway involving its translocation to the nucleus (Philbrick *et al.,* 1996). Clearly, much research is needed to define the receptors and signaling pathways by which PTHrP acts in skin. Only when this information is available will we be able to understand the mechanisms leading to the skin phenotypes that have been observed in the various transgenic models discussed earlier.

Pathophysiology of PTHrP

To date, PTHrP has not been clearly implicated in any diseases of the skin. It has been suggested that skin and skin appendage findings in the rescued PTHrP-null mice are reminiscent of a series of disorders collectively known as the ectodermal dysplasias (Foley *et al.,* 1998), but PTHrP has not been formally linked to any of these diseases. It has also been noted that psoriatic skin may downregulate PTHrP expression and a small trial of topically administered PTH suggested that stimulation of the PTH1R might improve the histological abnormalities in psoriatic plaques (Hollick *et al.,* 2003). However, these potentially exciting results will need to be verified in larger trials. Preliminary studies also suggest that topical application of PTH1R antagonists may be useful for stimulating hair growth, although there are no data to suggest that PTHrP is involved in the pathogenesis of alopecia(s) (Safer *et al.,* 2007). The most common tumors causing humoral hypercalcemia of malignancy (HHM) are those of squamous histology, but these tumors rarely arise from skin keratinocytes. In fact, the most common skin tumors, basal cell carcinomas, do not overexpress PTHrP and are not associated with hypercalcemia (Philbrick *et al.,* 1996). Although PTHrP appears to participate in the normal physiology of the skin, it is not clear at this juncture if it will be involved in skin pathophysiology.

MAMMARY GLAND

PTHrP was reported to be expressed in mammary tissue and to be secreted into milk very soon after its discovery (Thiede and Rodan, 1988; Budayr *et al.,* 1989). It is now appreciated that PTHrP is critically important for the proper development and functioning of the mammary gland throughout life. In addition, it has been implicated as an important modulator of the biological behavior of breast cancer. The mammary gland develops in several discrete stages and only reaches its fully differentiated state during pregnancy and lactation. PTHrP appears to serve different functions during these different stages of mammary development; therefore, we will organize our discussion around three principal stages of mammary development: embryonic development, adolescent growth, and pregnancy and lactation. For each stage, we will first outline the pertinent developmental events in rodents, as data regarding the function(s) of PTHrP largely come from studies in mice and rats. Next, we will discuss the localization of PTHrP and PTHrP receptors and the regulation of the expression of PTHrP and its receptors. Finally, we will address the function of PTHrP.

Embryonic Mammary Development

In mice, there are two phases of embryonic mammary development. The first involves the formation of five pairs of mammary buds, each of which consists of a bulb-shaped collection of epithelial cells surrounded by several layers of fibroblasts known as the mammary mesenchyme (Sakakura, 1987). After the formation of these buds, mouse mammary development displays a characteristic pattern of sexual dimorphism. In male embryos, in response to androgens, the mammary mesenchyme destroys the epithelial bud and male mice are left without mammary glands or nipples (Sakakura, 1987). In female embryos, however, the mammary buds remain quiescent until embryonic day 16 (E16) when they undergo a transition into the second step of embryonic development, the formation of the rudimentary ductal tree. This process involves the elongation of the mammary bud, its penetration out of the dermis and into a specialized stromal compartment known as the mammary fat pad, and the initiation of ductal branching morphogenesis. At the time of birth, the gland consists of a simple epithelial ductal tree consisting of 15–20 branched tubes within a fatty stroma (Sakakura, 1987). This initial pattern persists until puberty at which time the mature virgin gland is formed through a second round of branching morphogenesis, regulated by circulating hormones (discussed later).

The PTHrP gene is expressed exclusively within epithelial cells of the mammary bud, soon after it begins to form. PTHrP mRNA continues to be localized to mammary epithelial cells during the initial round of branching morphogenesis, as the bud grows out into the presumptive mammary fat pad and begins to branch (Dunbar *et al.,* 1998, 1999a; Wysolmerski *et al.,* 1998). At some point after birth, PTHrP gene expression is downregulated, and in the adult virgin gland, PTHrP mRNA is found only within specific portions of the duct system (Dunbar *et al.,* 1998). In contrast to the PTHrP gene, the PTH1R gene appears to be expressed within the mesenchyme, but its expression is widespread and is not limited to the developing mammary structures. Transcripts for the PTH1R gene are found within the mammary mesenchyme but also throughout the developing dermis (Dunbar *et al.,* 1999a; Wysolmerski *et al.,* 1998). It is not clear when the receptor gene is first expressed within the subepidermal mesenchyme. However, it is already present when the mammary bud begins to form and it continues to be expressed within fibroblasts surrounding the mammary ducts as they begin to extend and branch (Wysolmerski *et al.,* 1998; Dunbar *et al.,* 1998).

Epithelial expression of PTHrP and mesenchymal expression of the PTH1R are not unique to the developing mammary gland, and this pattern has long led to speculation that PTHrP and its receptor might contribute to the regulation of epithelial–mesenchymal interactions during organogenesis. There is now solid evidence that this is the case during embryonic mammary development, where PTHrP acts as an epithelial signal that influences cell fate decisions within the developing mammary mesenchyme. Data supporting this notion come from studies in several genetically altered mouse models. First, in PTHrP or PTH1R knockout mice, there is a failure of the normal androgen-mediated destruction of the mammary bud owing to the failure of the mammary mesenchyme to differentiate properly and to express androgen receptors (Dunbar *et al.,* 1999a). Second, in PTHrP or PTH1R knockout mice, the mammary buds fail to grow out into the fat pad and initiate branching morphogenesis, again due to defects in the mammary mesenchyme (Wysolmerski *et al.,* 1998; Dunbar *et al.,* 1998). Finally, in keratin 14 (K14)-PTHrP transgenic mice that ectopically overexpress PTHrP within all the basal keratinocytes of the developing embryo, subepidermal mesenchymal cells, which should acquire a dermal fate, instead react to the excess PTHrP by becoming mammary mesenchyme (Dunbar *et al.,* 1999a).

As demonstrated by these studies, PTHrP signaling is essential for mammary gland formation in rodents. When the mammary gland begins to form, the PTH1R is expressed in all of the mesenchymal cells underneath the epidermis, but PTHrP is expressed only within the mammary epithelial buds and not within the epidermis itself (Karmali *et al.,* 1992; Thiede and Rodan, 1988; Wysolmerski *et al.,* 1998). As the mammary bud grows down into the mesenchyme, PTHrP, produced by mammary epithelial cells, interacts over short distances with the PTH1R on the immature mesenchymal cells closest to the epithelial bud and triggers these cells to differentiate into mammary mesenchyme. Recent studies have demonstrated that PTHrP accomplishes this, at least in part, by upregulating expression of BMP receptors on the mesenchymal cells and sensitizing them to respond in an autocrine fashion to BMP4 expressed in the ventral surface of the embryo (Hens *et al.,* 2007). In this way, PTHrP acts as a patterning molecule contributing to the formation of small patches of mammary-specific stroma around the mammary buds and within the surrounding sea of presumptive dermis (Fig. 1; Foley *et al.,* 2001). The process of differentiation set in motion by PTHrP signaling is critical to the ability of the mammary-specific stroma to direct further morphogenesis of the epithelium and to inhibit hair follicle development in the vicinity of the developing nipple. In the absence of this signaling, the mesenchyme can neither destroy the epithelial bud in response to androgens nor trigger the outgrowth of the bud and the initiation of branching morphogenesis (Dunbar *et al.,* 1998, 1999a; Wysolmerski *et al.,* 1998).

Although the model described earlier was developed from studies in mice, PTHrP is also critical to the formation of breast tissues in human fetuses. Blomstrand's chondrodysplasia is a fatal form of dwarfism caused by null mutations of the PTH1R gene (Jobert *et al.,* 1998) (see Chapter 44). Affected fetuses have skeletal abnormalities similar to those caused by deletion of the PTHrP and PTH1R genes in mice (see Chapter 15) and, in addition, lack breast tissue or nipples (Wysolmerski *et al.,* 1999). In normal human fetuses, the PTHrP gene is expressed within the mammary epithelial bud, and the PTH1R gene

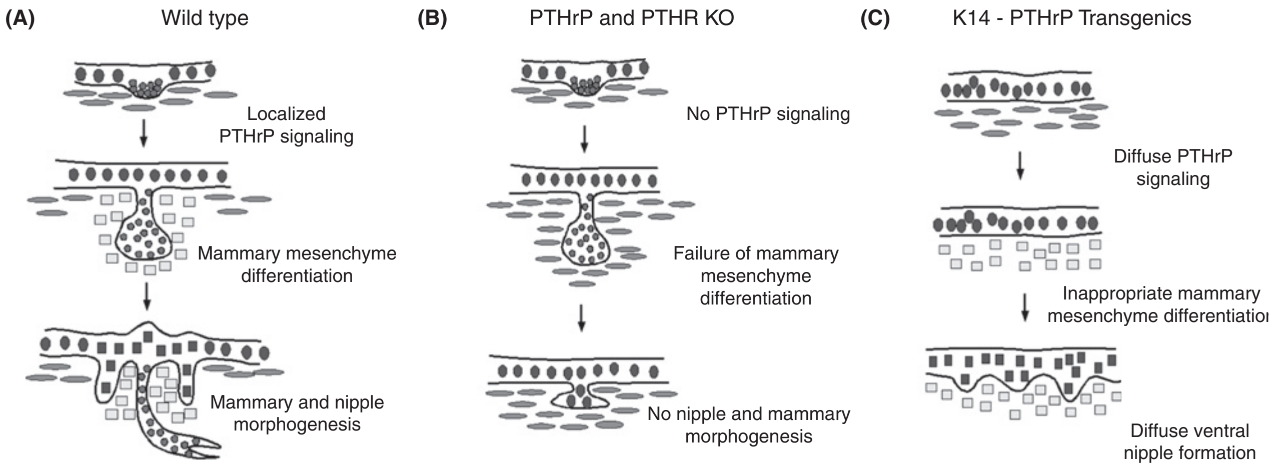
(A)
Wild type
Localized PTHrP signaling
Mammary mesenchyme differentiation
Mammary and nipple morphogenesis
(B)
PTHrP and PTHR KO
No PTHrP signaling
Failure of mammary mesenchyme differentiation
No nipple and mammary morphogenesis
(C)
K14 - PTHrP Transgenics
Diffuse PTHrP signaling
Inappropriate mammary mesenchyme differentiatioi
Diffuse ventral nipple formation

FIGURE 1 Model for the regulation of cell fate by PTHrP signaling during mammary gland and nipple development. **(A)** Normally, the mammary epithelial cells (small circles) express PTHrP after the bud starts to form. PTHrP signals to the dermal mesenchyme (ovals) near the developing bud and, as a result, these cells become mammary mesenchyme (light squares). The mammary mesenchyme maintains the mammary fate of the epithelial cells, triggers their morphogenesis, and induces the overlying epidermis (upright ovals) to become the nipple (dark squares). **(B)** In the absence of PTHrP signaling no mammary mesenchyme is formed. Therefore, the mammary epithelial cells revert to an epidermal fate, no morphogenesis occurs and the nipple does not form. **(C)** In the presence of diffuse PTHrP signaling, the entire ventral dermis becomes mammary mesenchyme and the ventral epidermis becomes nipple sheath. (Reproduced from Foley *et al.,* 2001, with permission.)

is expressed in surrounding mesenchyme (Wysolmerski *et al.,* 1999, Cormier *et al.*, 2003). Therefore, in humans, as in mice, epithelial-to-mesenchymal PTHrP to PTH1R signaling is essential to the formation of the embryonic mammary gland.

Adolescent Mammary Development

After birth, the murine mammary gland undergoes little development until the onset of puberty. At that point, in response to hormonal changes, the distal ends of the mammary ducts form specialized structures called terminal end buds. These structures serve as sites of cellular proliferation and differentiation during a period of active growth that gives rise to the typical branched duct system of the mature virgin gland (Daniel and Silberstein, 1987). Once formed, the ductal tree remains relatively unchanged until pregnancy when another round of hormonal stimulation induces the formation of the alveolar units that produce milk.

Similar to findings in the embryonic mammary gland, during puberty PTHrP is a product of mammary epithelial cells and the PTH1R is expressed in stromal cells (Dunbar *et al.,* 1998). However, the structure of the pubertal gland is more complex than that of the embryonic gland and, here, there are conflicting data regarding the localization of PTHrP and the PTH receptor. Although there is general agreement that PTHrP is expressed in epithelial cells in the postnatal gland, there is some disagreement regarding the specific epithelial compartments in which PTHrP is found. Studies employing *in situ* hybridization in mice have suggested that, after birth, the overall levels of PTHrP gene expression in mammary ducts are reduced except for in the terminal end buds during puberty (Dunbar *et al.,* 1998). In these structures, appreciable amounts of PTHrP mRNA were detected in the peripherally located cap cells. In other parts of the gland there was little, if any, specific hybridization for PTHrP. In contrast, studies looking at mature human and canine mammary glands using immunohistochemical techniques have suggested that PTHrP can be found in both luminal epithelial and myoepithelial cells throughout the ducts (Grone *et al.,* 1994; Liapis *et al.,* 1993). Furthermore, studies using cultured cells have suggested that PTHrP is produced by luminal and myoepithelial cells isolated from normal glands (Ferrari *et al.,* 1992; Seitz *et al.,* 1993; Wojcik *et al.,* 1999). There have been fewer reports looking at the localization of PTH1R expression in the postnatal mammary gland, but as in embryological development, it is expressed in the mammary stroma (Dunbar *et al.,* 1998). *In situ* hybridization studies have found the highest concentration of PTH1R mRNA in the stroma immediately surrounding terminal end buds during puberty (Dunbar *et al.,* 1998). This same study found lower levels of PTH1R mRNA distributed generally within the fat pad stroma, but very little expression in the dense stroma surrounding the more mature ducts. In addition, these investigators found no evidence of PTH1R mRNA in freshly isolated epithelial cells (Dunbar *et al.,* 1998). In contrast, other studies have suggested that PTH1R is expressed in cultured luminal epithelial and myoepithelial cells (Seitz *et al.,* 1993; Wojcik *et al.,* 1999), as well as in cultured breast cancer cell lines (Birch *et al.,* 1995). In summary, during puberty, PTHrP and its receptor are found predominantly within the terminal end buds, with PTHrP localized to the epithelium and PTH1R localized in the stroma. It remains an open and interesting question whether, at some time during mammary ductal development, epithelial cells express low levels of PTH1R.

Studies in transgenic mice have suggested that PTHrP regulates mammary morphogenesis during puberty. Overexpression of PTHrP in mammary epithelial cells using the K14 promoter results in an impairment of ductal branching morphogenesis (Wysolmerski *et al.,* 1995). There are two aspects to the defect. First, the terminal end buds advance through the mammary fat pad at a significantly slower rate than normal. Second, there is a severe reduction in the branching complexity of the ductal tree. As seen in Fig. 2, this results in a spare and stunted epithelial duct system. Experiments altering the timing and duration of PTHrP overexpression in the mammary gland using a tetracycline-regulated K14-PTHrP transgene have demonstrated that the two aspects of this pubertal phenotype appear to represent separate functions of PTHrP. The branching (or patterning) defect results from embryonic overexpression of PTHrP, whereas the ductal elongation defect is a function of overexpression of PTHrP during puberty (Dunbar *et al.,* 2001). These effects on ductal patterning provide further evidence of the importance of PTHrP as a regulator of embryonic mammary development. In addition, the localization patterns for PTHrP and PTH1R during puberty, combined with the effects of pubertal overexpression of PTHrP on ductal growth, suggest that PTHrP also functions later in mammary development. During puberty it appears to modulate epithelial–mesenchymal interactions that govern ductal elongation.

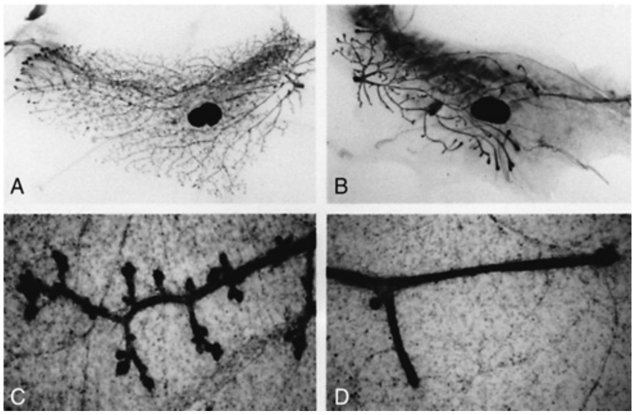

FIGURE 2 Overexpression of PTHrP in the mammary gland of K14-PTHrP transgenic mice antagonizes ductal elongation and branching morphogenesis during puberty. **A** and **B** represent typical whole-mount analyses of the fourth inguinal mammary glands from wild-type **(A)** and K14-PTHrP transgenic mice **(B)** at 6 weeks of age. The dark oval in the center of each gland is a lymph node. Growth of the ducts during puberty is directional and each gland is arranged so that the primary duct (the origin of the duct system) is toward the center of the figure. Note that overexpression of PTHrP results in an impairment of the elongation of the ducts through the fat pad as well as a dramatic reduction of the branching complexity of the ductal tree. **C** and **D** represent higher magnifications of a portion of the ducts from the wild-type **(C)** and transgenic **(D)** glands demonstrating the reduction in side branching caused by overexpression of PTHrP. (Modified from Wysolmerski *et al.,* 1995 with permission.)

Pregnancy and Lactation

Mammary epithelial cells only reach their fully differentiated state during lactation. Under hormonal stimulation during pregnancy, there is a massive wave of epithelial proliferation and morphogenesis that gives rise to multiple terminal ductules and alveolar units. During the later stages of pregnancy, the epithelial cells fully differentiate and then begin to secrete milk during lactation. By the time lactation commences, the fatty stroma of the mammary gland is almost completely replaced by actively secreting alveoli. Upon the completion of lactation, there is widespread apoptosis of the differentiated epithelial cells and the gland remodels itself into a duct system similar to that of the virgin animal (Daniel and Silberstein, 1987).

Localization studies in humans, rodents, and cows have all noted epithelial cells to be the source of PTHrP in the mammary gland during pregnancy and lactation (Liapis *et al.,* 1993; Wojcik *et al.,* 1998, 1999; Rakopoulos *et al.,* 1990). Based on the assessment of whole-gland RNA, PTHrP expression appears to be upregulated at the start of lactation under the control of both local and systemic factors (Philbrick *et al.,* 1996; Thiede and Rodan, 1988; Thiede, 1989; Thompson *et al.,* 1994; Buch *et al.,* 1992). Thiede and Rodan (1988) and Thiede (1989) originally reported that PTHrP expression in rats is dependent on suckling and on serum prolactin concentrations. However, prolactin must serve only as a permissive factor, for additional studies have shown that the suckling response is a local one and that PTHrP only rises in the milked gland (Thompson *et al.,* 1994). Furthermore, overall PTHrP expression increases gradually over the course of lactation, and in later stages, its expression becomes independent of serum prolactin levels (Bucht *et al.,* 1992). It is clear that much of the PTHrP made during lactation ends up in milk, in which levels of PTHrP are up to 10,000-fold higher than in the circulation of normal individuals and 1000-fold higher than in patients suffering from humoral hypercalcemia of malignancy (Philbrick *et al.,* 1996). PTHrP concentrations in milk have generally been found to mirror RNA levels in the gland, increasing over the duration of lactation and rising acutely with suckling (Thompson *et al.,* 1994; Yamamoto *et al.,* 1992a; Law *et al.,* 1991; Goff *et al.,* 1991). In addition, evidence shows that PTHrP levels may vary with the calcium content of milk (Yamamoto *et al.,* 1992a; Law *et al.,* 1991; Goff *et al.,* 1991; Vemura *et al.,* 1997; Kovacs and Kronenberg, 1997). In mice, the calcium-sensing receptor is expressed on the basolateral surface of mammary epithelial cells during lactation and regulates PTHrP production, such that increased delivery of calcium to the mammary gland decreases PTHrP production and secretion into milk

(VanHouten *et al.,* 2004; Ardeshirpour *et al.,* 2006). Finally, in mice, PTHrP mRNA levels are promptly downregulated during the early stages of involution and then increase to prelactation levels about a week into the remodeling process (M. Dunbar and J. J. Wysolmerski, unpublished data).

In contrast to PTHrP, there has been little study of the expression or regulation of PTHrP receptors during pregnancy and lactation. In early pregnancy, the PTH/PTHrP receptor is expressed at low levels in the stroma surrounding the developing alveolar units (Dunbar *et al.,* 1998). Studies using whole-gland RNA demonstrate a reciprocal relationship between PTH1R and PTHrP mRNA levels. That is, as PTHrP expression rises during lactation, PTH1R mRNA levels decrease, and as PTHrP mRNA levels fall during early involution, PTH1R expression increases to its former level (M. Dunbar and J. J. Wysolmerski, unpublished data). This may represent active downregulation of the receptor by PTHrP or may simply reflect the changing amount of stroma within the gland at these different stages. However, in a study of cells isolated from lactating rats, it was suggested that epithelial cells, as well as stromal cells, express this receptor (Wojcik *et al.,* 1999), so the regulation of PTH1R expression during pregnancy and lactation may be complex.

Initial reports of the presence of PTHrP in the mammary gland and in milk prompted a great deal of speculation regarding its functions in breast tissue during lactation. These proposals revolved around four general hypotheses: (1) PTHrP may be involved in maternal calcium homeostasis and the mobilization of calcium from the maternal skeleton; (2) PTHrP may be involved in regulating vascular and/or myoepithelial tone in the lactating mammary gland; (3) PTHrP may be involved in transepithelial calcium transport into milk; and/or (4) PTHrP may be involved in neonatal calcium homeostasis or neonatal gut physiology. The function of PTHrP in the lactating mammary gland in mice was addressed by disrupting the PTHrP gene solely in mammary epithelial cells during lactation (VanHouten *et al.,* 2003). This experiment has supplied evidence to support the role of PTHrP in regulating maternal and neonatal calcium and bone metabolism and has shown that PTHrP is not directly involved in mediating calcium transport into milk.

Milk production requires a great deal of calcium, and providing an adequate supply to the mammary gland stresses maternal bone and mineral metabolism (Kovacs and Kronenberg, 1997). Some of the calcium required for milk comes from the diet, as calcium intake is increased owing to the orexigenic effects of suckling (Smith and Grove, 2002). In addition, renal reabsorption of calcium is increased during lactation so that urinary losses are reduced. Finally, a significant proportion of the calcium transported into milk is derived from the maternal skeleton. Overall rates of bone turnover are elevated during lactation, but bone resorption outstrips bone formation so that bone mass declines rapidly. The average nursing woman loses between 5 and 10% of her bone mass over 6 months, whereas rodents lose up to one-third of their skeletal mass over 21 days of lactation (VanHouten and Wysolmerski, 2003). Mobilization of skeletal calcium during lactation does not rely on either of the established calcium-regulating hormones, PTH or 1,25(OH)$_2$D (Kovacs and Kronenberg, 1997). Recent experiments have shown that bone resorption is increased because of the combination of decreased systemic estradiol concentrations and elevated circulating PTHrP levels. Suckling directly inhibits hypothalamic GnRH secretion and induces hypogonadotropic hypogonadism (Smith and Grove, 2002). Bone loss correlates with the duration of amenorrhea in nursing women and rates of bone resorption correlate inversely with estradiol levels in lactating mice. Furthermore, estrogen replacement in lactating mice reduces bone loss by 60% (VanHouten and Wysolmerski, 2003). Many studies have now documented elevated circulating PTHrP levels in lactating humans and rodents (Kovacs and Kronenberg, 1997), and PTHrP levels correlate directly with biochemical markers of bone resorption and inversely with bone mass in lactating mice (VanHouten and Wysolmerski, 2003). In addition, circulating PTHrP levels have been shown to correlate with bone density changes in lactating humans (Sowers *et al.,* 1996). When the PTHrP gene was disrupted specifically in the lactating mammary gland in mice, circulating PTHrP levels declined, milk PTHrP became unmeasurable, rates of bone resorption decreased, and bone loss was reduced by 50% (VanHouten *et al.*, 2003). Thus, it is now clear that the lactating mammary gland secretes PTHrP both into milk and into the circulation. Systemic PTHrP from the breast acts together with estrogen deficiency to stimulate bone resorption and cause bone loss during lactation. As noted previously, the production of PTHrP by the lactating breast is decreased by stimulation of the calcium-sensing receptor on mammary epithelial cells (VanHouten *et al.*, 2004). Thus, as shown in Fig. 3, a classic endocrine feedback loop is created between bone and breast during lactation and, in effect, the mammary gland functions as an "accessory" parathyroid gland that uses PTHrP instead of PTH to ensure an adequate flow of calcium from the maternal skeleton to make milk. If calcium delivery to the gland falls, mammary epithelial cells produce more PTHrP, which stimulates increased bone resorption. The delivery of more calcium to the mammary gland from the skeleton then stimulates the CaR and decreases PTHrP production.

Genetic removal of PTHrP from the mammary gland does not alter milk calcium levels and PTHrP-null mammary epithelial cells are able to transport calcium *in vitro* at the same rate as wild-type mammary epithelial cells (VanHouten *et al.*, 2003). Thus, unlike the placenta (see later), it does not appear that PTHrP contributes to the regulation of transepithelial calcium transport in the mammary gland. However, the function of PTHrP in milk remains an open question. There is a dose–responsive

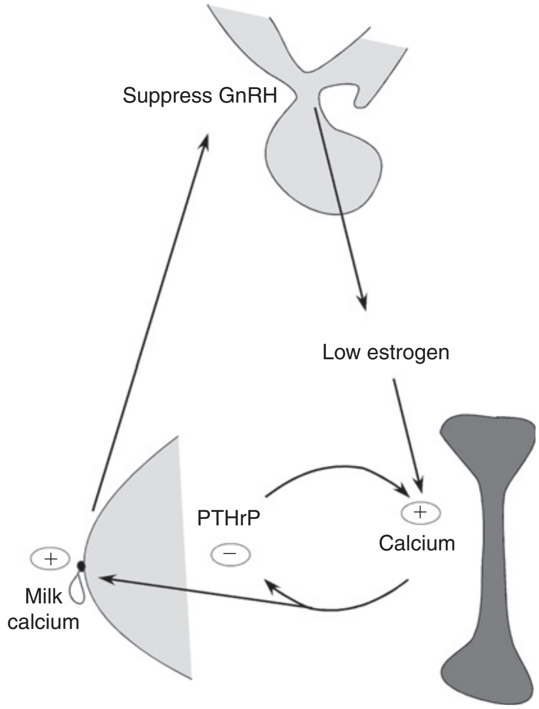

FIGURE 3 Interactions between breast, bone and brain during lactation. Suckling initiates neural reflexes that reduce GnRH secretion causing central hypogonadism. Low estrogen levels combine with PTHrP secreted from the breast to increase bone resorption. Calcium released from the skeleton feeds back to inhibit PTHrP secretion from breast cells.

nverse relationship between the milk PTHrP level and neonatal ash calcium content in mice (J. N. VanHouten and J. J. Wysolmerski, unpublished data). However, the mechanism(s) by which milk PTHrP modulates neonatal calcium and/or bone metabolism remains unexplored.

Another potential function of PTHrP during lactation is the regulation of vascular and/or myoepithelial cell tone. As discussed in Chapter 16, PTHrP has been shown to modulate smooth muscle cell tone in a variety of organs, including the vascular tree, where it acts as a vasodilator. Consistent with these effects, two studies have shown that PTHrP increases mammary blood flow during lactation (Davicco *et al.,* 1993; Thiede *et al.,* 1992). The injection of amino-terminal fragments of PTHrP into the mammary artery of dried ewes was shown to increase mammary blood flow and to override the vasoconstrictive effects of endothelin (Davicco *et al.,* 1993). Thiede and colleagues (1992) have demonstrated that the nutrient arteries of the inguinal mammary glands of rats secrete PTHrP and that its production is responsive to suckling and prolactin. Myoepithelial cells in the breast are similar, in some ways, to vascular smooth muscle cells and are thought to participate in the control of milk ejection by contracting in response to oxytocin (Daniel and Silberstein, 1987). Therefore, it is interesting that myoepithelial cells in culture have been shown to express PTH1R and to respond to PTHrP by elevating intracellular cAMP (Seitz *et al.,* 1993; Wojcik *et al.,* 1999). Furthermore, mirroring the effects of PTHrP on the endothelin-induced contraction of vascular smooth muscle, PTHrP has been shown to block the rise in intracellular calcium normally induced in response to oxytocin in myoepithelial cells (Seitz *et al.,* 1993).

Pathophysiology of PTHrP in the Mammary Gland

There are several instances in which PTHrP may contribute to pathophysiology in the human breast. First, as noted previously, fetuses afflicted with Blomstrand's chondrodystrophy lack nipples and breast tissue (Wysolmerski *et al.,* 1999). Second, some cases of lactational hypercalcemia have been reported to be associated with elevations in circulating levels of PTHrP (Khosla *et al.,* 1990; Reid *et al.,* 1992). One of these cases was caused by massive breast hyperplasia, and after reduction mammoplasty the patient's hypercalcemia and elevated PTHrP levels both resolved (Khosla *et al.,* 1990). Finally, the area with the greatest potential impact on human health is the relationship of PTHrP production to breast cancer. This is evolving into a complicated topic and will be addressed only briefly here. However, it will be reviewed in more depth in Chapter 43.

It is well documented that PTHrP is produced by a number of primary breast carcinomas and that this sometimes leads to classical humoral hypercalcemia of malignancy (Isales *et al.,* 1987). A potentially more widespread role may be the involvement of PTHrP in the osteotrophism of breast cancer (Guise *et al.,* 1996; Yin *et al.,* 1999). Animal models have suggested that PTHrP production by breast tumor cells is important to their ability to form skeletal metastases (Guise *et al.,* 1996; Yin *et al.,* 1999). However, there is conflicting evidence as to whether PTHrP production by a primary breast tumor is predictive of bone metastases in patients (Bundred *et al.,* 1996; Henderson *et al.,* 2001, 2006). The largest and most carefully controlled study suggested that PTHrP production by the primary tumor is actually a negative predictor, not a positive predictor, of skeletal metastases (Henderson *et al.,* 2006). It may be that PTHrP production does not enable a tumor cell to get into the skeleton, but once there, the ability of tumor cells to upregulate PTHrP production within the bone microenvironment becomes important to their ability to grow in the skeleton (Yin *et al.,* 1999). Several studies suggest that TGF-β released from the bone matrix during bone resorption may be particularly important in stimulating PTHrP production (Kakonen *et al.*, 2002). More PTHrP would, in turn, be expected to increase osteoclast activity and release more TGF-β, setting up a potential feed-forward vicious cycle that may be important in the pathogenesis of bone destruction around the metastatic tumor.

There is a growing literature that suggests that PTHrP may also be important in controlling breast cancer cell proliferation, apoptosis, migration, and/or invasion. Although it is unclear if normal mammary epithelial cells express significant levels of the PTH1R, it appears that many breast cancer cell lines do so (Birch *et al.,* 1995). Some of the effects ascribed to PTHrP appear to be mediated by this receptor, but some appear to be mediated by midregion PTHrP fragments acting either through intracrine mechanisms or perhaps via a specific midregion PTHrP cell-surface receptor (Shen *et al.*, 2004; Kumari *et al.*, 2006; Shen and Falzon, 2006; Dittmer *et al.*, 2006; Sirchia *et al.*, 2006). In either case, the effects of midregion PTHrP apparently require that this portion of the peptide enter the nucleus via a distinct bipartite nuclear localization signal. In particular, several studies have suggested that midregion PTHrP fragments may promote cell migration by regulating α6β4 integrin expression through nuclear effects (Shen *et al.*, 2003; Dittmer *et al.*, 2006; Shen and Falzon, 2006). Despite these intriguing findings, at this point it is not clear if these effects are artifacts of cell lines or if they will prove to be clinically relevant. Most of the studies in cell lines suggest that PTHrP expression by a breast tumor would contribute to increased aggressiveness or to a tendency to metastasize. However, in the large clinical study of Henderson and colleagues mentioned previously (Henderson *et al.,* 2006), PTHrP expression by a primary breast tumor was clearly a marker of a less aggressive course, correlating with estrogen and progesterone receptor positive status, better survival, lower rates of recurrence, and lower rates of all metastases, not just bone metastases. Similar results can be gleaned from gene expression surveys in breast cancer as well (www.oncomine.org; Rhodes *et al.,* 2004). Thus, it is difficult to reconcile these two lines of evidence and clarification will need to await experiments manipulating PTHrP expression in animal models of breast cancer *in vivo*.

REPRODUCTIVE TISSUES

PTHrP and Placental Calcium Transport

Nearly all of the calcium, and a large proportion of the inorganic phosphate (85%) and magnesium (70%), transferred from the mother to the fetus is associated with development and mineralization of the fetal skeleton (Grace *et al.,* 1986). The bulk of placental calcium transfer occurs rapidly over a short interval late in gestation, such that 80% occurs in the third trimester in humans (Givens and Macy, 1933), whereas 96% occurs in the last 5 days of gestation in the rat (Comar, 1956). The concentrations of both total and ionized Ca in all mammalian fetuses studied during late gestation have been observed to be higher than maternal levels. As a result of studies in which the sheep was used extensively for the study of fetal calcium control, one of the first suggested physiological roles of PTHrP was that of regulating the transport of calcium from mother to fetus in the mammal, thereby making calcium available to the growing fetal skeleton (Rodda *et al.,* 1988).

Immunoreactive PTH levels were found to be low in fetal lambs, whereas PTH-like biological activity in serum was high (Care *et al.,* 1985), suggesting the presence of another PTH-like substance. Similar findings of low PTH and high PTHrP have been demonstrated in multiple studies of fetal mice and human cord blood (Kovacs, 2003). Parathyroidectomy in the fetal lamb resulted in loss of the calcium gradient that exists between mother and fetus, as well as impairment of bone mineralization, implicating parathyroids as the source of the regulatory agent. Crude, partially purified, or recombinant PTHrP, but neither PTH nor maternal parathyroid extract that contained no immunoreactive PTHrP, restored the gradient (Rodda *et al.,* 1988). Thus, PTHrP appeared to be the active component of the fetal parathyroid glands responsible for maintaining fetal calcium levels and suppressing fetal PTH levels. In support of this hypothesis, immunoreactive PTHrP was found to be readily detectable in sheep fetal parathyroids from the time they form (MacIsaac *et al.,* 1991) and was also found in early placenta, suggesting that the latter tissue may be a source of PTHrP for calcium transport early in gestation.

The portion of PTHrP that appears to be responsible for regulating placental calcium transport lies between residues 67 and 86 (Care *et al.,* 1990), but the responsible receptor has not yet been identified. Although syncytiotrophoblasts are believed to be central in the transport of calcium to the fetus, cytotrophoblasts (which differentiate to form the syncytium) are believed to be the calcium-sensing cells, and raising the extracellular calcium concentration has been shown to inhibit PTHrP release from these cells (Hellman *et al.,* 1992). The calcium-sensing receptor (CaR) has been localized to cytotrophoblasts of human placenta (Bradbury *et al.,* 1997), and the work of Kovacs *et al.* (1998) has implicated it in placental calcium transport. Furthermore, a calreticulin-like, calcium-binding protein has been isolated from trophoblast cells and its expression is increased by treatment with PTHrP(67–84) but not with N-terminal PTHrP (Hershberger and Tuan, 1998).

Although these observations are strongly suggestive of involvement of PTHrP and the CaR, the mechanisms of placental calcium transport are still not fully understood. Support for the role of PTHrP also comes from the PTHrP gene knockout mouse in which placental calcium transport is severely impaired (Kovacs *et al.,* 1996). In mice homozygous for deletion of the PTHrP gene, fetal plasma calcium and maternal–fetal calcium gradient were significantly reduced. When fetuses were injected *in utero* with fragments of PTHrP or PTH, calcium transport was significantly restored only by treatment with a midmolecular region of

PTHrP that does not act via the PTH1R. Furthermore, in mice rendered null for the PTH1R gene, placental calcium transport was increased, and PTH1R null fetuses had plasma PTHrP levels more than 10 times higher than controls (Kovacs *et al.,* 2001). The circulating PTHrP in the fetal mice was found to be derived from several tissues, including liver and placenta, but the parathyroids were excluded as a source of PTHrP in this setting (Kovacs *et al.,* 2001). Additional studies confirmed that mice lacking parathyroid glands did not have an alteration in circulating PTHrP, nor was placental calcium transport reduced, although the absence of fetal PTH was associated with an even greater reversal of the maternal–fetal calcium gradient than that induced by loss of PTHrP (Kovacs *et al.,* 2001). These findings indicate that the fetal parathyroids may not be a source of circulating PTHrP, and are compatible with an earlier detailed examination of normal fetal rat parathyroids that found no detectable PTHrP by *in situ* hybridization, RT-PCR, and immunohistochemistry (Tucci *et al.,* 1996).

Thus, conclusions from the murine studies are similar in many respects to those in the sheep, namely that PTHrP contributes to fetal skeleton calcium supply by controlling maternal–fetal calcium transport through actions mediated by a midmolecule portion of the PTHrP molecule. The murine and sheep studies differ in that the parathyroids do not appear to be a dominant source of PTHrP in rodents; the placenta may be the relevant source of PTHrP that controls placental calcium transfer and the fetal–placental gradient.

Uterus and Extraembryonic Tissues

The uterus, both pregnant and nonpregnant, is another of the many sites of production and action of PTHrP. The relaxing effect of PTH on uterine smooth muscle had been long recognized (Shew *et al.,* 1984), and it was not surprising that PTHrP had the same effect (Shew *et al.,* 1991). The finding that expression of mRNA for PTHrP in the myometrium during late gestation in the rat was controlled by intrauterine occupancy by the fetoplacental unit raised the possibility of a role for PTHrP in regulating uterine muscle tone (Thiede *et al.,* 1990).

In studies in rats with or without estrogen treatment, protein and mRNA for PTHrP were localized not only in the myometrium, as had been shown in pregnancy (Thiede *et al.,* 1990), but also in the epithelial cells lining the endometrium and endometrial glands. Indeed, the strongest PTHrP production appeared to be in these sites (Paspaliaris *et al.,* 1992), suggesting that the endometrium and endometrial glands might be the major uterine site of PTHrP production and that PTHrP might be a local regulator of endometrial function and myometrial contractility. Estrogen treatment enhanced uterine production of PTHrP, but most significantly, the relaxing effect of PTHrP on uterine contractility *in vitro* was enhanced greatly by the pretreatment of noncycling rats with estrogen. In keeping with this observation, uterine horns from cycling rats in proestrous and estrous phases of the cycle showed a greater responsiveness to PTHrP than those from noncycling rats. These findings are consistent with a role for PTHrP as an autocrine and/or paracrine regulator of uterine motility and function. Furthermore they suggest that PTHrP belongs to a class of other locally acting peptides, such as oxytocin, vasoactive intestinal peptide, and relaxin, for which pretreatment of animals with estrogen increases the response of the uterus (Ottesen *et al.,* 1985; Mercado-Simmen *et al.,* 1982; Fuchs *et al.,* 1982).

Further evidence for a specific and regulated role of PTHrP in the uterus during gestation comes from the observation of a temporal pattern in the relaxation response to PTHrP by longitudinal uterine muscle during pregnancy in the rat, with maximal responses at times when estrogen levels would be high. In contrast, the circular muscle did not respond at any stage during gestation (Williams *et al.,* 1994). The inability of PTHrP to relax uterine muscle in the last stages of gestation does not support a direct role in the onset of parturition. Treatment of pregnant rats with i.p. injections of human PTHrP 1–34 resulted in a significant decrease in the expression of connexin-43 (mRNA and protein) and the oxytocin receptor mRNA in the myometrium, but it did not affect the timing of delivery, progesterone in maternal plasma, or levels of c-fos, fra-2, or PTH1R mRNA on any gestational day. These findings are compatible with the hypothesis that PTHrP may act to keep the myometrium quiescent at a time when progesterone levels are falling, but that the effects of PTHrP signaling are overridden by other factors that dictate the onset of labor (Mitchell *et al.,* 2003). This hypothesis is supported by the demonstration (Thiede *et al.,* 1990) that expression of mRNA was dependent on the presence of the fetus and that levels increased throughout pregnancy and decreased sharply after delivery. It seems likely, therefore, that the observed fall in PTHrP reflects the recontracted state of the uterine muscle, consistent with the observation in the bladder (Yamamoto *et al.,* 1992b), and that the level of expression is functionally related to contractility. The temporal expression of PTHrP in endometrial glands and blood vessels (Williams *et al.,* 1994) also supports roles in other regulated functions that might include uterine growth during pregnancy and the regulation of uterine and placental blood flow (Mandsager *et al.,* 1994).

Uterine growth restriction was induced in Wistar–Kyoto rats by ligating uterine vessels, and this resulted in a 15% decrease in fetal weight, a 21% decrease in fetal number, a 46% decrease in placental PTHrP content, but a 2.5-fold increase in uterine PTHrP content (Wlodek *et al.,* 2005). The increase in uterine PTHrP content may be compensatory to increase uteroplacental blood flow. Other studies have demonstrated that the vasodilatory effect of PTHrP on myometrial blood vessels is dependent upon the presence of functional endothelium, and that the effect is likely mediated by nitric oxide (Meziani *et al.*, 2005). Conversely, treatment of pregnant rats with a PTHrP antagonist (PTHrP 7–34) resulted in evidence of growth restriction (reduced fetal weight and placental weight) and apoptosis within placentas (Thoten *et al.*, 2005). Thus, there is some functional evidence to suggest that PTHrP plays a role in regulating uterine blood flow and growth.

Placenta and Fetal Membranes

PTHrP mRNA and protein have been detected in rat and human placenta in various cell types (Hellman *et al.*, 1992; Germain *et al.*, 1992; Bowden *et al.*, 1994). In addition, neoplastic cells of placental origin secrete PTHrP, including hydatidiform moles and choriocarcinomas *in vitro* (Deftos *et al.*, 1994). The presence of PTH/PTHrP receptor mRNA has been demonstrated in rat (Urena *et al.*, 1993), mouse (Kovacs *et al.*, 2002), and human (Curtis *et al.*, 1998) placenta, and infusion of PTHrP(1–34) into isolated human placental lobules stimulates cyclic AMP production (Williams *et al.*, 1991). Three further sets of observations lend support to the hypothesis that PTHrP is involved in placental/uterine interactions and that its most likely role in the placenta and placental membranes is related to the growth and maintenance of the placenta itself during pregnancy. First, PTHrP production by cultured amniotic cells has been shown to be regulated by prolactin, human placental lactogen, transforming growth factor-β (TGF-β), insulin, insulin-like growth factor, and epidermal growth factor (Dvir *et al.*, 1995). Second, PTHrP has been shown to regulate epidermal growth factor receptor expression in cytotrophoblast cultures (Alsat *et al.*, 1993), an event associated with placental development. Third, studies of vascular reactivity in isolated human placental cotyledons preconstricted with a thromboxane A_2 mimetic showed PTHrP to be a very effective vasodilator (Macgill *et al.*, 1997). The narrow concentration range to which the tissue responded, together with the desensitization in response to repeated PTHrP infusions, was consistent with a paracrine and/or autocrine action of PTHrP in human gestational tissues. Adequacy of the fetoplacental circulation is essential for the nutritional demands of the growing fetus, and both humoral and local factors are likely to be important in its control. It is possible that alterations of the expression, localization, and/or action of PTHrP might contribute to the genesis of conditions such as preeclampsia and intrauterine growth retardation in which placental vascular resistance is increased (Gude *et al.*, 1996). Another related and potentially interacting influence is angiotensin II, known to be a powerful enhancer of PTHrP production in the vasculature and in human placental explants (Li *et al.*, 1998). The ability of angiotensin II to stimulate estradiol production in human placental explants through actions upon its AT_1 receptor (Kalenga *et al.*, 1995) provides a further link with PTHrP control.

PTHrP has also been shown to regulate the differentiation of cells explanted from the murine placenta. PTHrP treatment reduced proliferation, inhibited apoptosis, and promoted differentiation into trophoblast giant cells. As well, PTHrP treatment induced the expression of transcription factors known to stimulate giant cell formation (Stra13 and AP-2gamma), and inhibited the formation of other trophoblast cell types by suppressing trophoblast progenitors and spongiotrophoblast-promoting factors (Eomes, Mash-2, and mSNA) (El-Hashashetd *et al.*, 2005). Thus, PTHrP likely plays a role in the differentiation of cells during placentation.

The most likely source of increased amniotic fluid PTHrP concentrations during pregnancy is the amnion itself, because PTHrP mRNA expression is also highest at term and greater in the amnion than in choriodecidua or placenta (Bowden *et al.*, 1994; Ferguson *et al.*, 1992; Wlodek *et al.*, 1996). In tissue from women with full-term pregnancies and not in labor, the concentration of N-terminal PTHrP has been found to be higher in amnion covering the placenta than in the reflected amnion covering the decidua parietalis (Bowden *et al.*, 1994). Nevertheless, the concentration of N-terminal PTHrP in reflected amnion (the layer apposed to the uterus) was inversely related to the interval between rupture of the membranes and delivery. The observation that PTHrP levels in the amnion decrease after rupture of the fetal membranes has led to the proposal that PTHrP derived from the membranes may inhibit uterine contraction and that labor may occur following loss of this inhibition. Plasma levels of PTHrP increase during pregnancy and at 6 weeks postpartum (Gallacher *et al.*, 1994; Ardawi *et al.*, 1991) with the likely sources being placenta and breast, respectively. Human fetal membranes have been shown to inhibit contractions of the rat uterus *in vitro* (Collins *et al.*, 1993) so this tissue does appear to produce factors that can modulate uterine activity. Furthermore, primary cultures of human amniotic cells secrete PTHrP into the medium (Germain *et al.*, 1992). Thus, although the physiological function(s) of amnion-derived PTHrP is currently unknown, preliminary evidence suggests that it may play a role in regulation of the onset of labor. It is also possible that amniotic fluid is a source of PTHrP ingested by the fetus, and that it acts as a growth factor in lung and/or gut development. Consistent with this hypothesis, mice lacking PTHrP have immature lungs associated with arrested type II alveolar cell development and reduced surfactant production *in vivo* and *in vitro* (Rubin *et al.*, 2004).

In summary, although many functional studies remain to be completed, potential roles for PTHrP produced by fetal membranes and placenta include transport of calcium across the placenta, accommodation of stretch of membranes, growth and differentiation of fetal and/or maternal tissues, vasoregulation, and the regulation of labor.

Implantation and Early Pregnancy

Some physiological functions other than control of myometrial activity were suggested by findings of Beck *et al.* (1993), who identified PTHrP mRNA as being limited to epithelial cells of implantation sites. This pregnancy-related expression appeared at day 5.5 in the rat fetus in the antimesometrial uterine epithelium of implantation sites, raising the possibility of a further function of PTHrP, playing a part in the localization of implantation or initial decidualization. Decidual cells produced mRNA for PTHrP both in normal gestation and after the induction of deciduomata. Expression of the gene in these cells followed epithelial expression by 48 hours. It was concluded from this work that the location of PTHrP gene expression in the uterus, together with the time of its expression, suggests that it plays a part in implantation of the blastocyst. Further evidence for a function of PTHrP in the implantation process came from Nowak *et al.* (1999), who showed that PTHrP and TGF-β were potent stimulators of trophoblast outgrowth by mouse blastocysts cultured *in vitro*. The TGF-β effect appeared to be mediated by PTHrP, which itself was acting through a mechanism distinct from the PTHR1.

Thus, both the timing and the localization of PTHrP gene expression suggested that it might play a part in the implantation of the blastocyst (Beck *et al.*, 1993). Upon finding substantial levels of immunoreactive PTHrP in uterine luminal fluid of estrogen-treated immature rats, and because the PTH/PTHrP receptor was known to be expressed in rat uterus (Urena *et al.*, 1993), Williams *et al.* (1998) investigated the role of PTHrP acting through this receptor in influencing early pregnancy in the rat. Infusion of either a PTHrP antagonist peptide or a monoclonal anti-PTHrP antibody into the uterine lumen during pregnancy resulted in excessive decidualization. The latter appeared to be the result of a decrease in the number of apoptotic decidual cells in the antagonist-infused horn. In pseudopregnant rats, infusion of receptor antagonist into the uterine lumen resulted in increases in wet weight of the infused horn compared with the control side, indicating an effect on deciduoma formation.

These observations suggest that activation of the PTH/PTHrP receptor by locally produced PTHrP might be crucial for normal decidualization during pregnancy in rats, probably not by being involved in the initiation of the decidual reaction, but rather in the maintenance of the decidual cell mass.

Summary

The multiple roles of PTHrP in the reproductive tissues and cycle and in the placenta largely reflect its roles as a paracrine/autocrine/intracrine regulator. Of the many functions it exerts in these systems, probably the only endocrine one is that in which PTHrP in the fetal circulation regulates placental calcium transport. There remains much to be learned of the place of PTHrP in reproductive and placental physiology and pathology.

ENDOCRINE PANCREAS

PTHrP and Its Receptors

The presence of PTHrP in the pancreatic islet became apparent shortly after the identification of PTHrP in 1987. Asa *et al.* (1990) demonstrated that PTHrP was present in islet cells and demonstrated that it was present in all four cell types of the islet, including the α, β, δ, and PP cells. PTHrP mRNA was shown to be present in isolated islet RNA as well (Drucker *et al.*, 1989), demonstrating that the peptide could be produced within the islet. Gaich and collaborators (1993) confirmed these findings, demonstrating that PTHrP was indeed present in islet cells of all four types and that it was also present in pancreatic ductular epithelial cells. The peptide is not present in adult pancreatic exocrine cells. Plawner and colleagues (1995) demonstrated that PTHrP is present in individual beta cells in culture and showed that PTHrP colocalized with insulin in the Golgi apparatus, as well as in insulin secretory granules. Interestingly, in a perifusion system employing a beta-cell line, PTHrP was shown to be cosecreted with insulin from beta cells following depolarization of the cell (Plawner *et al.*, 1995). The secreted forms of PTHrP included amino-terminal, midregion, and carboxy-terminal forms of PTHrP (see later).

With regard to receptors for PTHrP on beta cells, little direct evidence has been provided for the presence of the PTH1R, although its presence has not been sought rigorously. However, there can be no question as to the presence of some type of PTHrP receptor on the pancreatic beta cell, as it is clear that PTHrP(1–36) elicits prompt and vigorous responses in intracellular calcium in cultured beta-cell lines. For example, Gaich *et al.* (1993) have demonstrated that PTHrP(1–36) in doses as low as 10^{-12} M stimulates calcium release from intracellular stores. Interestingly, unlike events observed in bone and renal cell types where PTHrP receptor activation is associated with activation of cAMP/PKA, as well as the PKC/intracellular calcium pathways, only the latter is observed in cultured beta cells in response to PTH or PTHrP(1–36) (Gaich *et al.*, 1993). Whether this reflects the presence of a different type of receptor on beta cells or differential coupling of the PTHR1 to subsets of specific G-proteins or catalytic subunits in beta cells compared with bone and renal cells has not been studied.

Regulation of PTHrP and PTHrP Receptors

There is little information describing how or to what degree PTHrP or the PTH receptor family is regulated in the pancreatic islet. As will become clear from the sections that follow, there are physiological reasons why such regulation might occur under normal circumstances, but this area remains unexplored.

Biochemistry of PTHrP

PTHrP undergoes extensive post-translational processing as described in Chapters 3 and 4. Most of what is known or inferred regarding PTHrP processing is derived from studies in the rat insulinoma line, RIN-1038 (Soifer *et al.*, 1992; Yang *et al.*, 1994; Wu *et al.*, 1991). These cells have served as a model of PTHrP processing, because they have been shown to serve as a model for authentic processing of other human neuroendocrine peptides, such as insulin, proopiomelanocortin, glucagon, and calcitonin. Using a combination of untransfected RIN-1038 cells, RIN-1038 cells overexpressing hPTHrP(1–139), hPTHrP(1–141), or hPTHrP(1–173), and a panel of region-specific radioimmunoassays and immunoradiometric assays, RIN cells have been shown to secrete PTHrP(1–36), PTHrP(38–94), PTHrP(38–95), and PTHrP(38–101) (Soifer *et al.*, 1992; Yang *et al.*, 1994; Wu *et al.*, 1991). In addition, RIN 1038 cells have been shown to secrete a form of PTHrP that is recognized by a PTHrP (109–138) radioimmunoassay (Yang *et al.*, 1994), and another form that is recognized by a PTHrP(139–173) radioimmunoassay (Burtis *et al.*, 1992).

As described earlier, PTHrP(1–36) stimulates intracellular calcium increments in cultured beta cells (Gaich *et al.*, 1993). PTHrP(38–94) has also been shown to stimulate intracellular calcium release in these cells (Wu *et al.*, 1991). PTHrP(38–94) does not activate adenylyl cyclase in cultured beta cells, and other PTHrP species have not been explored in beta cells in functional terms.

Function of PTHrP

Pancreas development in rodents begins at approximately day E9–10, and by day E18–19, clusters of beta cells have begun to coalesce and form immature islets (Edlund, 1998). These islet cell clusters continue to increase in number, in size, and in density of beta cells in the week after delivery and then decline abruptly in number through a wave of beta-cell apoptosis (Finegood *et al.*, 1995).

The role of PTHrP in pancreatic cell development and function is poorly understood at present. The pancreas of PTHrP-null mice (Karaplis *et al.*, 1994) develops normally in anatomic terms (R. C. Vasavada and A. F. Stewart, unpublished observations), but nothing is known about the function of these islets. PTHrP-null mice die immediately after delivery, so nothing is known of islet function or development following birth in the absence of PTHrP. "Rescued" PTHrP mice do exist (Wysolmerski *et al.*, 1998) and they survive to adulthood. These mice have normal-appearing pancreata and islets (R. C. Vasavada and A. F. Stewart, unpublished observations), but they have dental abnormalities, are undernourished, and grow poorly. Therefore, it is difficult to characterize their islets in functional terms, as islet mass, proliferation, and function are heavily dependent on fuel availability. Streuker and Drucker (1991) have suggested that PTHrP may play a role in beta-cell differentiation, because it is upregulated in beta-cell lines in the presence of the islet-differentiating agent, butyrate.

In an effort to understand the role of PTHrP in the pancreatic islet, Vasavada and collaborators have developed transgenic mice that overexpress PTHrP under the control of the rat insulin-II promoter (RIP) (Vasavada *et al.*, 1996; Porter *et al.*, 1998). RIP-PTHrP mice display striking degrees of islet hyperplasia and an increase in islet number, as well as the size of individual islets. This increased islet mass is associated with increased function: RIP-PTHrP mice are hyperinsulinemic and hypoglycemic compared with their littermates (Vasavada *et al.*, 1996; Porter *et al.*, 1998). They become profoundly and symptomatically hypoglycemic with fasting. Interestingly, RIP-PTHrP mice are also resistant to the diabetogenic effects of the beta-cell toxin, streptozotocin. Following the administration of streptozotocin, normal mice readily develop diabetes, but RIP-PTHrP mice either fail to become diabetic or develop only mild hyperglycemia (Porter *et al.*, 1998).

The mechanism(s) responsible for the increase in islet mass in the RIP-PTHrP mouse remains undefined. There are two levels at which this question can be addressed: identification of the source of the cells responsible for the increase in islet mass and the signaling mechanisms that are responsible for the increase. With respect to the first, islet mass can, in theory, be increased by three pathways: (a) the recruitment of new islets from the pancreatic duct or its branches distributed throughout the exocrine pancreas, in a process referred to as "islet neogenesis"; (b) induction of proliferation of existing beta cells within islets; and/or (c) prolongation of the life span of existing beta cells. Of these options, there is evidence that PTHrP can drive beta-cell replication (Villanueva *et al.*, 1999; Fujinaka *et al.*, 2004) suggesting that beta-cell proliferation may account for at least part of the phenotype. The RIP promoter is restricted to expression in beta cells, and therefore unable to influence pancreatic cells prior to their differentiation into beta cells, suggesting that the neogenesis of beta cells from ductal or other precursors is not a likely contributor (Vasavada *et al.*, 1996). Finally, the bulk of evidence would support a dominant role for PTHrP in enhancing beta-cell survival, as occurs in other cell types (Cebrian *et al*, 2002).

At the signaling level, little is known regarding the mechanism of action of PTHrP on beta cells. Although it is known that PTHrP can stimulate intracellular calcium in cultured beta-cell lines (Gaich *et al.*, 1993; Wu *et al.*, 1991), it is not known whether this occurs *in vivo* in normal, nontransformed beta cells within intact islets. Nor is it known whether PTHrP stimulates adenylyl cyclase in normal beta cells *in vivo* or whether it participates in nuclear or intracrine signaling in beta cells as it appears to in chondrocytes, osteoblasts, vascular smooth muscle cells, or other cell types (Aarts *et al.,* 1999; Massfelder *et al.,* 1997; Lam *et al.,* 1999) (see Chapter 6). These processes, too, are under study.

Finally, and importantly, the results of overexpression studies do not demonstrate that PTHrP enhances beta-cell mass *in vivo* under normal circumstances. In the absence of meaningful data from knockout or rescued knockout mice, it is difficult to be sure if PTHrP is important in normal islet biology. This question, too, will need to await further studies such as the conditional or islet-specific deletion of the PTHrP gene.

Pathophysiology of PTHrP

From the discussion given earlier, it is clear that the normal physiological role of PTHrP in the pancreatic islet remains undefined. In contrast, PTHrP plays clear pathophysiological roles in at least some pancreatic islet neoplasms. PTHrP overexpression with resultant development of humoral hypercalcemia of malignancy has been demonstrated on multiple occasions in multiple investigators' hands (Asa *et al.,* 1990; Stewart *et al.,* 1986; Wu *et al.,* 1997; Skrabanek *et al.,* 1980). In the only large series of malignancy-associated hypercalcemia in which tumors have been fully subdivided based on histology (Skrabanek *et al.,* 1980), islet cell carcinomas, which are not particularly common, produce humoral hypercalcemia of malignancy fully as often as pancreatic adenocarcinomas, a very common neoplasm. Historically, islet tumors were among the first in which PTHrP bioactivity was identified (Stewart *et al.,* 1986; Wu *et al.,* 1997). Furthermore, patients with islet carcinomas regularly demonstrate increases in circulating PTHrP as determined by radioimmunoassay or immunoradiometric assays (Lansk *et al.,* 1996). When assessed by immunohistochemistry, these tumors also demonstrate increased staining for PTHrP (Asa *et al.,* 1990; Drucker *et al.,* 1989).

The significance of these findings for islet tumor oncogenesis is not known. Is this simply a random derepression of the PTHrP gene or is it a specific upregulation of the PTHrP gene? Is there a pathological role for PTHrP in the development of pancreatic islet tumors, corresponding to the mass enhancing effects of PTHrP in the islets of the RIP-PTHrP mouse? These questions remain interesting but unanswered at present.

CONCLUSION

Advances in mouse genetics and in transgenic technology have been a boon to the study of physiology. This has certainly been the case for the PTHrP field, where studies in genetically altered mice have provided a starting place for the study of the physiology of a protein that was discovered outside its natural context. This chapter outlined the current state of knowledge regarding the physiological roles of PTHrP in skin, the mammary gland, placenta, uterus, and pancreas. Much of this information (although not all) has come from studies performed in a variety of transgenic mice. These studies have shown that PTHrP is important to both the development and the physiological functioning of these organs. However, at this point, we continue to have as many questions as answers. There are many experiments to be done before we comprehend all the nuances of the functions of PTHrP at these sites. The next several years promise to see continued progress in the investigation of the nonskeletal effects of this remarkable molecule.

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Chapter 34

Vascular, Cardiovascular, and Neurological Actions of Parathyroid-Related Protein

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VASCULAR AND CARDIOVASCULAR ACTIONS

Historical Perspectives

The origins of parathyroid hormone (PTH) as a putative cardiovascular regulatory factor date to the early 1900s when the calcemic properties of the hormone were first identified. In classic studies, Collip and Clark (1925) demonstrated that systemic injection of extracts of parathyroid glands lowered systemic blood pressure in dogs (Fig. 1). The first formal characterization of the cardiovascular activity of PTH was conducted by Charbon (1968a,b, 1969) in the early 1960s. This investigator quantified the vasodilatory effects of a purified parathyroid extract in the rabbit and cat and demonstrated that a synthetic N-terminal fragment displayed similar actions in the dog. Relaxant activity was not blocked by pharmacological antagonists of other known vasoactive agents, suggesting a direct action of the hormone. Numerous studies have now unequivocally established the hypotensive/vasodilatory and cardiac effects of PTH (Mok *et al.,* 1989) that can be broadly summarized as follows: First, the hypotensive and vasorelaxant actions of PTH occur in the absence of a change in blood calcium and are mediated by PTH activation of the type 1 PTH/PTH-related protein (rP) receptor (PTH1R) expressed in the smooth muscle layer of the vessel wall. Second, although all vascular beds are relaxed by PTH, resistance vessels appear to be more responsive than conduit vessels. Third, PTH can reduce the pressor effects of other vasoactive agents that exert their action through different mechanisms. Finally, PTH exerts both ionotropic and chronotropic effects on the heart (Ogino *et al.,* 1995).

Although the cardiovascular effects of PTH are undisputed, their physiological significance has been frequently debated. This is in part because the concentrations of PTH required to produce vasodilation (10−100nM) are substantially greater than normal circulating levels (low pM). Consequently, it has been difficult to conceptualize how physiological levels of this systemic hormone, which is synthesized only in the parathyroid gland, could function in the local control of vascular tone. Also enigmatic is the fact that patients with primary hyperparathyroidism and elevated circulating PTH levels often have high (not low) blood pressure that sometimes returns to normal after parathyroidectomy. A plausible explanation for the seemingly enigmatic regulatory effects of PTH on the cardiovascular system emerged with the discovery of PTHrP in 1987.

As discussed in Chapter 33, PTHrP was identified as the factor responsible for the paraneoplastic syndrome termed humoral hypercalcemia of malignancy. Almost immediately after its cloning, expression of PTHrP was detected in many normal fetal and adult tissues but was undetectable in the circulation, suggesting that the protein functioned in an autocrine/paracrine mode. Although many functions have been ascribed for PTHrP, three main

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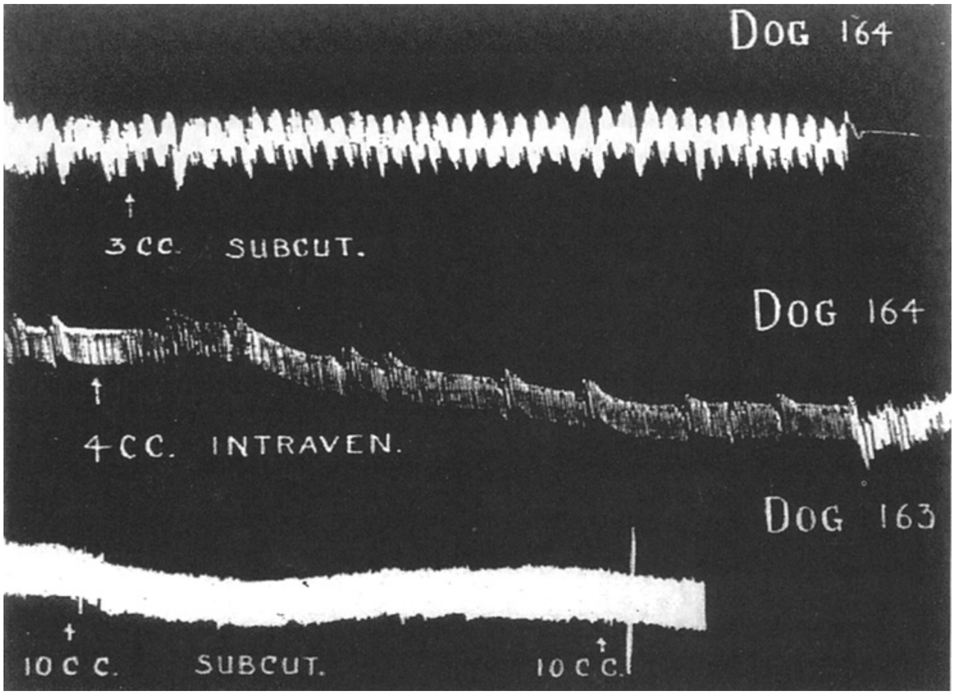

FIGURE 1 Effects of extracts of parathyroid glands on blood pressure in thyroparathyroidectomized dogs. From Collip and Clark (1925), with permission.

physiological themes have emerged. Observations in gene knockout mice have demonstrated that PTHrP is required for the development of cartilage, morphogenesis of the mammary gland, and tooth eruption (reviewed in Philbrick *et al.,* 1996; Lanske and Kronenberg, 1998; and elsewhere in this volume). PTHrP also appears to participate in maternofetal calcium transfer across the placenta. The third physiological role for PTHrP is in smooth muscle in which the protein functions to regulate contractility and proliferation. Although this chapter focuses on the physiology of PTHrP in *vascular* smooth muscle, it is relevant to begin with a brief review of its physiological effects in other smooth muscle-containing tissues.

In all smooth muscle cell beds studied to date, induction of PTHrP expression occurs in close association with normal physiological stimuli. In the smooth muscle layer of the chicken oviduct, induction of PTHrP expression coincides temporally with egg movement and its arrival in the shell gland (Thiede *et al.,* 1991). In the rat uterus, PTHrP expression is localized to the myometrium and is markedly upregulated by fetal occupancy (Thiede *et al.,* 1990) or by mechanical distention of the uterine horn with a balloon catheter (Daifotis *et al.,* 1992). PTHrP expression is increased in prelabor human amnion and abruptly falls with the onset of labor and rupture of the amniotic sac (Ferguson *et al.,* 1992). In the urinary bladder, induction of PTHrP mRNA occurs during filling in proportion to bladder distension (Yamamoto *et al.,* 1992). Finally, as discussed in detail later, PTHrP is also expressed in vascular smooth muscle, in which it is induced by vasoconstrictor agents and mechanical stimuli. In each of these smooth muscle beds, application of PTHrP to precontracted smooth muscle preparations induces relaxant activity, precisely mimicking the actions described previously for PTH. It would therefore appear that PTHrP rather than PTH represents the physiologically important regulator of smooth muscle tone. Consequently, the remainder of this chapter focuses primarily on the physiology of PTHrP in the cardiovascular system.

PTHrP in the Vasculature

Vascular Anatomy and Contractile Mechanisms

Blood vessels are composed of three principal cell types: the intima, which consists of a single epithelial cell layer; the muscularis layer, made up of vascular smooth muscle cells embedded in a connective tissue matrix; and an outer adventitial layer, which receives input from the cholinergic and adrenergic nervous system. The relative composition and contribution of each of these cell types to vascular growth and tone varies during development and among different vascular beds. For example, during development, blood vessels initially form as simple tubular structures consisting entirely of endothelial cells into which smooth muscle cells migrate to form the vascular wall. In the mature mammal, the large conduit vessels (e.g., aorta) are highly elastic to accommodate high-capacity blood flow, whereas resistance vascular beds (e.g., mesentery) typically contain more smooth muscle cells and are densely innervated. Changes in the cellular and connective tissue constituents within the vasculature occur with normal aging and in particular during pathological conditions such as atherosclerosis. The regulation of vascular growth, remodeling, and smooth muscle cell tone is achieved through a coordinated network of both systemic and local factors, as well as input from adrenergic, cholinergic, peptidergic, and sensory neurons.

Mechanisms regulating vascular smooth muscle cell contractility have been studied in detail (reviewed in Somlyo *et al.,* 1999). The intracellular-free-calcium concentration is the major determinant of vascular tone. Depolarization of vascular smooth muscle cells enables calcium to enter the cell via L-type voltage-sensitive calcium channels (L-VSCCs). These events trigger the release of much larger quantities of calcium from the sarcoplasmic reticulum. Alternatively, pharmacological or ligand activation of G protein receptors (e.g., angiotensin II) activate phospholipase (PLC), which catalyzes phosphoinositol hydrolysis and causes calcium release from intracellular stores. The increases in cytoplasmic calcium achieved by either of these mechanisms activate myosin light chain kinase through the calcium–calmodulin complex and phosphorylation of the 20-kDa regulatory light chain of myosin, with subsequent cross-bridge cycling and force development. The mechanisms of vascular smooth muscle cell relaxation are less well understood. In the simplest scheme, a reduction of cytoplasmic calcium with a fall in myosin light chain kinase activity would suffice to account for dephosphorylation of the regulatory light chain and relaxation. However, other mechanisms have been implicated in cyclic nucleotide-dependent relaxation in vascular and other smooth muscle tissues (McDaniel *et al.,* 1994).

The demonstration of a calcium-sensing receptor in vascular smooth muscle with pharmacological properties similar to those of the parathyroid calcium-sensing receptor (discussed in Chapter 26) has prompted speculation that it might also participate in the regulation of contractile events (Bukoski *et al.,* 1995). Alterations of extracellular calcium over the physiological concentration range depress contractility of precontracted vascular smooth muscle. This effect of extracellular calcium has been shown to be mediated by activation of a calcium-dependent potassium channel and is associated with alterations in myofilament calcium sensitivity. These activities were mimicked by gadolinium, neomycin, and lanthanum, all factors that activate the calcium-sensing receptor. However, the structure of this putative calcium-sensing receptor is unknown and it remains unclear whether it bears homology to the renal or parathyroid or kidney calcium-sensing receptor.

Expression and Regulation of PTHrP

PTHrP is expressed in blood vessels in essentially all vascular beds from a broad range of species, including rodent and human fetal blood vessels (Moniz *et al.,* 1990), adult rat aorta (Burton *et al.,* 1994; Pirola *et al.,* 1994), vena cava (Burton *et al.,* 1994), kidney afferent arterioles, artery, and microvasculature (Nickols *et al.,* 1990), the arterial and venous supply of the mammary gland (Thiede, 1994), the serosal arterioles in avian egg shell gland (Thiede *et al.,* 1991), and blood vessels of the rat penis (Lang *et al.,* 1999). The protein appears to be expressed predominantly in the smooth muscle layer of the vessel, although its expression has also been reported in cultured endothelial cells (Rian *et al.,* 1994; Ishikawa *et al.,* 1994a). The regulation of PTHrP mRNA expression has been studied in detail by using cultured vascular smooth muscle cells. In primary rat aortic vascular smooth muscle cells, expression of PTHrP is induced rapidly (2−4 hours) but transiently by exposure of quiescent cells to serum (Hongo *et al.,* 1991) (Fig. 2). This mode of tight regulation is reminiscent of the behavior of cytokine mRNAs and would appear to constitute a mechanism that would restrict the activity of PTHrP to a narrow window of time. Among the most potent inducers of PTHrP are vasoconstrictors, including angiotensin II, seritonin, endothelin, norepinephrine, bradykinin, and thrombin, each of which induces PTHrP mRNA and protein levels over the same time course as that observed for serum (Pirola *et al.,* 1993). The induction of PTHrP mRNA by angiotensin II depends on protein kinase C activation and is mediated by both transcriptional and post-transcriptional mechanisms (Pirola *et al.,* 1993). Prior addition of saralaysin and captopril, which inhibit angiotensin II action or generation, respectively, inhibit the serum-induced increase in PTHrP

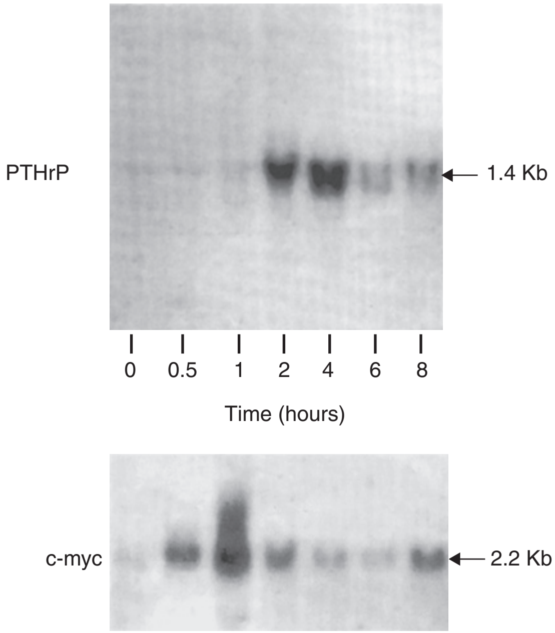

FIGURE 2 Time course of serum induction of PTHrP mRNA in aortic vascular smooth muscle cells. From Hongo *et al.* (1991), with permission.

in vascular smooth muscle cells. This finding suggests that the angiotensin II present in serum represents a significant component of the serum induction of PTHrP.

PTHrP is also induced in vascular smooth muscle in response to mechanical stimuli. PTHrP mRNA is increased transiently in rat aorta following distension with a balloon catheter (Pirola *et al.,* 1994). Fluid flow-induced mechanical events induced by rocking or rotation of monolayer cultures of rat aortic vascular smooth muscle cells result in increased PTHrP mRNA expression (Pírola *et al.,* 1994; Noda *et al.,* 1994). The inductive effects of mechanical stretch and angiotensin II on PTHrP mRNA appear to be synergistic, suggesting that they occur through distinct mechanisms (Noda *et al.,* 1994). PTHrP mRNA is also produced in capillaries of slow-twitch soleus and fast-twitch skeletal muscle, and its expression is increased in response to low-frequency stimulation (Schneider *et al.,* 1999). This maneuver was associated with enhanced capillarization of the muscle, indicating that PTHrP might function to promote new capillary growth in response to increased contractile activity.

Vascular Actions of PTHrP

Shortly after the identification of PTHrP, a number of studies demonstrated that synthetic N-terminal fragments of the peptide replicated many of the vascular actions of PTH, including its vasorelaxant actions in the aorta (Crass and Scarpace, 1993), portal vein (Shan *et al.,* 1994), coronary artery (Nickols *et al.,* 1989), renal artery (Wingulst *et al.,* 1987; Musso *et al.,* 1989), placenta (Macgill *et al.,* 1997; Mandsager *et al.,* 1994), and mammary gland (Prosser *et al.,* 1994). In general, the vasodilatory potency of PTHrP is comparable to that of PTH when examined in organ bath systems. In contrast, in mouse portal vein preparations, PTHrP(1–34) was shown to be a more potent vasorelaxant than PTH(1–34) (Shan *et al.,* 1994). In perfused rabbit kidney (Musso *et al.,* 1989) and in rat aorta (Nickols *et al.,* 1989) the vasorelaxant effects of PTHrP do not appear to require the presence of an intact endothelium. However, in mouse aortic rings, endothelium denudation attenuates the relaxant activity of PTHrP markedly (Sutliff *et al.,* 1999), possibly reflecting a species difference.

In addition to its effects on vascular tone, PTHrP also modulates vascular smooth muscle cell proliferation. The peptide decreases serum- and platelet-derived growth factor (PDGF)-activated DNA synthesis in primary arterial vascular smooth muscle cells (Hongo *et al.,* 1991; Jiang *et al.,* 1995) and in A10 vascular smooth muscle cells stably expressing the PTH1R receptor (Maeda *et al.,* 1996). In both of these cell types, antimitogenic effects require the PTH-like N-terminal portion of the molecule and are mimicked by dibutyryl cAMP or forskolin. The mechanism for the antiproliferative effect of PTHrP involves the induction of the cyclin-dependent kinase inhibitor, $p27^{kip1}$, and impairment of phosphorylation of the retinoblastoma gene product (Rb), which results in cell cycle arrest in mid-G_1 phase (Maeda *et al.,* 1997). However, Massfelder *et al.* (1997) reported that overexpression of PTHrP in A10 vascular smooth muscle cells was associated with an increase in DNA synthesis. This apparent discrepancy likely results from the presence of an alternate CUG translation initiation site downstream of PTHrPs secretion signal sequence. PTHrP translated from this second site is transported via β1-importin to the nucleus where it stimulates the proteasomal degradation of $p27^{kip1}$ and hyperphosphorylation of Rb (Fiaschi-Taesch *et al*., 2004, 2006). Similar nuclear trafficking has also been described in chondrocytes, whereby nuclear PTHrP enhances chondrocyte survival (Henderson *et al.,* 1995). It is important to note that, in these studies, exogenous application of PTHrP inhibited A10 cell growth in agreement with the studies cited earlier. Therefore, the ability of PTHrP to influence the proliferation of vascular smooth muscle cells either positively or negatively appears to depend on where the protein is trafficked in the cell. Cellular levels of PTHrP fluctuate during the cell cycle and reach their highest levels in G_2/M (Okano *et al.,* 1995). It is possible that the protein is directed to the nucleus in the later stages of the cell cycle to participate in mitotic events. The mechanism regulating differential usage of translation initiation sites remains undefined.

PTHrP also inhibits PDGF-directed migration of vascular smooth muscle cells *in vitro* (Ishikawa *et al.,* 1998). The antimigratory effects of PTHrP are mediated through a cAMP-dependent mechanism that leads to diminished PDGF signaling through the PI3 kinase cascade.

The effects on vascular smooth muscle cell growth and migration *in vitro* are likely to be physiologically relevant to conditions under which vascular smooth muscle cell (VSMC) growth and migratory behavior is altered *in vivo.* For example, Ozeki *et al.* (1996) have reported that PTHrP protein and mRNA expression were upregulated markedly in neointimal smooth muscle in rat carotid arteries following experimental balloon injury. Moreover, immunoreactive PTHrP is increased in human arterial tissue removed from patients undergoing angioplasty. In light of the possibility of opposing effects of PTHrP on vascular smooth muscle cell growth cited earlier, these observations can be viewed in one of two ways: either upregulation of PTHrP is a primary stimulus for growth under these conditions or, alternatively, it represents an antiproliferative signal. Consistent with the latter possibility, the local administration of PTHrP(1−34) dose-dependently inhibits cuff-induced neointimal formation in the rat femoral artery (Ishikawa *et al.,* 2000), whereas 3′,5′-cyclic AMP or the phosphodiesterase inhibitors aminophylline or amrinone have similar effects following experimental balloon injury of rat carotid arteries (Indolfi *et al.,* 1997). Moreover, other studies using a similar model of arterial injury showed high levels of $p27^{kip1}$ expression in media within 2 weeks of angioplasty (Tanner *et al.,* 1998).

PTHrP may also function in the calcification of vascular tissue. The overexpression of the molecule in atherosclerotic lesions increases the expression of MCP-1 and enhances the migration of circulating monocytes (Martin-Ventura *et al.,* 2003). The accumulation of inflammatory cytokines within the plaque likely leads to the expression of osteoinductive molecules and ultimately calcification (reviewed by Whitfield, 2005). However, *in vitro* studies suggest that PTHrP inhibits the calcification of bovine vascular smooth muscle cells. PTHrP secretion decreases as vascular smooth muscle cells produce a calcified matrix following treatment with a source of inorganic phosphate, and the addition of the molecule inhibits calcification of VSMC via the activation of both phosphokinase A (PKA) and phosphokinase (PKC) (Jono *et al.,* 1997). Thus, the exact role of PTHrP in the calcification of vascular tissue remains elusive.

The ability of PTHrP to modulate vascular smooth muscle cell growth suggests that the protein might function during the development of the cardiovascular system. Although the cardiovascular system appears to develop normally in the PTHrP knockout mouse, homologous deletion of the PTH1R receptor results in a higher incidence of early fetal death at approximately embryonic day 9–10, coincident with development of the heart and major blood vessels (Lanske *et al.,* 1996). Furthermore, transgenic mice expressing high levels of PTHrP and its receptor in vascular smooth muscle, created by crossing the ligand- and receptor-overexpressing mice, die at day E9.5 with severe thinning of the ventricle and disruption of ventricular trabeculae (Qian *et al.,* 1999) (Fig. 3; see also color plate). Additional anecdotal evidence for a role of PTHrP in heart and vascular development is evident from the abnormalities seen in patients with the rare fatal condition known as Blomstrand chondrodysplasia caused by an inactivating mutation of PTH1R receptor (Karaplis *et al.,* 1998). These patients die prenatally with coarctation of the aorta and hydrops fetalis, the latter condition typically caused by high-output heart failure.

PTHrP and Angiogenesis

As suggested earlier, PTHrP might function in the development of new blood vessels. The overexpression of the peptide by many tumor cell types and the observation that PTHrP enhances the angiogenic behavior of endothelial cells *in vitro* led to the hypothesis that PTHrP is a proangiogenic factor (Akino *et al.,* 2000). Indeed, PTHrP may be able to influence angiogenesis by modulating the expression of interleukin-8 (Guijal *et al.,* 2001). However, two recent studies suggest that PTHrP is a negative regulator of angiogenesis. Adenoviral-mediated overexpression of PTHrP or treatment with N-terminal fragments of the peptide disrupts angiogeneis in chick chorioallantoic membranes (Bakre *et al.,* 2002). Whereas PTHrP acts on VSMCs to produce its vasodilatory effects, *in vitro* studies suggest that PTHrP inhibits endothelial cell migration to affect angiogenesis. The activation of PKA inhibits Rac activity and thus cell migration (Bakre *et al.,* 2002). Consistent with these findings, the fetal skin of mice lacking either PTHrP or the PTH1R receptor exhibits increased blood vessel diameter and length, whereas overexpression of PTHrP produced reciprocal changes (Diamond *et al.,* 2006).

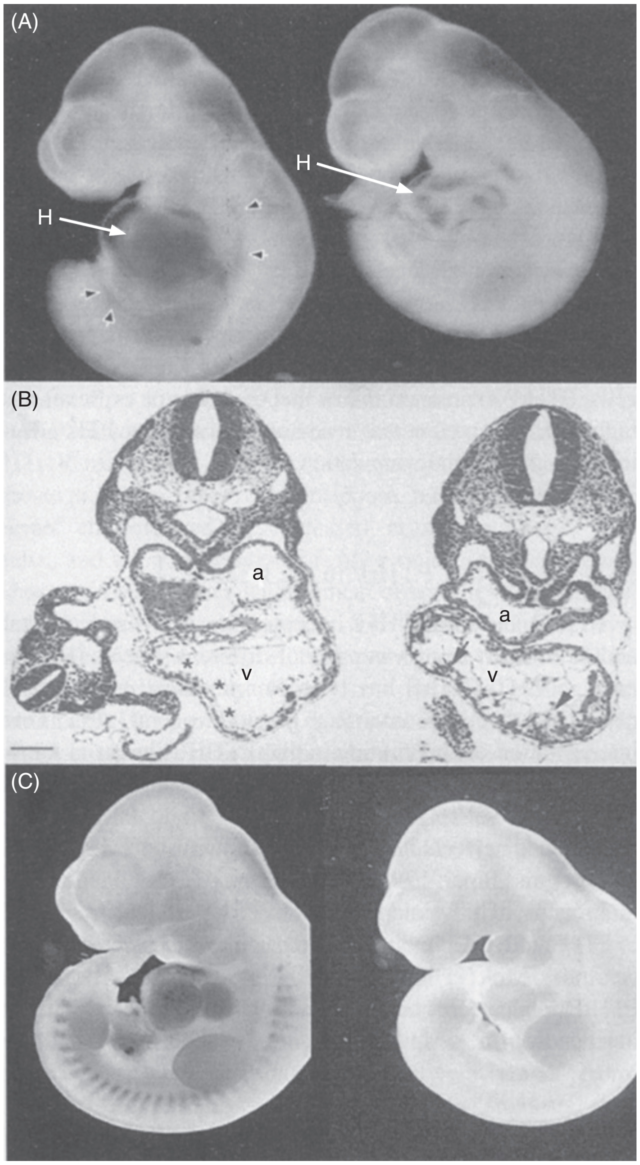

FIGURE 3 Overexpression of PTHrP and PTH1R disrupts heart development. **(A)** Whole mounts at E9.5 of double transgenic (*left*) and wild-type (*right*) embryos. The double transgenic embryo exhibits a greatly enlarged heart with pericardial effusion and vascular pooling (*arrows*). **(B)** Histological sections of double transgenic (*left*) and wild-type (right) embryos at E9.5. Trabeculae within the ventricular cavity (v) of the wild-type embryo are prominent (*large arrows*), whereas in the double transgenic embryo, trabeculae are reduced severely or absent (*asterisks*). Prominent gaps are also evident between the cardiomyocytes in the double transgenic hearts (*small arrowheads*), a, atria. Bar: 100 μm. (**C**, left). Localization of SMP8 lacZ transgene in a 9.5-day-old embryo. Staining is apparent in heart, hind gut, and somites. (*Right*) An unstained control. From Qian *et al.* (1999), with permission.

Mode of Action/Receptor Interactions

PTHrP exerts its vasodilatory actions by activating the PTH1R. This receptor is expressed in rat vascular smooth muscle beds (Urena *et al.,* 1993), and relaxation of aortic preparations is accompanied by an increased accumulation of cAMP (Ishikawa *et al.,* 1994b). Cultured rat aortic smooth muscle cells also express the PTH1R and respond to N-terminal PTHrP peptide fragments with an increase in cAMP formation (Wu *et al.,* 1993). Moreover, relaxation responses to PTH in aortic strip preparations are potentiated by phosphodiesterase inhibitors and forskolin (Nickels and Cline, 1987). Although the PTH1R receptor appears to be primarily coupled to adenylate cyclase, linkage to calcium-phosphoinositol pathways is suggested by studies by Nyby *et al.* (1995), who demonstrated a transient increase in cytosolic calcium and cAMP in response to PTHrP(1−34) in primary arterial rat vascular smooth muscle cells. However, other studies using similar preparations of primary rat aortic smooth muscle cells showed that PTHrP consistently stimulated cAMP accumulation but had no effect on intracellular calcium (Wu *et al.,* 1993). Furthermore, in A10 embryonic aortic vascular smooth muscle cells stably expressing recombinant PTH1R receptors, PTHrP induced large increases in cAMP accumulation but did not increase cytoplasmic calcium (Maeda *et al.,* 1996) despite the presence of detectable levels of expression of G_q, known to be required for functional coupling of the receptor to the PLC-phosphoinositide calcium pathway. However, when G_q was overexpressed in these cells, PTHrP evoked a calcium transient. It therefore appears that under most conditions the PTH1R receptor couples preferentially to G_s and adenylate cyclase to raise intracellular cAMP, which would be consistent with the established vasodilatory properties of this cyclic nucleotide. This does not, however, preclude the possibility that under certain physiological conditions (or in specific vascular smooth muscle cell beds), PTHrP might also activate PLC, which could mediate other as yet unidentified activities of the protein.

Vasorelaxation induced by cyclic nucleotides in arterial smooth muscle has also been reported to be associated with a reduction in intracellular calcium. In addition, in rat tail artery, PTH relaxes KCl-induced contraction; this effect is inhibited by nifedipine, suggesting an inhibition of the L-VSCC (Wang *et al.,* 1991a). Subsequent patch-clamp experiments (Wang *et al.,* 1991b) confirmed a decrease in L-type voltage-dependent calcium currents in vascular smooth muscle cells in response to PTH. Although not yet formally tested, it is likely that PTHrP also inhibits the L-VSCC activity in vascular smooth muscle, as is the case in cultured neuroblastoma cells.

As discussed in detail elsewhere in this volume (see Chapter 33), PTHrP is subject to post-translational processing to produce both N-terminal peptides, midregion PTHrP fragments, and possibly also C-terminal forms. PTHrP peptides that lack the PTH-like N-terminal region likely activate receptors distinct from the PTH1R and would be expected to exhibit a biological profile different from N-terminal PTHrP peptides. To date, however, there is no evidence that these midregion or C-terminal forms of PTHrP are biologically active either in cultured vascular smooth muscle cells (Wu *et al.,* 1993) or in intact vessel preparations (Sutliff *et al.,* 1999).

Although PTHrP is capable of relaxing vascular preparations devoid of endothelium, studies in mouse aortic preparations suggest that the endothelial layer may serve to amplify relaxant effects of PTHrP and PTH (Sutliff *et al.,* 1999). The mechanism accounting for the endothelium-dependent relaxant effects of PTH and PTHrP remains unclear, but does not appear to require nitric oxide formation. The recent demonstration of expression of a novel PTH-2 receptor (PTH2R; see later) in endothelial and smooth muscle cells in blood vessels and heart (Usdin *et al.,* 1996) suggests an additional pathway through which PTH-related peptides could alter vascular reactivity.

As with other G-coupled receptors, prolonged exposure of vessel preparations (Nyby *et al.,* 1995) or cultured aortic smooth muscle cells (Okano *et al.,* 1994) to PTHrP is associated with desensitization. Angiotensin II, which induces PTHrP expression in cultured aortic vascular smooth muscle cells, also rapidly desensitizes cells to PTHrP and downregulates the PTH1R receptor mRNA expression (Okano *et al.,* 1994), indicating crosstalk in the signaling circuitry among these vasoactive peptides.

From the studies just summarized, it is possible to construct a simple model for the mode of PTHrP action in vascular smooth muscle (Fig. 4; see also color plate). In response to mitogenic, vasoconstrictor, or mechanical signals, PTHrP is released and acts locally via a short feedback loop to activate the PTH1R receptor and stimulate adenylate cyclase in adjacent cells. Effector pathways downstream of cAMP impact specific sets of genes, which function to oppose the pressor (contraction coupling) and mitogenic (cell cycle) events. As mentioned earlier, induction of $p27^{kip1}$ with consequent inhibition of Rb phosphorylation would represent one such target for cAMP-induced cell cycle arrest. With regard to relaxant activity, stimulation of cAMP-dependent PKA is associated with a reduction in cytoplasmic calcium and attenuated myosin light chain kinase activity (McDaniel *et al.,* 1994).

Because PTH and PTHrP activate the same receptor, how does the smooth muscle PTH1R receptor distinguish between these two ligands? A likely possibility is that the sensitivity of a given tissue to PTH or PTHrP is governed by the relative abundance of each ligand and the number of PTH1R receptors. For example, in tissues such as vascular smooth muscle, which express high levels of PTHrP but relatively low numbers of the PTH1R receptor, the fraction of receptor occupancy must be high in order to achieve a

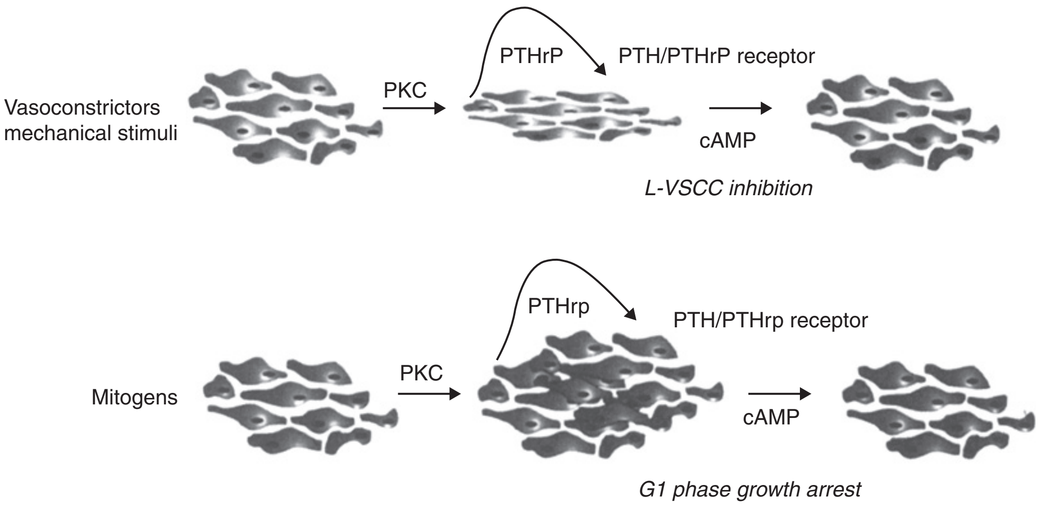

FIGURE 4 Model for PTHrP production and action in vascular smooth muscle cells (see text for description).

response, thus favoring the local (PTHrP) regulator. In contrast, in bone cells, PTHrP expression is low and the receptor expression is high, enabling preferential receptor activation by PTH arriving from the systemic circulation.

PTHrP in the Heart

PTHrP and the PTH1R receptor are expressed in fetal and adult heart from a number of different species (Burton *et al.*, 1994). PTHrP has been immunolocalized to atrial natriuretic peptide-containing granules of rat atria. One interpretation of this finding is that PTHrP, like atrial natriuretic peptide, is released in response to stretch, but this concept has yet to be tested. Both PTH and PTHrP exert pronounced effects on cardiac function (reviewed in Schluter and Piper, 1998).

Infusion of physiological levels of N-terminal fragments of PTH and PTHrP induce hypotension and tachycardia in intact rats (*Mok et al.*, 1989). In isolated perfused hearts, PTHrP induces chronotropic and ionotropic effects that are independent of perfusion pressure (Nickols *et al.*, 1989). Additionally, it has been established that the ionotropic activity of PTHrP occurs indirectly in response to increased coronary blood flow (Ogino *et al.*, 1995). The mechanisms responsible for the chronotropic effects of PTH and PTHrP have been examined in cultured cardiomyocytes (reviewed in Schluter and Piper, 1998). In neonatal cardiomyocytes, PTH increases beating frequency through a cAMP-dependent pathway. These effects are associated with increased L-type calcium currents, precisely the opposite of what is observed in vascular smooth muscle cells. In contrast, in adult rat cardiac myocytes, both PTH(1−34) and PTHrP(1–34) increase the rate of spontaneous contraction, but only PTHrP was found to stimulate cAMP accumulation. The reason for this difference is unclear but may relate to the coupling of the PTH1R receptor to different G proteins. PTH has also been shown to elicit a hypertrophic response in adult rat cardiomyocytes characterized by increased protein synthesis, cell mass, and the reexpression of embryonic cardiac proteins. These effects, together with clinical observations of patients with elevated PTH and increased left ventricular mass, have been interpreted as evidence for a pathogenic role of PTH in ventricular hypertrophy. Finally, as discussed earlier, the timing (E9–10) of embryonic death occurring in PTH/PTHrP receptor-null mice suggests that PTHrP functions during heart development. Consistent with this hypothesis, disruption of the receptor is associated with cardiomyocyte death likely as a result of calcium dysregulation (Qian *et al.*, 2003).

Insight into the global actions of PTHrP in the cardiovascular system has come from studies in genetically manipulated mice. Transgenic mice overexpressing either PTHrP or PTH1R in smooth muscle have reduced systemic blood pressure consistent with the prediction that PTHrP acts as a local vasodilator (Qian *et al.*, 1999). In aortic ring preparations from PTHrP-overexpressing mice, the relaxant effects of both PTHrP and acetylcholine seen in nontransgenic mice were attenuated markedly in aortas from PTHrP-overexpressing mice. This finding suggests that local overexpression of PTHrP not only desensitizes the vasculature to PTHrP, but also dampens relaxation to acetylcholine and perhaps other vasorelaxants. Thus, it appears that prolonged stimulation of the PTH1R and the consequent increase in cAMP converge on signaling circuitry used by acetylcholine.

PTH-Related Proteins and Hypertensive States

Several lines of circumstantial evidence suggest that PTH and PTHrP alter vascular tone in hypertensive humans and animals. For example, primary hyperparathyroidism is commonly associated with hypertension that may be corrected upon removal of the parathyroid lesion (Young *et al.,* 1988). However, because alterations in circulating PTH also influence other regulators of vascular tone (e.g., ionized calcium), it is probable that the hypertension seen in long-term hyperparathyroidism is a secondary event. Alternatively, prolonged exposure to elevated PTH concentrations in these patients could desensitize vascular tissue to PTH or PTHrP, thereby increasing vascular tone (Nyby *et al.,* 1995). A similar scenario appears to occur in two rat models of hypertension. For example, removal of the parathyroid glands in the spontaneously hypertensive rat (SHR) and the desoxycorticosterone acetate (DOCA) salt hypertensive rat attenuates the development of hypertension (Schleiffer, 1992). Moreover, the PTH-induced changes in urinary cAMP, magnesium, calcium, and phosphorus responses are blunted in the SH rats, again suggesting a desensitization of the PTH1R receptor. The apparent resistance to PTH and PTHrP in humans and rats with hypertension described earlier prompted Pang and coworkers (1991) to propose the existence of an additional "hypertensive" factor made in the parathyroid gland. However, despite more than a decade of work on this putative hypertensive factor, its precise structure is still unknown.

NEUROLOGICAL ACTIONS

Introduction

As noted earlier, interest in potential regulation of excitable cells by PTH/PTHrP began with Collip and Clark's demonstration in 1925 that parathyroid extracts had hypotensive effects in the dog. For the next 60 years, PTH was the focus of work in both vascular and nonvascular smooth muscle and in neurons. In smooth muscle, it now seems quite clear that the physiological regulator is actually PTHrP, acting on the PTH1R. The best functional evidence suggests that very similar trends are apparent in the central nervous system (CNS), in that PTHrP acts on the PTH1R. Additionally, the pivotal target of PTH/PTHrP regulation is the L-VSCC, because PTH and/or PTHrP appear to be capable of either inhibiting of stimulating L-VSCC-mediated Ca^{2+} influx, depending on the cell/tissue in question (Wang *et al.,* 1991a). However, in the CNS PTH may influence pituitary function, and the recently described TIP39 acting on the PTH2R may prove to be an important CNS regulatory system.

PTH/PTHrP Gene Family Expression in the CNS

The CNS was one of the first sites to be examined in detail for PTHrP gene expression, and the gene was found to be widely expressed in neurons of the cerebral cortex, hippocampus, and cerebellum (Weir *et al.,* 1990) (Fig. 5). This work was extended by a second survey, which included the PTH1R as well as PTHrP (Weaver *et al.,* 1995). Both were found to be widely expressed, and they colocalized in a number of sites. It was noted at the time that the hot spots for PTHrP gene expression are neuronal populations that have a number of features in common, including high-density L-VSCC expression as well as high-density

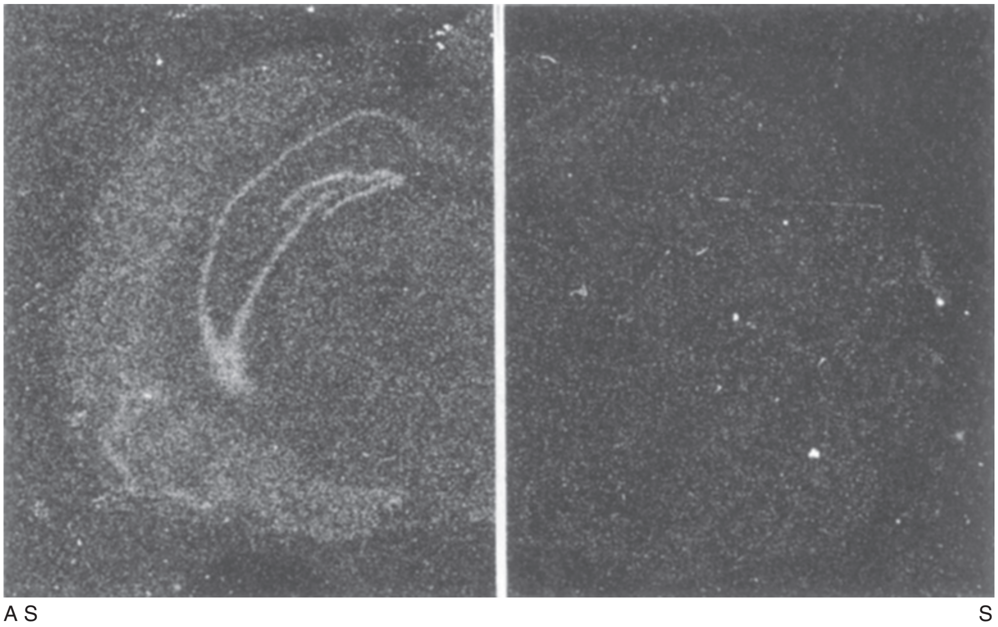

FIGURE 5 In situ hybridization histochemistry of PTHrP mRNA in the CNS of the rat using antisense (AS) and sense (S) oligonucleotides. The dentate gyrus and CA fields of the hippocampus are strongly positive. There are also many PTHrP-positive neurons scattered throughout the cerebral cortex. From Weir *et al.* (1990), with permission.

expression of excitatory amino acid receptors and a known susceptibility to excitotoxicity. The implications of these common features will become clear later in this chapter.

Although early histochemical studies suggested that PTH might be present in a number of neuronal populations, the most careful and reproducible work has localized PTH to nuclei on the hypothalamus with projections into the portal system (Pang *et al.*, 1988; Harvey and Fraser, 1993). The implication is that PTH may regulate pituitary function, specifically including prolactin secretion (Harvey and Fraser, 1993).

Usdin and colleagues identified the PTH2R in a cerebral cortical cDNA library by homology screening in 1995. This receptor is sensitive to PTH(1−34) (EC_{50} about 1 nM) but is unresponsive to PTHrP(1–36). The PTH2R is expressed most abundantly in several basal forebrain nuclei and hypothalamic nuclei (Usdin *et al.*, 1996). This group has succeeded in purifying the natural ligand for this receptor by using a staggering 50 lbs of bovine hypothalamus as starting material (Usdin *et al.*, 1999). This ligand is a small unmodified peptide of 39 amino acids referred to as tuberoinfundibular peptide 39 (TIP39), and it bears only 9 of 39 amino acids that are identical to those of bovine PTH. Only limited structure–function work has been done, but TIP39 is at least as potent as PTH(1–34) at the PTH2R and may be one or two orders of magnitude more potent than PTH, depending on the species of origin of the PTH2R (Usdin *et al.*, 1999). The sites of PTH2R expression imply potential TIP39 function in regulating the pituitary and in modulating pain sensitivity.

An additional neuroendocrine role for PTHrP has been proposed in osmoregulation. PTHrP is localized to the paraventricular (PVN) and supraoptic nuclei (SON), the population of neurons that synthesize arginine vasopressin (AVP) (Weaver *et al.*, 1995; Tokunaga *et al.*, 1997; Yamamoto *et al.*, 2002). Axonal projections from the PVN and SON terminate in the posterior pituitary where AVP is released in response to changes in plasma osmolality and volume. In response to these changes, AVP stimulates renal tubular water reabsorption and exerts vasoconstrictive effects.

The central administration of PTHrP results in the secretion and expression of AVP from the PVN and SON (Yamamoto *et al.*, 1998). AVP and PTHrP expression are also mutually regulated in dissociated SON neuronal cultures. PTHrP stimulates the intracellular accumulation of cAMP in SON neurons and the secretion of AVP in a PKA-dependent manner. However, the inability of PTH(1−34) to exert any effect on AVP secretion suggests that PTHrP acts via a novel adenylyl cyclase-coupled receptor subtype in SON neurons. In contrast, stimulation of PTHrP by AVP is PKC-dependent and is mediated via the type 1 AVP receptor (V1a and V1b), which couple to phospholipase signaling and the generation of inositol 1,4,5-triphosphate and diacylglycerol (Yamamoto *et al.*, 2002).

Thus, three ligands and at least two receptors of the PTH/PTHrP gene family are expressed in the CNS. Two of the ligands (PTH and TIP39) are expressed in highly discrete locations, whereas PTHrP is widely expressed in neuronal populations throughout the brain.

Calcium Channels, Neuromodulation, and Signaling Microdomains

Calcium Channels

Calcium channels are heteromeric associations of four or five subunits (Walker and de Waard, 1998). The α_1 subunit is the pore-forming structure that is responsible for permeation as well as gating function of the channel. There are a half-dozen classes of calcium channels, each defined by a specific α_1 gene. Given the number of different genes for each subunit and alternate splicing of these gene products, the combinatorial possibilities are enormous (perhaps 1000).

In brief, calcium channels are either L-type or non-L-type (e.g., N, P/Q, T, and R channels) (Walker and de Waard, 1998). L-type channels mediate large and long-lasting Ca^{2+} fluxes (therefore “L”) and are composed of three subclasses, defined by their α_1 subunits, as well as by the locations in which they were initially identified. These are S (“skeletal,” α_1s), C (“cardiac,” α_1c), and D (neuroendocrine, α_1d). L channels are dihydropyridine sensitive, and there are a number of classes of these widely used drugs (e.g., nifedipine, diltiazem).

Virtually every class of calcium channel is expressed in the CNS (Walker and de Waard, 1998). N and P/Q channels are expressed in both pre- and postsynaptic locations and are involved in the regulation of synaptic transmission. L channels are widely expressed in neurons throughout the brain and are found only in postsynaptic locations, specifically on cell bodies and proximal dendrites (Hell *et al.*, 1993). This localization is crucial to L channel function. These channels appear to regulate cytosolic Ca^{2+} levels in the soma and proximal dendrites of neurons as a function of the integrated excitatory synaptic input into these locations (Hell *et al.*, 1993). Given the location and gating of these channels, it is quite clear that their Ca^{2+} currents are not involved in neurotransmission, but rather with fundamental aspects of neuronal cell biology such as regulation of cellular signaling pathways and regulation of gene expression.

Neuromodulation

The clustering of L-VSCCs on neuronal cell bodies is also characteristic of the location of neuropeptide/growth factor receptors. This clustering of receptors is strategically convenient to the nucleus as well as to the regulation of channels of all sorts and the capacity of peptides and growth factors to crosstalk with each other (Hökfelt, 1991). This kind of short-range autocrine/paracrine signaling to the soma and proximal processes of neurons is referred to as "neuromodulation" to emphasize that the regulation and signaling involved are very different from neurotransmission (Hökfelt, 1991).

Signaling Microdomains

Even a generation ago, it was clear that signal transduction corresponded to more than cells simply serving as bags of rising and falling tides of cyclic nucleotides and Ca^{2+}, but the biochemical details that account for the exquisite specificity of signal transduction have become clear only in the past decade. The work of Ghosh and Greenberg (1995) has provided insight into the specificity of neuronal Ca^{2+} signaling. Depending on the specific route of entry into a neuron, Ca^{2+} has highly specific and differential effects on a wide variety of neuronal processes, such as gene expression, learning and memory, modulation of synaptic strength, and Ca^{2+}-mediated cell death (Ghosh and Greenberg, 1995). For example, Ca^{2+} entry via L-VSCCs elicits an entirely different response in terms of gene expression than Ca^{2+} entry mediated via *N*-methyl D-aspartate (NMDA) receptors (Ghosh and Greenberg, 1995). Clearly, every calcium ion entering the cytosol of a neuron is not perceived in the same way. Similarly, cAMP generated in a neuron by a voltage-sensitive adenylate cyclase as opposed to a G protein coupled to a hormone receptor is not perceived by the cell in the same way.

A major advance in understanding the specificity of signaling has come from the recognition that microdomains exist at the cell surface that cluster together the receptor/channel in question, the PKA and/or PKC transducers, and the target to be modified. The key players that account for this clustering of specific signaling components are the A kinase anchoring proteins (AKAPs) and the receptors for activated C kinase (RACKs) (Mochly-Rosen, 1995). In certain cases, a single AKAP is capable of binding both PKA and PKC, thus serving as a scaffold that brings together all of the early components of a complex regulatory system. The net result of this tethering of signaling receptor, transducer, and target into a microdomain is a tremendous resolution in terms of specificity and speed.

PTHrP Is Neuroprotective

PTHrP Gene Expression in Neurons Is Regulated by L-VSCC Ca^{2+} Influx

It is now apparent that the regulation of PTHrP gene expression in cerebellar granule cells is a classic example of the kind of specificity of Ca^{2+} signaling described in the previous section. Cerebellar granule cells are a hot spot of PTHrP and PTH1R expression *in vivo* (Weir *et al.*, 1990; Weaver *et al.*, 1995), and cultured cerebellar granule cells are a commonly used neuronal model system *in vitro*. PTHrP gene expression in these cells is a direct function of depolarization, which triggers L-VSCC Ca^{2+} influx that tracks to the PTHrP gene via the calmodulin-CaM kinase cascade (Holt *et al.*, 1996; Ono *et al.*, 1997). Ca^{2+} entry into granule cells by any other means (e.g., veratridine treatment) has no influence whatsoever on the PTHrP gene. In granule cells, as in most other cells that express the PTHrP gene, PTHrP is a constitutive secretory product, so that the quantity of PTHrP secreted by the cell is a linear function of the level of PTHrP mRNA expression. PTHrP immunolocalizes principally to the granule cell soma (Holt *et al.*, 1996) so that it is presumably secreted by the cell bodies themselves, acting in the autocrine/paracrine fashion typical of a neuromodulatory peptide.

PTHrP Inhibits L-VSCC Ca^{2+} Influx, Defining a Protective Feedback Loop

Overstimulation can lead to neuronal injury or death, a process referred to as excitotoxicity, which may be brought about in two conditions. High concentrations of the excitatory amino acid glutamate cause a generalized influx of cations and a collapse in mitochondrial function leading to almost immediate necrosis (Ankarcrona *et al.*, 1995). Lower concentrations of glutamate or exposure to other excitotoxins, such as kainic acid, trigger Ca^{2+} entry via L-VSCCs, which leads to excitotoxicity characterized by a long latency (6–24 hours to cell death) (Ankarcrona *et al.*, 1995; Weiss *et al.*, 1990).

The granule cell system is subject to both immediate and latent forms of excitotoxicity. A low concentration of kainic acid produces about 50% granule cell death at 24 hours, and the calcium channel blocker nitrendipine is capable of fully protecting these cells, thereby defining the central importance of L-VSCC Ca^{2+} influx in long-latency excitotoxicity (Brines *et al.*, 1999). It will be recalled that PTH has been shown to inhibit L-VSCCs in smooth muscle and neuroblastoma cells (Wang *et al.*, 1991; Pang *et al.*, 1990). This led to the working hypothesis that PTHrP might be capable of inhibiting L-VSCC Ca^{2+} influx in cerebellar granular cells, which proved to be the case. PTHrP was found to be fully neuroprotective in kainic acid-treated granule cells (Fig. 6) and was as effective as nitrendipine in reducing kainic acid-induced L-VSCC Ca^{2+} influx (Brines *et al.*, 1999). Pang *et al.* (1990) used whole-cell patch-clamp techniques to demonstrate that PTH is capable of inhibiting L-VSCC Ca^{2+} influx in mouse neuroblastoma cells and one of us (AEB) has used patch-clamp techniques to demonstrate the same findings with PTHrP in these cells. This effect is mediated by the PTH1R, but nothing is yet known of the mechanism by which the channel is actually regulated.

Although the PTHrP knockout mouse dies at birth as a result of systemic chondrodystrophy, a genetic strategy has been used to generate a mouse that is PTHrP sufficient in chondrocytes but PTHrP null in all other sites (Wysolmerski *et al.,* 1998). There are no CNS abnormalities per se in the rescued mouse, but it displays a sixfold increase in sensitivity to kainic acid. Thus, as might be predicted from the findings in cultured cerebellar granule cells described earlier, PTHrP appears to be provided with a defense against excitotoxicity that is operative *in vivo* (Chatterjee *et al.,* 2002).

Taken together, these findings indicate that PTHrP serves as an endogenous L-VSCC regulator that functions in a neuroprotective feedback loop of the sort depicted in Fig. 7. As shown, the L-VSCC itself is the fulcrum of this loop, and the rheostat is L-VSCC Ca^{2+} entry. This loop would provide neuroprotection to individual (autocrine) and neighboring (paracrine) neurons.

Other Potential Calcium Channel Effects

PTHrP increases L-VSCC activity and thereby enhances dopamine secretion in PC-12 cells (Brines and Broadus, 1999). Additionally, PTH and/or PTHrP has been reported to increase calcium channel-like activity in snail neurons (Kostyuk *et al.,* 1992) and in rat hippocampal neurons (Hirasawa *et al.,* 1998; Fukayama *et al.,* 1995), but these effects are slow and perhaps involve channels other than the classic L-VSCC. UMR-106 osteoblast-like cells contain L-VSCCs that are stimulated by PTH treatment (Barry *et al.,* 1995). L-VSCCs are also widely expressed in a great many other excitable and nonexcitable cells that have thus far not been examined with respect to PTHrP regulation.

PTHrP in the Peripheral Nervous System

PTHrP as a Regulator of Schwann Cell Differentiation

One of the most well characterized functions of PTHrP is its role in regulating the differentiation state of chondrocytes during endochondrial bone development and growth. The growth plate is an exceptional example of a tissue that provides a “snapshot” of cells progressing through its own developmental program. An increase in PTHrP production by growth plate chondrocytes delays the differentiation and maturation of these cells, maintaining a proliferative phenotype and slowing chondrocyte differentiation so that premature ossification of bone does not occur during development (Kronenberg, 2006).

Although the development of tissues such as bone may seem a world away from the peripheral nervous system (PNS), the potential biological significance of PTHrP as a regulator of Schwann cell differentiation following nerve injury has recently been described (Macica *et al.*, 2006). Neurons of the PNS are able to regenerate after nerve injury, resulting in recovery of sensory and motor function (Ide, 1996). Just as the maintenance of an immature pool of chondrocytes is important during periods of bone growth, the dedifferentiation of Schwann cells to an immature phenotype in response to nerve injury is paramount to successful nerve regeneration.

Schwann cells, which are quiescent in normal myelinated and unmyelinated nerves, dedifferentiate and expand into phenotypically immature Schwann cells in response to nerve injury (Hall, 1999). Whereas myelin genes are downregulated upon denervation, a number of genes associated

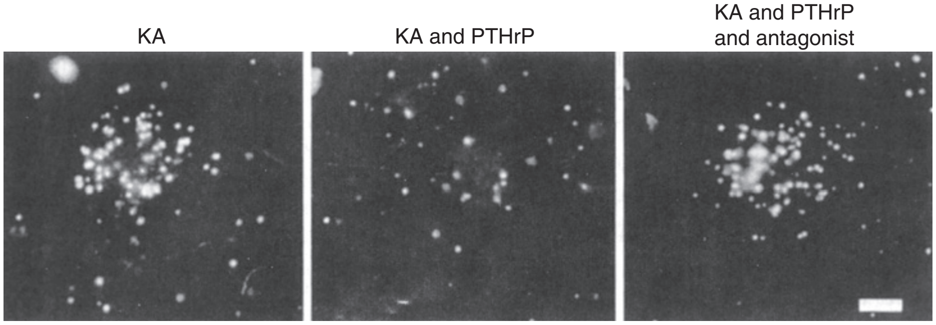

FIGURE 6 Cell death assessed by propidium iodide staining. Propidium iodide can bind to nuclear DNA only when the cell membrane is not intact; each bright dot therefore represents the nucleus of a dead cell. Kainic acid (KA) alone (*left*), KA plus PTHrP (*center*), and KA together with PTHrP and a 10-fold molar excess of a competitive antagonist of PTHrP binding (*right*). Percentage kill (±SEM) under these three conditions was 23 ± 3% ($n = 10$), 2 ± 2% ($n = 11$, $P < 0.001$ with respect to KA alone), and 23±2% ($n = 10$), respectively. Scale bar: 25 μm. From Brines *et al.* (1999), with permission.

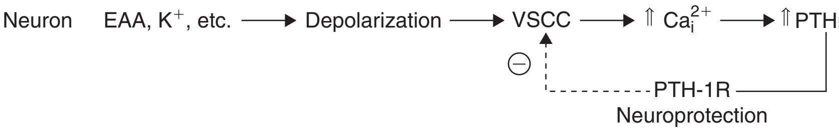

FIGURE 7 Schema of autofeedback loop in which PTHrP, triggered by L-VCSS Ca^{2+} influx, feeds back via the PTH1R to dampen L-VCSS Ca^{2+} currents.

with the immature nonmyelinating Schwann cells are upregulated including a change in the profile of adhesion molecules, growth factors, and growth factor receptors (Ide, 1996; Wanner, 2002). PTHrP expression strongly correlates with the immature Schwann cell phenotype and suggests that PTHrP may regulate the Schwann cell differentiation program following injury (Macica *et al.*, 2006).

PTHrP is localized to the PNS and is upregulated following sympathetic ganglion axotomy (Weaver *et al.*, 1995; Boeshore *et al.*, 2004). PTHrP mRNA is also upregulated *in vivo* following sciatic nerve injury and is a constitutively secreted product of dedifferentiated Schwann cells. Furthermore, consistent with reports that nuclear c-jun in Schwann cells is induced in injured sciatic nerve, c-jun was highly upregulated in nuclei of PTHrP-positive Schwann cells in crushed sciatic nerve (Shy *et al.*, 1996; Soares *et al.*, 2001; Macica *et al.*, 2006). These data suggest that loss of axonal contact may be a stimulus for Schwann cell-derived PTHrP mRNA upregulation. Indeed, by using purified Schwann cell cultures, PTHrP is a constitutively secreted product of immature Schwann cells. Conversely, when grown under conditions that stimulate Schwann cell differentiation and quiescence, PTHrP secretion is inhibited (Macica *et al.*, 2006).

PTHrP also potentiates the migration of Schwann cells in dorsal root ganglion (DRG) explants. Dedifferentiated Schwann cells retain the ability to migrate, a role that fulfills two functions important for successful regeneration. First, they migrate along regrowing axons from the proximal stump and segregate axons into groups of clustered fibers (Bunge, 1987). They ultimately form a one-to-one relationship with an axon, followed by myelin ensheathment and basal lamina deposition. Schwann cells also ensheath and segregate small, unmyelinated axons into separate troughs. Further, Schwann cells from both the proximal and distal stump form columns of cells along the basal lamina (bands of Büngner) that provide both a physical conduit and growth-promoting factors to regrowing axons (Ide, 1996). PTHrP can dramatically modulate the migration of Schwann cells along regrowing axons in DRG explants, a model of both nerve regeneration and Schwann cell migration that retains the physiological relationship between DRG axons and Schwann cells within an extracellular matrix (Macica *et al.*, 2006; Tonge *et al.*, 1997; Salzer and Bunge, 1980).

PTHrP also affects axonal bundling by Schwann cells. During development and following injury, immature Schwann cells migrate along outgrowing axons where they segregate axons into groups of clustered fibers (Bunge, 1987). Bundling of axons by migrating Schwann cells likewise occurs in DRG explants. DRG explants treated with exogenous PTHrP reveal a striking potentiation of axonal bundling by migrating Schwann cells. In contrast, explant cultures immunoneutralized with PTHrP antibody show significantly smaller axonal bundles and are only rarely associated with Schwann cells owing to the relative decrease in Schwann cell migration (Macica *et al.*, 2006).

Thus, PTHrP may play a pivotal role in Schwann cell dedifferentiation, one of the earliest events following loss of axonal contact and one that precedes Schwann cell proliferation. PTHrP may therefore promote Schwann cell dedifferentiation by downregulating genes or gene products that are typical of differentiated Schwann cells.

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Chapter 35

$1\alpha,25(OH)_2$ Vitamin D_3

Vitamin D Nuclear Receptor (VDR) and Plasma Vitamin D-Binding Protein (DBP) Structures and Ligand Shape Preferences for Genomic and Rapid Biological Responses

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INTRODUCTION TO VITAMIN D AND $1\alpha,25(OH)_2D_3$

Structure

The term vitamin D designates a group of closely related secosteroids (Fig. 1). The two most prominent members of this group are cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2) (Norman *et al.*, 2006). Cholecalciferol is produced from 7-dehydrocholesterol that is present in skin of humans and most animals; it is the natural form of vitamin D that since the 1950s has been widely employed for food fortification (Norman 2003; Norman *et al.*, 2007) and as a vitamin supplement (Norman *et al.*, 2006). Ergocalciferol is derived from a common plant steroid, ergosterol, and is the form that was employed for vitamin D fortification of foods in the United States from the 1940s to 1960s.

The chemical structures of vitamin D and its daughter metabolites are closely related structurally to their provitamin forms as well as to the four-ring nucleus of other classical steroids that are derived from the cyclopentanoperhydrophenanthrene ring system (see Fig. 1). The official nomenclature proposed for vitamin D by the International Union of Pure and Applied Chemistry (IUPAC) relates it to the steroid nucleus, which is numbered as shown in Figure 1 for provitamins (The Commission on the Nomenclature of Biological Chemistry, 1960). The carbons in vitamin D retain the same number as designated in the provitamins. No vitamin D biological activity becomes apparent until ring B of the provitamins is opened. Thus, vitamin D and its metabolites are simply steroids with a broken B ring as a consequence of rupture of the carbon–carbon bond between C-9 and C-10. The presence of the nonintact B ring in the steroid nucleus is officially designated by the use of the term "seco." The formal chemical name of vitamin D_3 is 9,10-secocholesta-5,7,10,(19)-trien-3β-ol and of $1\alpha,25(OH)_2D_3$ is 9,10-secocholesta-5,7,10,(19)-trien-1α,3β,25-triol. Vitamin D_3 is the form of vitamin D obtained when radiant energy from the sun strikes the skin and converts the precursor 7-dehydrocholesterol into vitamin D_3. Because the body is capable of producing cholecalciferol, vitamin D technically does not meet the classical definition of a vitamin, i.e., a substance required by the body, but which can not be made by the body. A more accurate description of vitamin D is that it is a prohormone. It has been shown that vitamin D is metabolized to a biologically active form, $1\alpha,25(OH)_2$vitamin D_3 [$1\alpha,25(OH)_2D_3$] that functions as a steroid hormone (Haussler *et al.*, 1968; Myrtle *et al.*, 1970; Norman *et al.*, 1971; Brickman *et al.*, 1972). However, because the parent vitamin D was first recognized as an essential nutrient, it continues to be classified among the fat-soluble vitamins. Indeed, even in the first decade of the twenty-first century there are classical examples of vitamin D deficiency in many countries as documented by low serum concentrations of $25(OH)D_3$ (e.g., DeLucia *et al.*, 2003; Rejnmark *et al.*, 2004; Hanley *et al.*, 2005; Hirani *et al.*, 2005; Tareen *et al.*, 2005).

Conformational Flexibility

Vitamin D_3 and all its daughter metabolites, including $1\alpha,25(OH)_2D_3$, are unusually conformationally flexible (Fig. 2). The structure of $1\alpha,25(OH)_2D_3$ is shown in Figure 2A.Three key aspects of the $1\alpha,25(OH)_2D_3$ molecule confer a unique range of conformational mobility on this molecule.

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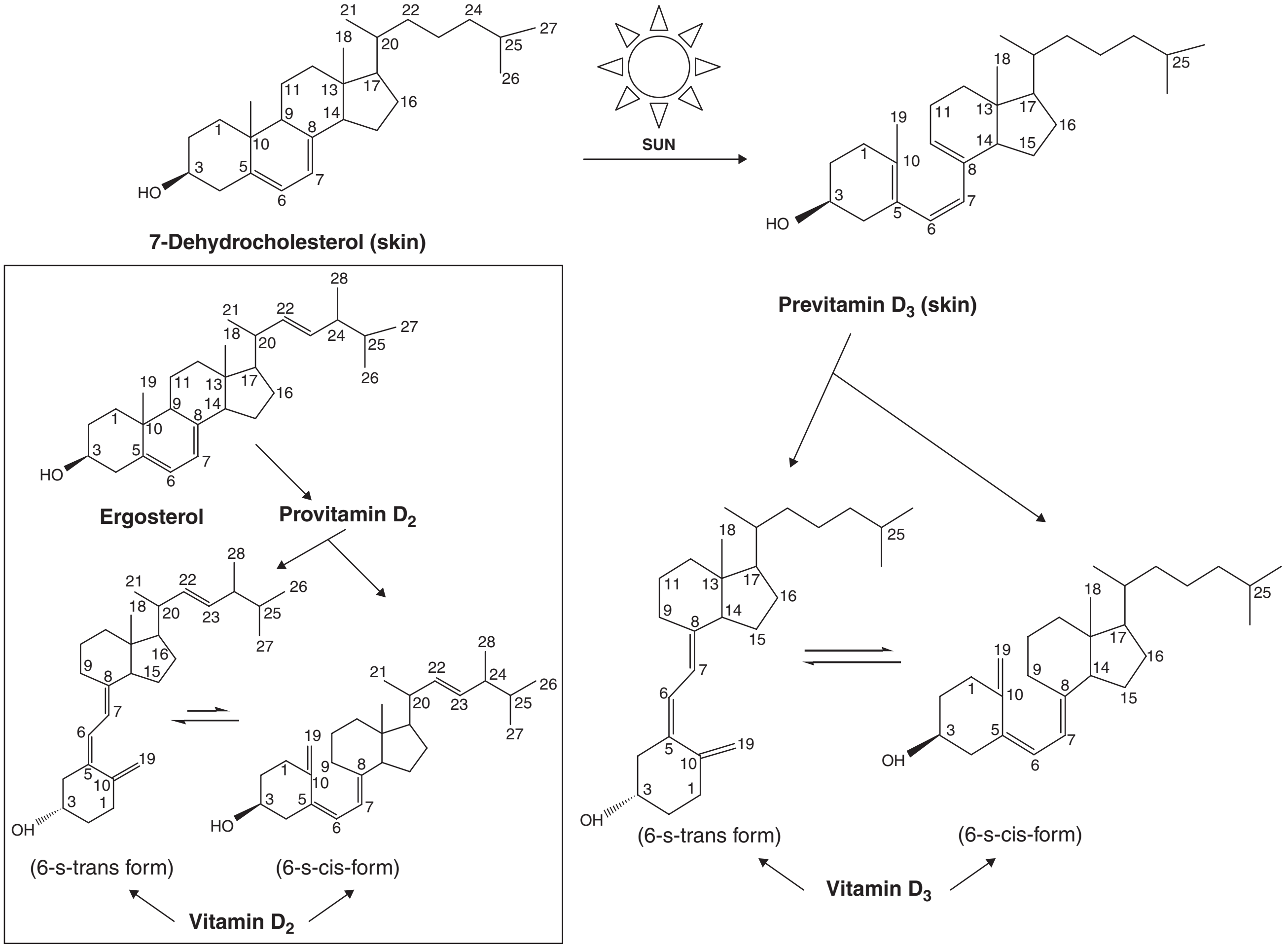
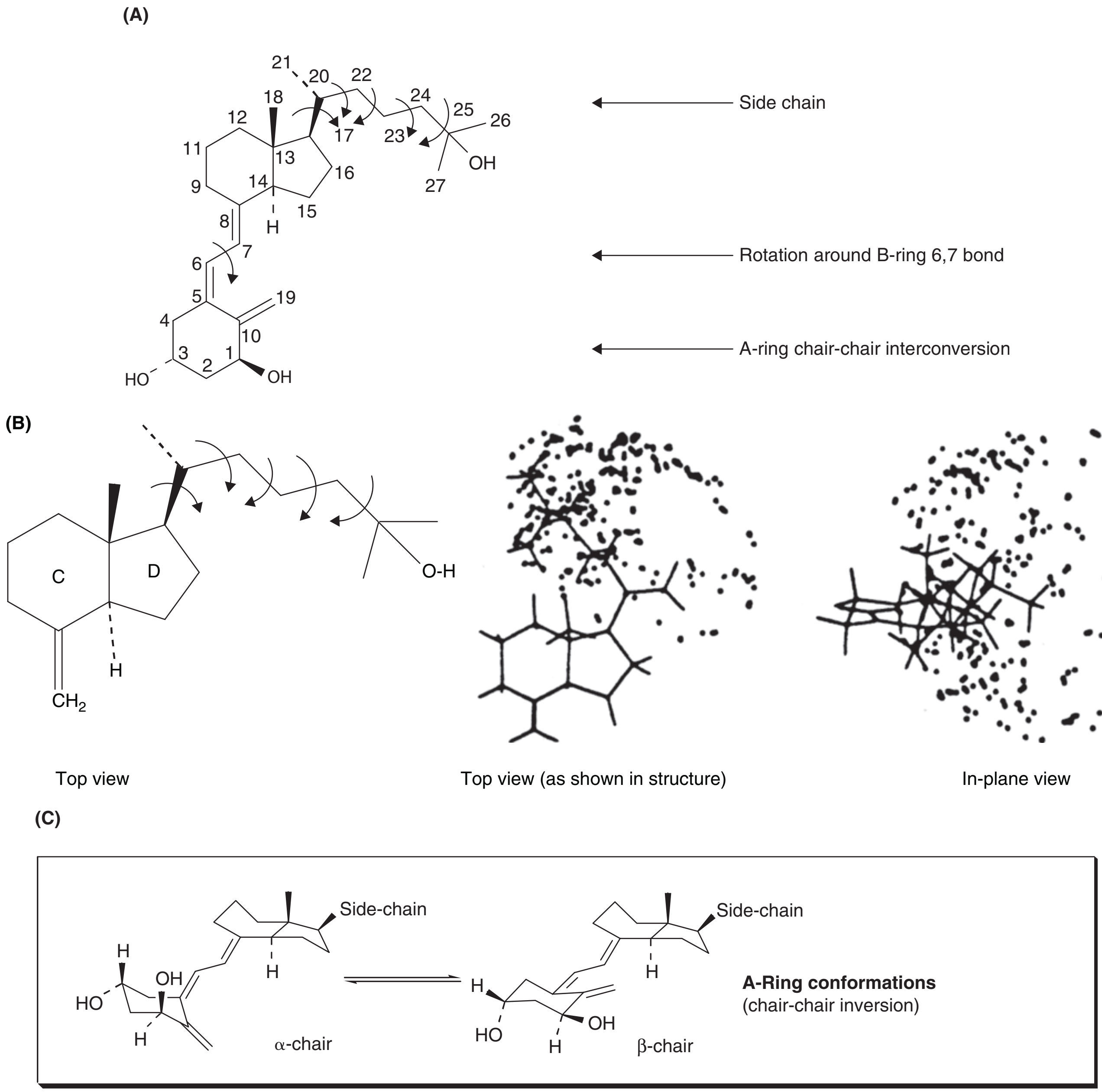


FIGURE 1 Chemistry and irradiation pathway for production of vitamin D3 (a natural process) and vitamin D_2 (a commercial process). In each instance, the provitamin, which is characterized by the presence in the B ring of a Δ5, Δ7-conjugated double-bond system, is converted to the seco-B previtamin steroid, where the 9,10 carbon–carbon bond has been broken. Then the previtamin D, in a process independent of ultraviolet light, thermally isomerizes to the vitamin form, which is characterized by a Δ5,6, Δ7,8, Δ10,19-conjugated triple bond system. In solution (and in biological systems) vitamin D is capable of assuming a large number of conformational shapes because of rotation about the 6,7 carbon–carbon single bond of the B ring.

(1) The intact 8-carbon side chain of vitamin D and related secosteroids can easily assume numerous shapes and positions in three-dimensional space by virtue of rotation about its five carbon–carbon single bonds (see Fig. 2B). A discussion of the consequences of the side-chain conformational mobility has been previously presented (Okamura *et al.*, 1992; Midland *et al.*, 1993). (2) The cyclohexane-like A ring is free to rapidly interchange (many thousands of times per second) between a pair of chair–chair conformers (see Fig. 2C); this has the consequence of changing the orientation of the key 1α− and 3β-hydroxyls between either an equatorial or an axial orientation (Wing *et al.*, 1974). (3) Rotational freedom about the 6–7 carbon–carbon bond of the seco-B ring allows conformations ranging from the more steroidlike 6-s-*cis* conformation to the open and extended 6-s-*trans* form of the hormone (see Fig.2D) (Norman *et al.*, 1993b). In general, it is accepted that this conformational mobility of vitamin D secosteroids is displayed by the molecules in both an organic solvent as well as an aqueous environment similar to that encountered in biological systems. Thus, receptors for 1α,25$(OH)_2D_3$ have had to accommodate to the reality of binding a highly conformationally flexible ligand.

VITAMIN D ENDOCRINE SYSTEM

The concept of the existence of the vitamin D endocrine system is firmly established (Reichel *et al.*, 1989; Bouillon *et al.*, 1995) (Fig. 3). The key organ in this endocrine system is the kidney where the renal proximal tubule is responsible for producing the hormonal 1α,25$(OH)_2D_3$ that circulates in the blood in accordance with strict physiological signals (Henry, 2000). The parent vitamin D_3 is metabolized to 25$(OH)D_3$ (by the liver) and then to 1α,25$(OH)_2D_3$ and 24R,25$(OH)_2D_3$ (by the endocrine gland and the kidney) as well as to at least 34


(A)
21
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18
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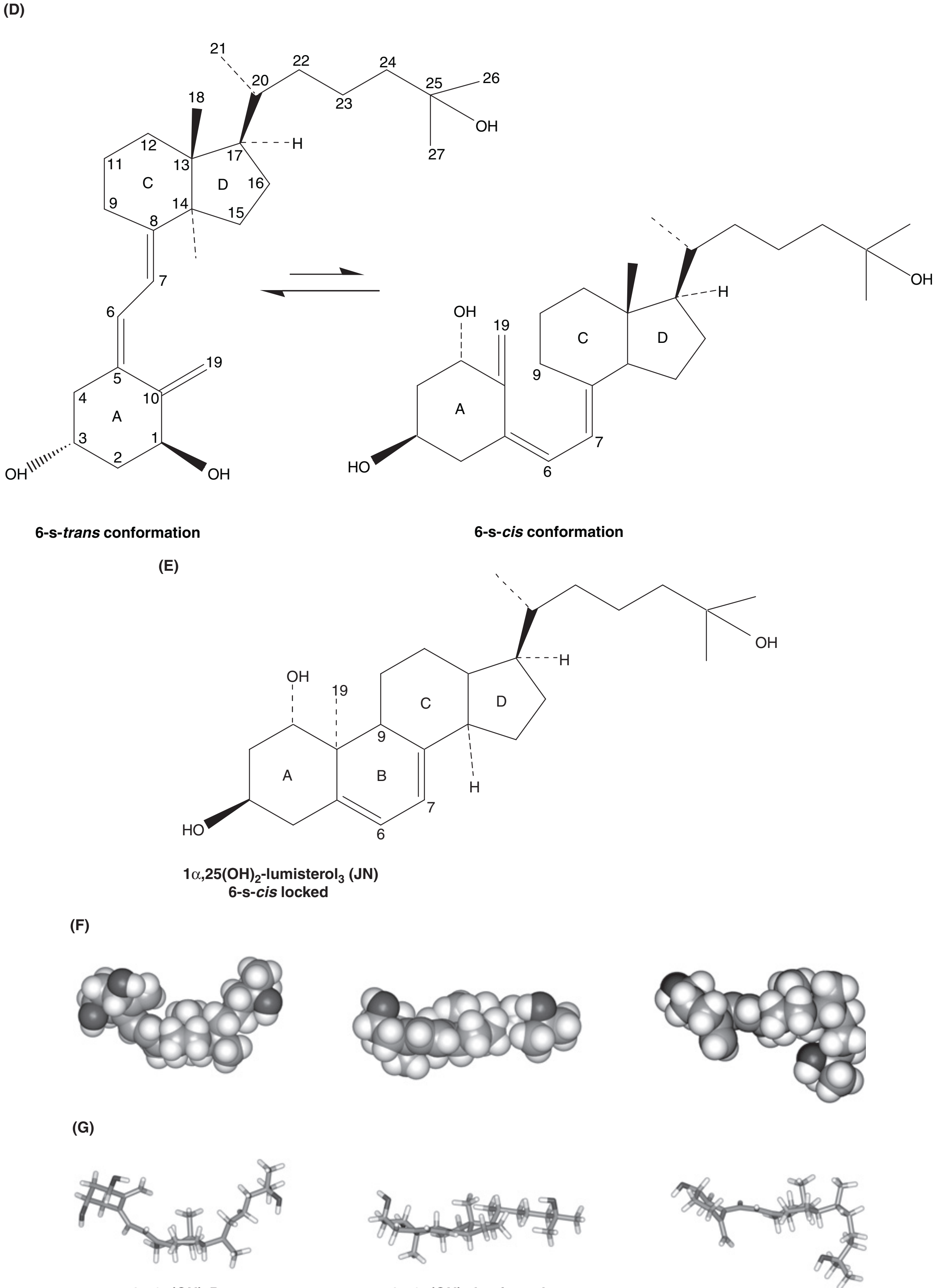
25
26
12
17
23
11
13
16
OH
9
14
27
15
8
H
7
6
5
19
4
10
3
1
2
HO
OH
Side chain
Rotation around B-ring 6,7 bond
A-ring chair-chair interconversion
(B)
C
D
O-H
H
CH_2
Top view
Top view (as shown in structure)
In-plane view
(C)
Side-chain
Side-chain
H
OH
HO
H
H
H
H
HO
H
OH
α-chair
β-chair
A-Ring conformations
(chair-chair inversion)

FIGURE 2 Structure of $1\alpha,25(OH)_2D_3$ illustrating the three structural aspects of vitamin D secosteroids that contribute to the conformational flexibility of these molecules. **(A)** Structure of $1\alpha,25(OH)^2D^3$ with indication of the three structural features of the molecule that confer conformational flexibility on this molecule. **(B)** The dynamic rotation of the cholesterol-like side chain of $1\alpha,25(OH)_2D_3$ with 360° rotations about the five single carbon–carbon bonds indicated by the curved arrows. The dots indicate the position in three-dimensional space of the 25-hydroxyl group for some 394 readily identifiable side-chain conformations. A discussion of the consequences of the side-chain conformational mobility has been presented (Okamura *et al*., 1992; Midland *et al*., 1993; Okamura *et al*., 1994). **(C)** The rapid (millions of times per second) chair–chair interconversion of the cyclohexane-like A ring of the secosteroid between chair conformer A and chair conformer B; this effectively equilibrates the 1α- and 3β-hydroxyls between the axial and equatorial orientations. **(D)** Rotational freedom about the 6–7 carbon–carbon single bond of the seco-B ring allows conformations ranging from the open and extended 6-s-*trans* conformation, utilized by the VDR for genomic responses, to the more steroid-like 6-s-*cis* form of the hormone believed to be used by the VDR for RR. **(E)** Structure of $1\alpha,25(OH)_2$-lumisterol (JN) that is chemically locked in a permanent 6-s-*cis* shape and has potency equivalent to $1\alpha,25(OH)_2D_3$ in mediating RR (see text). Space-filling **(F)** and stick representations **(G)** illustrating the clear differences in their shapes of the three optimal ligands for the nuclear localized VDR (left), the membrane-caveolae-localized VDR (*center*), and the plasma vitamin D-binding protein (*right*). The nuclear VDR ligand $[1\alpha,25(OH)_2D_3]$ is in a bowl-shaped, twisted 6-s-*trans* conformation with the A ring and side chain both 300 above the plane of the C/D ring. The relatively planar 6-s-*cis* locked $[1\alpha,25(OH)_2$-lumisterol$]$ is an optimal ligand for the membrane VDR. The DBP ligand $[25(OH)D_3]$ is in a twisted 6-s-*trans* orientation with the A ring C10-C19 exocyclic alkene 300 below the plane of the C/D ring and the side chain almost 900 below the C/D ring plane. Each of the illustrated molecules has its 25-OH pointed to the "right" and the A ring to the "left." The shapes of the nuclear VDR (Rochel *et al*., 2000) and DBP (Verboven *et al*., 2002) ligands were learned from the separate determination of the x-ray structure of each protein, whereas the shape of the membrane VDR ligand was determined by computer modeling.


(D)
21
22
24
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25
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OH
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17
H
11
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C
D
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5
4
10
A
3
1
2
OH
OH
H
OH
19
C
D
9
A
7
HO
6
(E)
OH
H
OH
19
C
D
9
A
B
7
H
HO
6
(F)
(G)

6-s-*trans* conformation

6-s-*cis* conformation

1α,25(OH)$_2$-lumisterol$_3$ (JN)
6-s-*cis* locked

1α,25(OH)$_2$D$_3$
VDR$_{nuc}$

1α,25(OH)$_2$-lumisterol
VDR$_{mem}$

25(OH)D$_3$
DBP

FIGURE 2 (*Continued*)

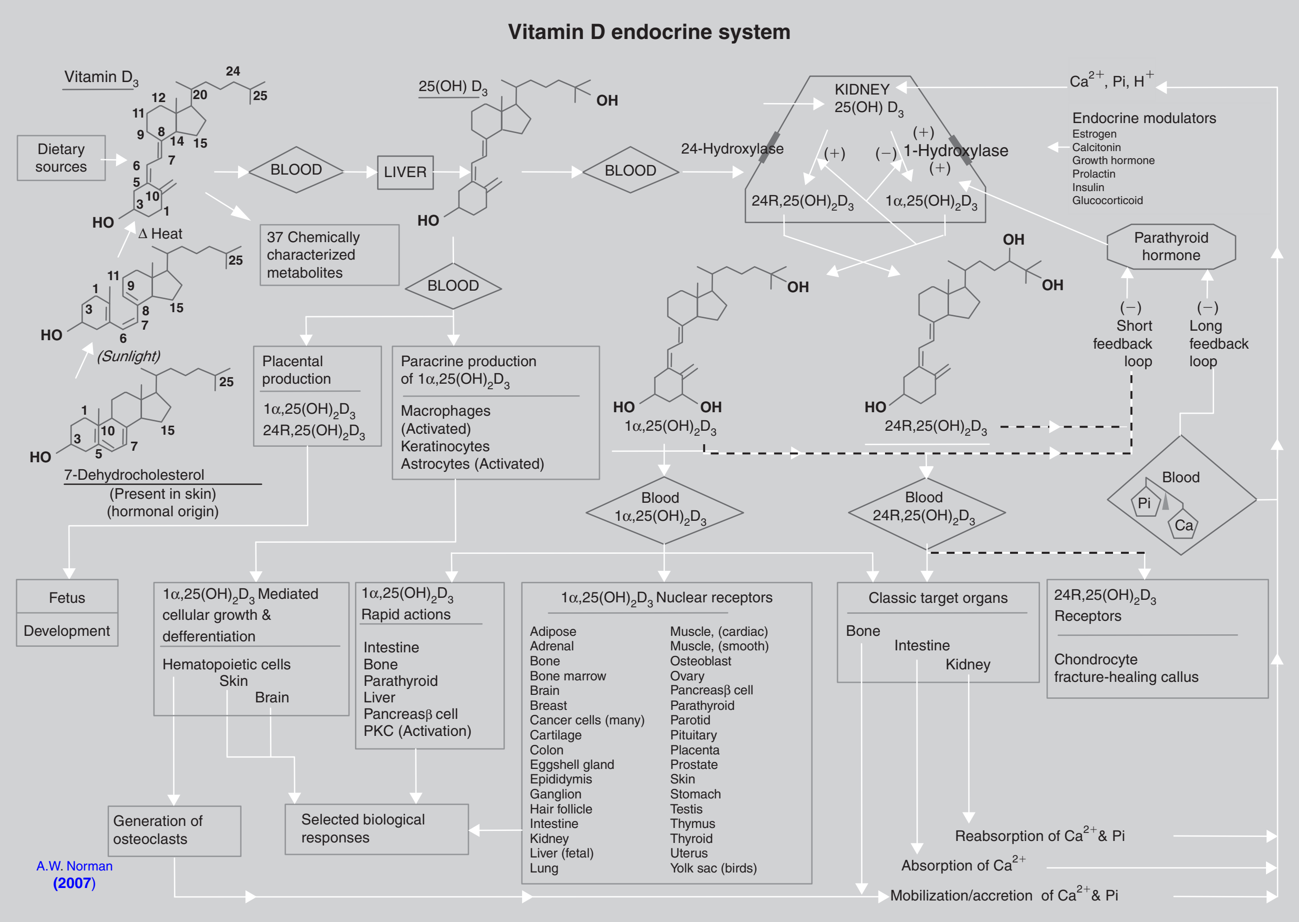
Vitamin D endocrine system
Vitamin D_3
Dietary sources
BLOOD
LIVER
25(OH) D_3
BLOOD
KIDNEY 25(OH) D_3
24-Hydroxylase
1-Hydroxylase
24R,25(OH)$_2$D$_3$
1α,25(OH)$_2$D$_3$
Ca^{2+}, Pi, H^+
Endocrine modulators
Estrogen
Calcitonin
Growth hormone
Prolactin
Insulin
Glucocorticoid
Parathyroid hormone
(−) Short feedback loop
(−) Long feedback loop
Δ Heat
(Sunlight)
37 Chemically characterized metabolites
7-Dehydrocholesterol
(Present in skin)
(hormonal origin)
BLOOD
Placental production
1α,25(OH)$_2$D$_3$
24R,25(OH)$_2$D$_3$
Paracrine production of 1α,25(OH)$_2$D$_3$
Macrophages (Activated)
Keratinocytes
Astrocytes (Activated)
1α,25(OH)$_2$D$_3$
24R,25(OH)$_2$D$_3$
Blood 1α,25(OH)$_2$D$_3$
Blood 24R,25(OH)$_2$D$_3$
Blood
Pi
Ca
Fetus
Development
1α,25(OH)$_2$D$_3$ Mediated cellular growth & defferentiation
Hematopoietic cells
Skin
Brain
1α,25(OH)$_2$D$_3$ Rapid actions
Intestine
Bone
Parathyroid
Liver
Pancreasβ cell
PKC (Activation)
1α,25(OH)$_2$D$_3$ Nuclear receptors
Adipose
Adrenal
Bone
Bone marrow
Brain
Breast
Cancer cells (many)
Cartilage
Colon
Eggshell gland
Epididymis
Ganglion
Hair follicle
Intestine
Kidney
Liver (fetal)
Lung
Muscle, (cardiac)
Muscle, (smooth)
Osteoblast
Ovary
Pancreasβ cell
Parathyroid
Parotid
Pituitary
Placenta
Prostate
Skin
Stomach
Testis
Thymus
Thyroid
Uterus
Yolk sac (birds)
Classic target organs
Bone
Intestine
Kidney
24R,25(OH)$_2$D$_3$ Receptors
Chondrocyte fracture-healing callus
Generation of osteoclasts
Selected biological responses
Reabsorption of Ca^{2+}& Pi
Absorption of Ca^{2+}
Mobilization/accretion of Ca^{2+}& Pi
A.W. Norman (2007)

FIGURE 3 Overview of the vitamin D endocrine system. The figure lists 34 target organs and cells types that are known to contain the nuclear vitamin D receptor (VDR$_{nuc}$). When the VDR$_{nuc}$ is occupied by its cognate ligand, the naturally occurring steroid hormone 1α,25(OH)$_2$D$_3$, this receptor-ligand complex is able to modulate genomic events (Hannah *et al.*, 1994; Carlberg, 2003; MacDonald *et al.*, 2004; Whitfield *et al.*, 2005; Norman, 2006). In addition 1α,25(OH)$_2$D$_3$ generates biological effects via rapid response pathways; these sites are listed in Table 2 and are discussed in the following references: Norman (1997), Norman *et al.* (2004), and Norman (2006) . There is also emerging evidence that 24R,25(OH)$_2$D$_3$ may also have important biological effects (Seo *et al.*, 1997a; Boyan *et al.*, 2001).

other metabolites (Bouillon *et al.*, 1995). The secosteroid 1α,25(OH)$_2$D$_3$ has been shown to initiate biological responses via regulation both of gene transcription as well as via a membrane-receptor that initiates rapid responses. As will be discussed later, the vitamin D nuclear receptor (VDR), which is present in the nucleus and responsible for regulation of gene transcription, is also known to be localized to membrane caveolae where it can initiate in rapid responses (Norman *et al.*, 2002b; Huhtakangas *et al.*, 2004). The rapid responses can, for example, involve opening of voltage-gated Ca^{2+} channels (Caffrey *et al.*, 1989) or the rapid stimulation of intestinal Ca^{2+} absorption known as transcaltachia (Nemere *et al.*, 1984b) (Fig. 4).

An additional key participant in the operation of the vitamin D endocrine system is the plasma vitamin D-binding protein (DBP), which carries vitamin D$_3$ and all its metabolites to their various target organs. The DBP is known to have a specific ligand-binding domain for vitamin D-related ligands that is different in specificity from the ligand-binding domain of the nuclear vitamin D receptor (Bishop *et al.*, 1994). The structure of these two key proteins of the vitamin D endocrine system, the VDR and DBP, is presented later in this chapter.

SIGNAL TRANSDUCTION PATHWAYS UTILIZED BY 1α,25(OH)$_2$D$_3$ TO GENERATE BIOLOGICAL RESPONSES

Figure 3 summarizes the 33 target organs that are known to possess the VDR$_{nuc}$ and in which there is an impressive amount of detail concerning regulation of gene transcription (Hannah *et al.*, 1994; Sutton *et al.*, 2003; Carlberg, 2004; Pike *et al.*, 2005; Carlberg *et al.*, 2006).

Figure 4 presents a schematic model that postulates that the various biological responses generated by

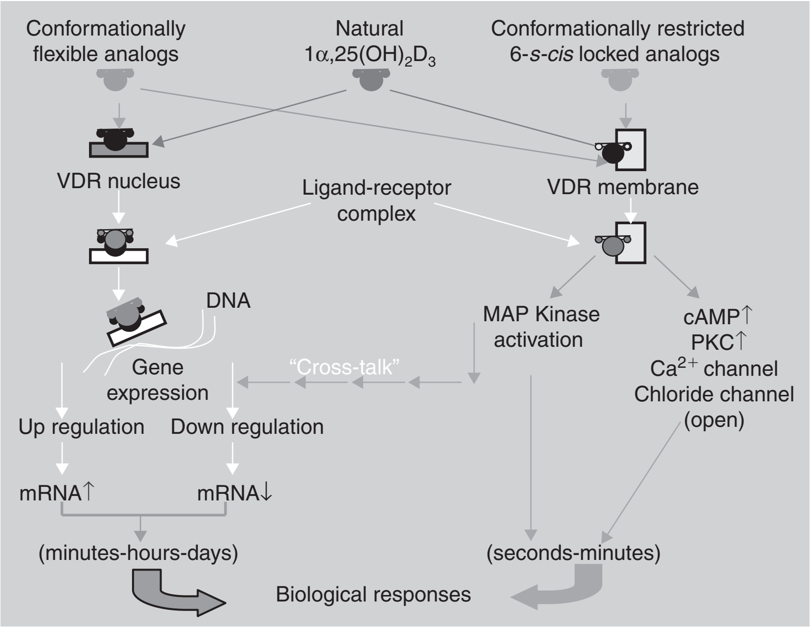

FIGURE 4 Proposed signal transduction pathways utilized by $1\alpha,25(OH)_2D_3$ (hat icon) and analogs of $1\alpha,25(OH)_2D_3$ to generate biological responses. Different shapes of $1\alpha,25(OH)_2D_3$ and its conformationally flexible analogs (spiked hat icon) may interact with either the VDR_{nuc} or the VDR_{mem} to initiate different signal transduction pathways that result in genomic responses or rapid responses. In contrast, the conformationally restricted 6-s-*cis* analogs restricted (half hat icon) (see Figure 1; JM, JN) only can interact with VDR_{mem}. In the genomic pathway (*left side*), occupancy of the nuclear receptor for $1\alpha,25(OH)_2D_3$ (VDR_{nuc}) by a ligand leads to an up- or downregulation of genes subject to hormone regulation. More than 50 proteins are known to be transcriptionally regulated by $1\alpha,25(OH)_2D_3$ [see Table I and Hannah *et al.*, 1994]. In the membrane-initiated pathway (*right side*), occupancy of a putative membrane receptor for $1\alpha,25(OH)_2D_3$ by a ligand is believed to rapidly lead to activation of a number of signal transduction pathways, including adenylate cyclase, phospholipase C, protein kinase C (PKC), mitogen-activated protein kinase (MAP kinase), and/or opening of voltage-gated L-type Ca^{2+} channels, which are either individually or collectively coupled to generation of the biological response(s).

$1\alpha,25(OH)_2D_3$ depend on two types of receptors. These are the classic nuclear receptor for $1\alpha,25(OH)_2D_3$ (designated as VDR_{nuc}) and a membrane receptor (designated as VDR_{mem}). It has been postulated that the conformationally flexible steroid hormone $1\alpha,25(OH)_2D_3$ generates biological responses using different shapes so as to selectively activate the two general signal transduction pathways (Norman, 1997; Norman *et al.*, 2002a; Norman, 2005). (1) One shape of $1\alpha,25(OH)_2D_3$ interacts with the VDR_{nuc} to form a competent receptor–ligand complex that interacts with other nuclear proteins to create a functional gene transcription complex to increase or decrease mRNA coding for selected proteins (see Fig. 4, left). (2) A different shape of $1\alpha,25(OH)_2D_3$ interacts with the VDR_{mem} that promptly stimulates signal transduction events that activate the rapid appearance of biological responses (see Fig. 4, right). Also see Fig.2E, to see the structure of the 6-s-*cis*-locked $1\alpha,25(OH)_2$-lumisterol (analog JN) that is a full agonist for rapid responses. Figure 2E and F compares the shapes of the optimal ligands for genomic responses (VDR) and rapid responses and for binding to the DBP.

A wide array of rapid responses stimulated by $1\alpha,25(OH)_2D_3$ have been reported over the past 15 years; a summary is given in Table I. Recent additions to the list include demonstrations that $1\alpha,25(OH)_2D_3$ can stimulate opening of chloride channels (Zanello *et al.*, 1996, 2006) and activation of mitogen-activated protein (MAP) kinase (Buitrago *et al.*, 2006; Song, 1998). MAP kinase belongs to the family of serine/threonine protein kinases and can be activated by phosphorylation on a tyrosine residue induced by mitogens or cytodifferentiating agents (Pelech *et al.*, 1992). MAP kinase integrates multiple intracellular signals transmitted by various second messengers and regulates many cellular functions by phosphorylation of a number of cytoplasmic kinases and nuclear transcription factors including the epidermal growth factor (EGF) receptor, c-Myc, and c-Jun (Lange-Carter *et al.*, 1993). These rapid actions of $1\alpha,25(OH)_2D_3$ have been postulated to regulate cell biological function and potentially to interact with other membrane-mediated kinase cascades or to crosstalk with the cell nucleus to control genomic responses associated with cell differentiation and proliferation (Berry *et al.*, 1996) and modulation of osteoblast function (Vertino *et al.*, 2005).

NUCLEAR RECEPTOR (VDR) FOR $1\alpha,25(OH)_2D_3$

The $1\alpha,25(OH)_2D_3$ receptor was originally discovered in the intestine of vitamin D-deficient chicks (Haussler *et al.*, 1969; Tsai *et al.*, 1972). A timeline summarizing the important developments in our understanding of the

TABLE I Distribution of Rapid Responses to 1α,25$(OH)_2D_3$

Organ/cell/system	Response studied	Reference
Intestine	Rapid transport of intestinal Ca^2 (transcaltachia)	Nemere *et al.* (1984b); De Boland ***et al.*** (1990a, 1990b)
	CaCo-2 cells, PKC, G proteins	Khare *et al.* (1994)
	Activation of PKC	De Boland *et al.* (1990a); Bissonnette ***et al.*** (1994)
	Activation of MAP-kinase	De Boland *et al.* (1998)
	Stimulation of phospholipase C	Khare *et al.* (1997)
Colon	PKC effects	Bissonnette *et al.* (1995)
	Subcellular distribution	Simboli-Campbell *et al.*(1992, 1994)
	Regulation of 25$(OH)D_3$- 24-hydroxylase	Mandla *et al.* (1990)
Osteoblast	ROS 17/2.8 cells	
	Ca2+ channel opening	Caffrey *et al.* (1989)
	Cl^- channel opening	Zanello *et al.* (1996)
Chondrocytes	PKC activation	Sylvia *et al.* (1996); Schwartz *et al.* (2000)
	Phospholipase A2 activation	Boyan *et al.* (1998)
Liver	Lipid metabolism	Baran ***et al.*** (1989, 1990)
	Activation of PKC and MAP-kinase	Beno *et al.* (1995)
Muscle	PKC and Ca^{2+} effects	De Boland *et al.* (1993); Morelli *et al.* (1993); Vazquez *et al.* (1996)
	Phospholipase D	Fernandez *et al.* (1990)
Promyelocytic leukemia cells	Aspects of cell differentiation	Bhatia *et al.* (1995, 1996); Miura *et al.* (1999)
	PKC effects	Biskobing *et al.* (1993)
	Activation of MAP-kinase	Berry *et al.* (1996); Song ***et al.*** (1998)
Keratinocytes	Alter PKC subcellular distribution	Yada *et al.* (1989); Gniadecki *et al.* (1997)
	Sphingomyelin hydrolysis	Gniadecki (1996)
	Activation of Src and Raf	Gniadecki (1996, 1998a)
Pancreas B cells	Intracellular calcium changes	Sergeev *et al.* (1995)
	Insulin secretion	Kajikawa *et al.* (1999)
Parathyroid cells	Phospholipid metabolism	Bourdeau *et al.* (1990)
	Cytosolic Ca^{2+}	
Lipid bilayer	Activation of highly purified PKC	Slater *et al.* (1995)

The reader should compare the information in this table with the concepts illustrated in Figures 4 and 7 which summarize the vitamin D endocrine system and signal transduction pathways utilized by 1α,25$(OH)_2D_3$ for generation of biological responses.

vitamin D receptor (VDR) and its historical involvement in genomic responses and now, more recently, its proposed involvement in rapid responses is presented in Table II. The interval from its discovery in 1969 to 1984 established that the cell nucleus-localized VDR, like those for the classical ligand-occupied steroid hormone receptors (the estrogen, progesterone, androgen, glucocorticoid, and mineralocorticoid receptors) is intimately involved with selective gene transcription appropriate to the biology of each system. The VDR is known to be present in more than 30 target tissues in humans (Reichel *et al.*, 1989; Bouillon *et al.*, 1995; Norman, 2001; Norman *et al.*, 2006) (see Figure 3). Given the long-standing understanding that vitamin D acting via 1α,25$(OH)_2D_3$ is principally responsible for calcium homeostasis via actions in the intestine, bone, and kidney; the other additional VDR-containing target organs collectively reflect "new" genomic response assignments for 1α,25$(OH)_2D_3$ and its VDR. Some of the new genomic frontiers include the immune system's B and T lymphocytes, the hair follicle, muscle, adipose tissue, bone marrow, and cancer cells.

The VDR has been extensively characterized and the cDNA for the nuclear receptor has been cloned and sequenced (McDonnell *et al.*, 1987; Baker *et al.*, 1988). To date only a single form of the receptor has been identified. The 1α,25$(OH)_2D_3$ receptor is a DNA-binding protein with a molecular mass of about 50,000 Da. It binds 1α,25$(OH)_2D_3$ with high affinity with a K_D in the range

TABLE II Vitamin D Receptor Timeline: A Historical Past (Genomic Responses) and New Assignments for the Future (Rapid Responses)

Year	Comment	Reference
1968–1971	Discovery of $1\alpha,25(OH)_2D_3$ as a new steroid hormone and its chemical characterization	Haussler *et al.* (1968); Myrtle *et al.* (1970); Norman *et al.* (1971)
1969	Discovery of the nuclear receptor for $1\alpha,25(OH)_2D_3$	Haussler *et al.* (1969); Tsai *et al.* (1973b)
1973	Actinomycin D, an inhibitor of DNA-directed RNA synthesis, blocks the biological actions of both vitamin D_3 and $1\alpha,25(OH)_2D_3$	Norman (1965); Tsai *et al.* (1973a)
1982	The $1\alpha,25(OH)_2D_3$ unoccupied receptor is partitioned between the cytosol and nucleus of the cell	Walters *et al.* (1980)
1985–1995	Appreciation that $1\alpha,25(OH)_2D_3$ is a very conformationally flexible molecule/receptor ligand as a consequence of its eight-carbon side chain, 360° rotation about its 6,7 single carbon and A-ring chair–chair interconversion	Okamura *et al.* (1974); Wing *et al.* (1974)
1984	Discovery of $1\alpha,25(OH)_2D_3$ rapid response of transcaltachia	Nemere *et al.* (1984a)
1984	Appreciation that the VDR is present in more than 30 target tissues of humans, including classical (intestine, kidney, cartilage, and bone) as well as nonclassical (pancreas β-cell, hair follicle, many cancer cells, and activated B and T lymphocytes) target organs of vitamin D through its daughter steroid hormone, $1\alpha,25(OH)_2D_3$	Norman *et al.* (1982b); Reichel *et al.* (1989)
1994	Postulate of a membrane receptor for $1\alpha,25(OH)_2D_3$ involved in rapid responses	Nemere *et al.* (1994)
1997	Demonstration for $1\alpha,25(OH)_2D_3$ that a 6-s-*cis* shape is the rapid responses agonist, whereas a 6-s-*trans* shape is the genomic responses agonist	Norman *et al.* (1997)
2000	The first x-ray structure of the VDR ligand-binding domain is reported.	Rochel *et al.* (2000)
2003	Evidence that annexin II is not a membrane receptor for $1\alpha,25(OH)_2D_3$	Mizwicki *et al.* (2003a)
2003	Discovery of a $[^3H]$ $1\alpha,25(OH)_2D_3$-binding protein in caveolae membrane fractions of chick intestinal cells	Norman *et al.* (2002b)
2004	The vitamin D receptor is present in caveolae-enriched plasma membranes and binds $1\alpha,25(OH)_2$-vitamin D_3 *in vivo* and *in vitro*	Huhtakangas *et al.* (2004)
2004	A putative $1\alpha,25(OH)_2D_3$ membrane-binding protein (1,25D-MARRS or membrane-associated, rapid-response steroid-binding), linked to intestinal phosphate transport is found to be identical to the protein ERp57	Nemere *et al.* (2004)
2004	The rapid response effects of $1\alpha,25(OH)_2D_3$ requires a functional VDR	Nguyen *et al.* (2004)
2004	Identification of an alternative ligand-binding pocket in the VDR and its functional importance in $1\alpha,25(OH)_2D_3$ signaling emphasizes the importance of a VDR receptor ensemble model	Norman *et al.* (2004); Mizwicki *et al.* (2004)
2004	Rapid modulation of osteoblast ion channel rapid responses is abrogated in VDR knockout mice emphasizing that $1\alpha,25(OH)_2D_3$-mediated rapid responses require the presence of a functional VDR	Zanello *et al.* (2004)
2005	Presence of a truncated form of the VDR in a strain of VDR KO mice	Bula *et al.* (2005)

of 1–50 × 10^{-10} M (Wecksler *et al.*, 1980a, 1980b). The exquisite ligand specificity of the nuclear $1\alpha,25(OH)_2D_3$ receptor is illustrated in Table III. This table tabulates the Relative Competitive Index (RCI) of eight key analogs of the reference compound, namely $1\alpha,25(OH)_2D_3$; the RCI of $1\alpha,25(OH)_2D_3$ is set to 100%. Thus, changing the 3β-hydroxyl to a 3α-hydroxyl reduces the RCI to 24%, whereas removal of the 25-hydroxyl or 1α-hydroxyl reduces the RCI to 0.15%. The parent vitamin D_3 with a RCI of 0.0001% has no capability to bind to the VDR.

The impact of $1\alpha,25(OH)_2D_3$-mediated rapid response (RR) on the VDR from 1994 to the present are summarized in Table I. The concept of the existence of a membrane receptor for $1\alpha,25(OH)_2D_3$ has its origins in the study of the

TABLE III Ligand Specificity of the Nuclear 1α,25$(OH)_2D_3$ Receptor

Ligand	Structural modification	RCI[a] (%)
1α,25$(OH)_2D_3$		100
1α,25$(OH)_2$-24-nor-D_3	Shorten side chain by 1 carbon	67
1α,25$(OH)_2$-3-epi-D_3	Orientation of 3β-OH altered	24
1α,25$(OH)_2$-24a-dihomo-D_3	Lengthen side chain by 2 carbons	24
1β,25$(OH)_2D_3$	Orientation of 1α-OH changed	0.8
1α$(OH)D_3$	Lacks 25-OH	0.15
25$(OH)D_3$	Lacks 1α-OH	0.15
1α,25$(OH)_2$-7-dehydrocholesterol	Lacks a broken B ring; is not a secosteroid	0.10
Vitamin D_3	Lacks 1α- and 25-OH	0.0001

[a] *The Relative Competitive Index* (RCI) *is a measure of the ability of a nonradioactive ligand to compete, under* in vitro *conditions, with radioactive* 1α,25$(OH)_2$ D_3 *for binding to the nuclear* 1α,25$(OH)_2D_3$ *receptor* (VDR).

Source: From Bouillon et al. (1995).

process of 1α,25$(OH)_2D_3$-mediated response of transcaltachia, or the rapid hormonal stimulation of intestinal Ca^{2+} absorption in the perfused chick intestine (Nemere *et al.*, 1984b). The most potent agonist is the natural hormone 1α,25$(OH)_2D_3$, which can stimulate the transport of calcium across the intestine within 4–5 minutes of its injection in the celiac artery that perfuses the duodenum (Nemere *et al.*, 1984b). It now appears that the classical VDR normally associated with the cell nucleus and gene transcription can also be resident near to or associated with caveolae present in the plasma membrane (Norman *et al.*, 2002b; Huhtakangas *et al.*, 2004; Bula *et al.*, 2005). Caveolae are flask-shaped membrane invaginations that are enriched in sphingolipids and cholesterol, which are commonly found in both caveolae and/or lipid rafts (Razani *et al.*, 2002). It has also been reported that the estrogen receptor (ER), androgen receptor (AR), and aldosterone receptor are associated with caveolae of (Norman *et al.*, 2004).

There are several recent reviews describing the properties of the VDR. See the following: Sutton *et al.* (2003); MacDonald *et al.* (2004); Kato *et al.* (2004); Norman (2005); Pike *et al.* (2005); and Carlberg *et al.* (2006).

The protein superfamily to which the VDR belongs includes receptors for glucocorticoids (GR); progesterone (PR); estrogens (ER); aldosterone; androgens; thyroid hormone (T3R); hormonal forms of vitamin A (RAR, RXR); the insect hormone, ecdysone; the peroxisome proliferator–activator receptor, PPAR; and several orphan receptors including the estrogen-related receptor, ERR, and the cholic acid lipid-sensing receptor, LXR (Evans 1988; Mangelsdorf *et al.*, 1995; Carlberg, 1996).To date, biochemical evidence has been obtained for the existence of about 24 small molecule/steroid receptors. Based on evaluation of the human genome database, it is believed that there are a total of 48 members of the steroid receptor superfamily (Mangelsdorf *et al.*, 1995). Figure 5 describes the hypothesized evolutionary relationship of the superfamily of nuclear receptors.

Figure 6 presents a schematic model of the domains of the VDR in comparison with the domains of the vitamin D-binding protein (DBP); both of these proteins bind 1α,25$(OH)_2D_3$ specifically and with high affinity, but they have a vastly different protein structure and biological functions. A discussion of the ligand-binding domain of DBP and its protein structure, compared with that of the VDR, is presented somewhat later.

The DNA-binding domain C is the most conserved domain throughout the family of all the nuclear receptors. About 70 amino acids fold into two zinc finger-like motifs in which conserved cysteines coordinate a zinc ion in a tetrahedral arrangement. The first finger, which contains four cysteines and several hydrophobic amino acids, determines the hormone response element (HRE) specificity; an HRE is a specific nucleotide sequence located in the promoter region of the gene to be regulated by the receptor and cognate ligand (Beato, 1989; McKenna *et al.*, 2002a; McKenna *et al.*, 2002b). The second zinc finger, which contains five cysteines and many basic amino acids, is also necessary for DNA binding and is involved in receptor dimerization (Green *et al.*, 1988; Rastinejad *et al.*, 1995). The zinc fingers identify the receptor cognate HRE and physically interact with the HRE to form a receptor + ligand + HRE–DNA complex.

The next most conserved region for the nuclear receptors is paradoxically the steroid-binding domain (region E). This region contains a hydrophobic pocket for ligand binding and also contains signals for several other functions including dimerization (Bourguet *et al.*, 1995; Renaud *et al.*, 1995), nuclear translocation, and hormone-dependent transcriptional activation (Weatherman *et al.*, 1999). Given the wide diversity in the chemistry and structure of all the steroid, thyroid, and retinoid ligands, it would not have been predicted that the same fundamental amino acid sequence of domain E would specifically and exquisitely define the strict high-affinity binding of each specific ligand only with its cognate receptor. Thus, the conserved amino acid residues of the E domain create the three-dimensional framework of the ligand-binding domain, whereas the nonconserved amino acid residues confer upon the ligand-binding domain (LBD) its ability for sharp discrimination in favor of its cognate steroid hormone.

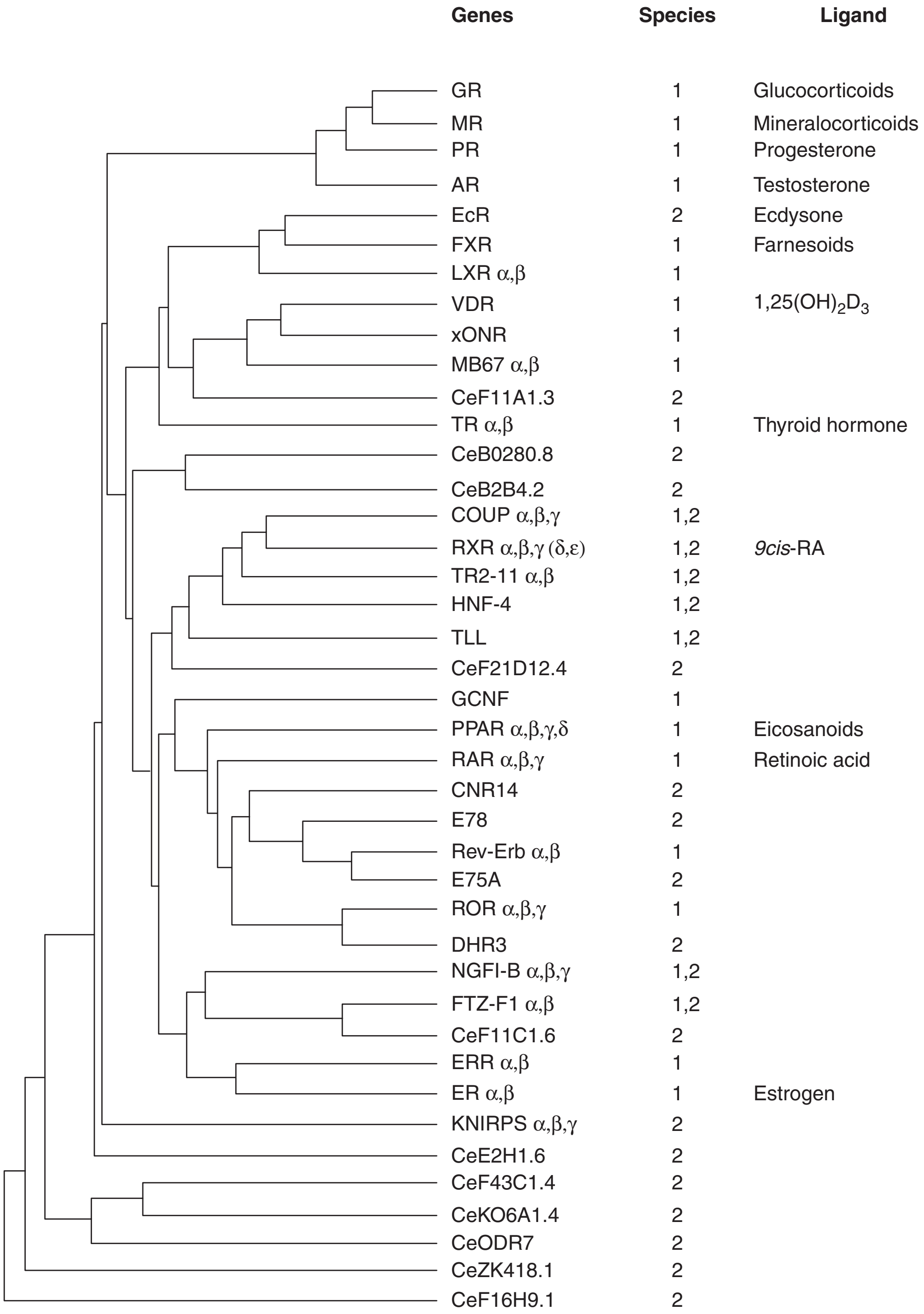
Genes
Species
Ligand
GR 1 Glucocorticoids
MR 1 Mineralocorticoids
PR 1 Progesterone
AR 1 Testosterone
EcR 2 Ecdysone
FXR 1 Farnesoids
LXR α,β 1
VDR 1 1,25(OH)2D3
xONR 1
MB67 α,β 1
CeF11A1.3 2
TR α,β 1 Thyroid hormone
CeB0280.8 2
CeB2B4.2 2
COUP α,β,γ 1,2
RXR α,β,γ (δ,ε) 1,2 9cis-RA
TR2-11 α,β 1,2
HNF-4 1,2
TLL 1,2
CeF21D12.4 2
GCNF 1
PPAR α,β,γ,δ 1 Eicosanoids
RAR α,β,γ 1 Retinoic acid
CNR14 2
E78 2
Rev-Erb α,β 1
E75A 2
ROR α,β,γ 1
DHR3 2
NGFI-B α,β,γ 1,2
FTZ-F1 α,β 1,2
CeF11C1.6 2
ERR α,β 1
ER α,β 1 Estrogen
KNIRPS α,β,γ 2
CeE2H1.6 2
CeF43C1.4 2
CeKO6A1.4 2
CeODR7 2
CeZK418.1 2
CeF16H9.1 2

FIGURE 5 Nuclear receptor superfamily. The figure summarizes the hypothesized evolutionary relationships for the extended family of known nuclear receptors and related orphan receptors based on the extent of homology for the nucleotide sequence of the cDNA of the individual protein. This figure was modified from that presented by Mangelsdorf *et al*. (1995).

The A/B (transactivation) domain, which is quite small in the VDR (25 amino acids), is poorly conserved across the nuclear receptor superfamily and its function has not yet been clearly defined. An independent transcriptional activation function is located within the A/B region (Green *et al*., 1988; Weatherman *et al*., 1999) which is constitutive in receptor constructs lacking the ligand-binding domain (region E). The relative importance of the transcriptional activation by this domain depends on the receptor, the context of the target gene promoter, and the target cell-type (Weatherman *et al*., 1999).

Domain D of the nuclear receptors is the hinge region between the DNA-binding domain and the ligand-binding domain. The hinge domain must be conformationally

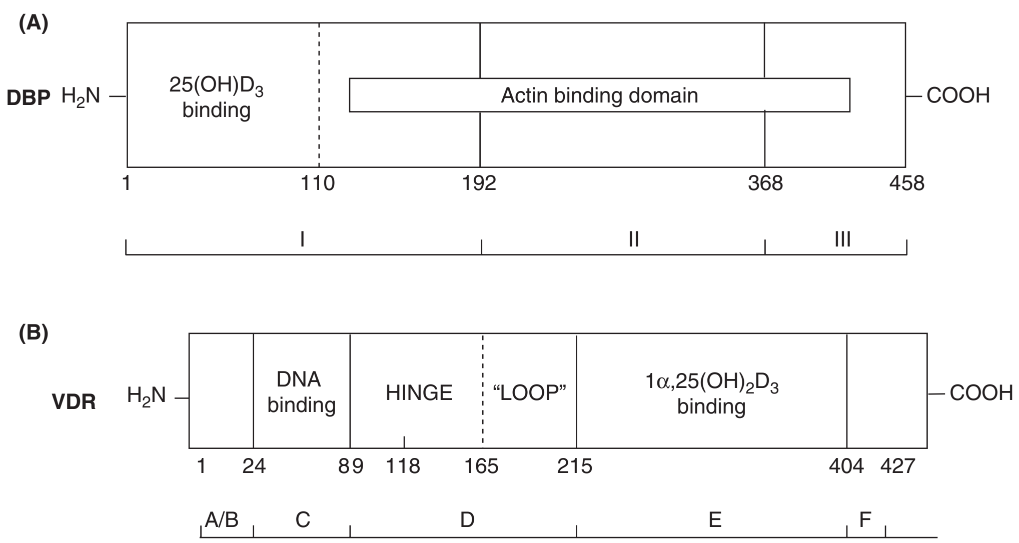

FIGURE 6 Schematic models of the vitamin D-binding protein (DBP) **(A)** and the vitamin D receptor (VDR) **(b)**. **(a)** The DBP consists of 458 amino acid residues and is divided into three domains (I, II, and III). The numbers below the DBP indicate the amino acid residue boundaries for the various domains. The domains I, II, and III have been postulated to have evolved from a progenitor that arose from the triple repeat of a 192-amino-acid sequence (Gibbs *et al*., 1987a). However, domain III is significantly truncated at the C terminus. The 25(OH)D_3 binding cleft is associated with the first six α-helices or residues 1–110 of domain I. The actin-binding property of DBP is associated with a portion of domains I and III that clamp the actin while it rests on domain II (Verboven et al., 2003). **(B)** The VDR comprises 427 amino acid residues that are divided into six domains (A–F). The numbers below the VDR indicate the amino acid residue boundaries for the various domains. The VDR belongs to a superfamily of nuclear receptors all of which have the same general A–F domain organization. The C domain, the most highly conserved, which contains the DNA-binding domain, defines the superfamily; it contains two zinc finger motifs. The E domain or ligand-binding domain (LBD) is less conserved and is responsible for binding 1α,25$(OH)_2D_3$ or its analogs and transcriptional activation. The A/B domain of the VDR is much smaller than other members of the superfamily. The portion of the intact VDR that was crystallized and subjected to x-ray crystallographic analysis included residues 118–427 but with deletion of the "loop" region of the hinge domain D, residues 165–215 (Rochel *et al*., 2000; Tocchini-Valentini *et al*., 2001, 2004).

flexible because it allows the DNA-binding domains and ligand-binding domain some flexibility for their proper interactions with DNA and ligand, respectively. The VDR hinge region contains 65 amino acids and has immunogenic properties.

Receptor Dimerization

The superfamily of nuclear receptors has been classified into subgroups based on their dimerization properties, DNA-binding site preferences, and cellular localization. Group I includes the receptors for glucocorticoids, estrogen, mineralocorticoids, progesterone, and androgens. These receptors bind as homodimers to palindromic DNA response elements. Group II includes the receptors for VDR, T3R, RAR, RXR, ecdysone, and several orphan receptors. These receptors bind as homodimers or heterodimers to direct repeats, palindromic, and inverted palindromic DNA response elements. Group III includes the orphan receptors for reverb A, ROR, SF-1, and NGFI-B. No ligands have yet been identified for these receptors and they bind DNA response elements as monomers or heterodimers.

As a class, the group II receptors bind nonsteroid conformationally flexible ligands (where vitamin D is classified as a secosteroid, rather than as a steroid). The group II receptors have more flexibility in the types of DNA response elements they can recognize and in the types of dimeric interactions they participate in than the group I receptors. All of the group II receptors can form heterodimers with RXR (Yu *et al*., 1991; Kliewer *et al*., 1992), and other heterodimeric interactions have also been reported (Carlberg, 1993). The VDR_{nuc} can bind to DNA response elements as homodimers and as heterodimers with RAR, RXR, and T3R (Schräder *et al*., 1993; Carlberg, 1993). The ability to form heterodimers with other receptors allows for enhanced affinity for distinct DNA targets, generating a diverse range of physiological effects as shown in Figure 7.

The first zinc finger determines the sequence specificity of the DNA element. The second zinc finger is aligned by the binding of the first finger to the DNA and is involved in the protein–protein contacts responsible for the cooperativity of binding. The spacing of nucleotides between the two half-sites is important for the DNA-binding specificity because of the asymmetric dimer interface formed by the DNA-binding domains of a heterodimer pair. Ligand binding may function to modulate receptor dimerization. In fact, VDR_{nuc} has been shown to exist as a monomer in solution either in the presence or absence of ligand. When DNA is present, in the absence of ligand,

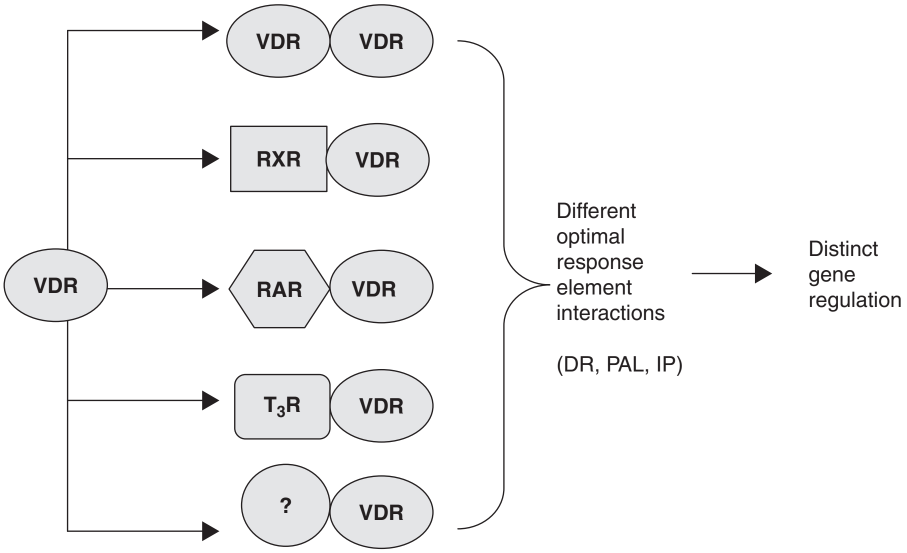

FIGURE 7 Schematic of possible dimeric interactions of VDR_{nuc} with other receptor members of the superfamily (see Fig. 5). VDR_{nuc} can bind to DNA as a homodimer or as a heterodimer with a variety of other group II receptors, i.e., RXR (retinoid X receptor), RAR (retinoid A receptor), T3R (thyroid receptor), and perhaps other receptors or factors not yet identified. Each dimer pair has an enhanced affinity for distinct DNA targets allowing a small family of receptors to generate a diverse range of physiological effects.

the VDR_{nuc} binds to the DNA both as monomers and homodimers. The addition of ligand stabilizes the bound monomer, which favors the formation of VDR_{nuc}-RXR (or other) heterodimers. The presence of the ligand decreases the rate of monomer-to-homodimer conversion. The presence of the RXR ligand, 9-*cis*-retinoic acid, has the opposite affect on heterodimerization formation; it enhances the binding of RXR homodimers to DR + 1 elements (Cheskis *et al.*, 1994). Ligand bound to VDR_{nuc} enhances the binding of RXR-VDR_{nuc} heterodimers to DR + 3 elements. There are also other possible protein–protein interactions that can involve VDR_{nuc} including association with AP-1, EE1A/TFIID, TFIIB, and coactivator proteins (Zhang *et al*,. 2003; MacDonald *et al.*, 2004). These protein–protein interactions can be determined by the concentration of the protein partner and/or by the concentration of ligand or both as well as by the nature of the DNA target site itself.

Hormone Response Elements

Each zinc finger appears to be encoded by separate exons as shown by the genomic structure of the ER (Ponglikitmongkol *et al.*, 1988), PR (Huckaby *et al.*, 1987), and the VDR_{nuc} (Freedman 1992). Most of the knowledge of how zinc fingers interact with DNA response elements has been gained by studies of GR and ER. The palindromic nature of GR and ER response elements suggested that these hormone receptors would bind to DNA as symmetrical dimers. Subsequent studies have confirmed that both GR and ER bind as homodimers to their response elements (Schwabe *et al.*, 1990; Picard *et al.*, 1990). The principal ER dimerization domain is in its ligand-binding domain (Kumar *et al.*, 1988). Both the ER and GR contain additional residues in the DNA-binding domain that are also important for dimerization. When the GR and ER DNA-binding domain are translated, they cannot dimerize alone but, in the presence of the correct palindromic response element, they bind to DNA as a dimer in a cooperative manner (Hard *et al.*, 1990). The five-amino-acid stretch between the first two coordinating cysteines of the second zinc finger is designated the “D” box (Umesono *et al.*, 1988) and mediates spacing requirements critical for cooperative dimer binding to palindromic HREs probably through a dimer interface involving these residues in each monomer (Jonat *et al.*, 1990; Schule *et al.*, 1990; Diamond *et al.*, 1990).

Using the GR and ER as models of receptor–DNA interactions, the binding of VDR_{nuc} to DNA has also been examined. Because VDR_{nuc} can bind to DNA as a heterodimer, often with RXR, VDR_{nuc} and other group II receptors seem to display more variety in how they bind to their response elements (Forman *et al.*, 1989; Jones, 1990; Freedman, 1992). The primary response element for the group II receptors is a direct repeat instead of an inverted palindrome; the protein–protein contacts are nonequivalent. There is an asymmetrical dimerization interface. Amino acid residues, designated the T/A box in the hinge region (domain D) just adjacent to the DNA-binding domain, are involved. The T/A-box residues form an α-helix making backbone and minor groove interactions that are involved in intramolecular packing against residues in the tip of the first zinc finger and determine the spacing requirements for the heterodimer pair.

TABLE IV Hormone Response Elements for the Nuclear Vitamin D Receptor (VDR_{nuc})

Gene	Hormone response element	Reference
hOsteocalcin	GGGTGA acg GGGGCA	Morrison *et al.* (1989)
rOsteocalcin	GGGTGA atg AGGACA	Terpening *et al.* (1991)
MOsteopontin	GGTTCA cga GGGTCA	Noda *et al.* (1990)
RCalbindin D_{9k}	GGGTGA cgg AAGCCC	Darwish *et al.* (1993)
MCalbindin D_{28k}	GGGGGA tgt GAGGAG	Gill *et al.* (1993)
24R-Hydroxylase	AGGTGA gtg AGGGCG	Hahn *et al.* (1994)
DR + 3	AGGTCA agg AGGTCA	Umesono *et al.* (1991)
CONSENSUS	GGGTGA nnn GGGNCNAA	

Note: A comparison of reported VDREs. The two half-sites are listed as upper case letters. The sequences are −500 to −486 of human osteocalcin, −456 to −438 of rat osteocalcin, −758 to −740 of mouse osteopontin, −488 to −474 of rat calbindin D_{9K}, *−199 to −184 of mouse calbindin* D_{28K}.

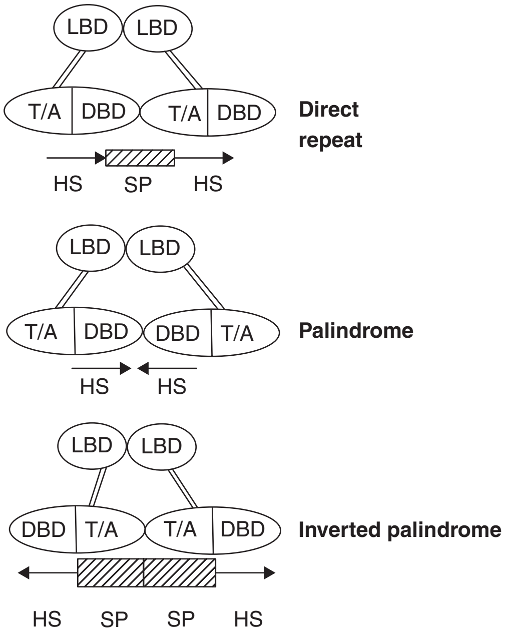

FIGURE 8 Mechanism of steroid nuclear receptor superfamily dimers binding to DNA response elements. Group II receptors can bind to three types of response elements: direct repeats, palindromes, and inverted palindromes. The spacing (SP), number of base pairs between half-sites (HS), is determined by steric constraints of the T/A box. The orientation of the DNA half-sites is shown with arrows. The flexible hinge domain allows the formation of the same dimerization interface between ligand-binding domains (LBD) regardless of the orientation of the DNA half-sites.

Eventually it is to be anticipated that a x-ray crystallographic structure of a full-length nuclear receptor bound to its cognate DNA promoter with all the associated transcription factors and coactivator proteins necessary for a functional transcriptional complex will be elucidated. There are two recent papers available that provide the first steps in this arena (Taatjes *et al.*, 2004a; Taatjes *et al.*, 2004b).

Table IV summarizes examples of hormone response elements for VDR_{nuc} and Figure 8 illustrates how receptor dimers can bind to the DNA hormone response elements. The natural response elements for the group II receptors appear to consist of a direct repeat of the hexamer AGGTCA. The spacing of the direct repeat determines the receptor preference: VDR_{nuc} prefers a three base-pair space, T3R prefers four base pairs, and RAR prefers five base pairs (Umesono *et al.*, 1991). The RXR, RAR, T3R, and VDR_{nuc} spacing optimum on a palindrome is no nucleotides between the half-sites. Spacing on inverted palindromes depends on the overhang of the dimeric partners: 11 for VDR_{nuc}-RAR; 7 to 8 is predicted for VDR_{nuc}-RXR, but actually is 9; RXR appears to use a slightly different contact interface when it heterodimerizes with VDR_{nuc} than with other receptors (Schräder *et al.*, 1994). Free rotation around the hinge (domain D) enables the same interaction of the ligand-binding domains of both receptors on each response element. The steric requirements of the T/A boxes gives the receptor its asymmetry when binding to direct repeats and inverted palindromes and determines the optimal spacing (see Fig. 8).

Ligand-Binding Domain

The ligand-binding domain of group II receptors as exemplified by the VDR has been further dissected (see Fig. 6). Subdomains ligand1 and ligand2 are nearly identical. Surprisingly, there is greater homology between the ligand-binding subdomains of RAR (α, β, γ) and T3R (α, β) than there is between RAR and RXR. The τi subdomain is highly conserved among all nuclear hormone receptors and is a putative transcriptional inactivating domain. Inactivation of this domain is relieved by ligand binding.

The dimerization domain consists of eight to nine heptad repeats of hydrophobic amino acids. The heptads contain leucine or other hydrophobic residues such as Ile, Val, Met, or Phe at positions 1 and 8 or charged amino acids with hydrophobic side chains such as Arg or Gln in position 5. In an ideal coiled-coil α-helix, these amino acids would form a hydrophobic surface along one face of the helix that would act as a dimerization interface R (Fawell *et al.*, 1990). Deletion/mutation analysis of the VDR_{nuc} ligand-binding domain has shown that Asp-258 and Ile-248 are involved in heterodimerization with RXR. Leu-254 and -262 are critical for heterodimerization. A mutant that is truncated at amino acid 190 becomes constituitively transcriptionally active.

Other amino acids identified as being important for heterodimerization are 325–332, 383–390, and 244–263. Residues 403–427 are particularly important for ligand side-chain binding [$1\alpha,25(OH)_2D_3$] (Nakajima *et al.*, 1994).

Figure 9 presents a schematic model of the VDR_{nuc} interaction with its heterodimer partner and their subsequent interaction with the promoter of genes selected for modulation, as well with other proteins (coactivators, TATA-binding protein, etc.) so as to generate a competent transcriptional complex. Over the past decade there has been a continuing evolution of understanding and complexity concerning the details of what constitutes a "competent transcriptional complex." Additional viewpoints and information can be found in several recent articles (Weatherman *et al.*, 1999; Glass *et al.*, 2000; Pathrose *et al.*, 2002; Pike *et al.*, 2005; Kim *et al.*, 2005) .

VDR Receptor Structure

A dramatic advance in understanding the three-dimensional structure of the LBD of steroid receptors occurred from 1995 to 1999 when the x-ray crystallographic structure determination of the LBD of five hormone nuclear receptors was achieved. These include the LBDs of the thyroid hormone (TR), retinoic acid (RAR), estrogen (ER), progesterone (PR), and the PPARγ [see the review by Weatherman *et al.* (1999)]. Also an x-ray structure is available for the LBD of the unoccupied 9-*cis*-retinoic acid receptor RXR (Bourguet *et al.*, 1995). Further, ER LBD x-ray structures are known for a ligand (raloxifene) that can act as an antagonist of the transcriptional activation function (Brzozowski *et al.*, 1997; Shiau *et al.*, 1998).

The crystal structure of an engineered version of the ligand-binding domain of the nuclear receptor for vitamin D, bound to $1\alpha,25(OH)_2D_3$, was determined in 2000 at a 1.8-Å resolution (Rochel *et al.*, 2000). A follow-up x-ray crystallographic report compared the VDR LBD and bound ligand for $1\alpha,25(OH)_2D_3$ with that of four superagonist analogs of $1\alpha,25(OH)_2D_3$ (Tocchini-Valentini *et al.*, 2001). Other x-ray structures of the VDR, which are all very similar to the original report, have appeared in the following: Tocchini-Valentini *et al.* (2001), Shaffer *et al.* (2002), Sicinska *et al.* (2004), Ciesielski *et al.* (2004), Vanhooke *et al.* (2004), and Tocchini-Valentini *et al.* (2004).

Figure 10 compares the three-dimensional structure of the vitamin D receptor (VDR) with bound $1\alpha,25(OH)_2D_3$ (see Fig. 10A and B) with the three-dimensional structure of the vitamin D-binding protein (DBP) with bound $25(OH)D_3$ (see Fig 10C and D).

The x-ray structure of the LBD of the human VDR_{nuc} spans amino acid residues 143–427 (COOH terminus), but without residues 165–215, which were in an "undefined" loop in the hinge region of domain D (see Fig. 10A

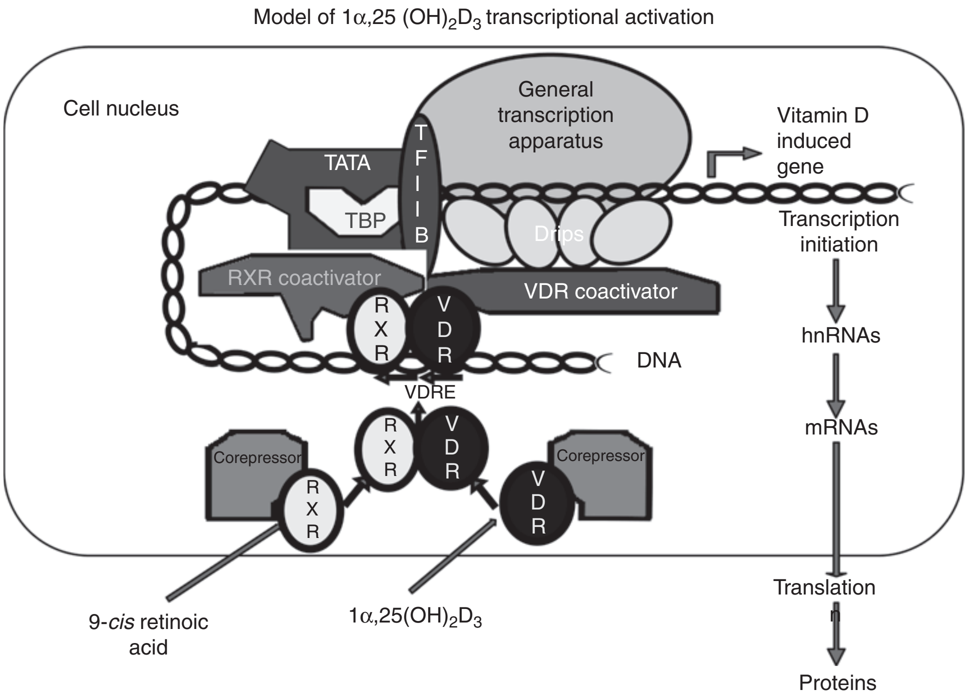

FIGURE 9 Model of 1α,25(OH) and VDR_{nuc} activation of transcription. The VDR, after binding its cognate ligand $1\alpha,25(OH)_2D_3$, forms a heterodimer with RXR. This heterodimer complex then interacts with the appropriate VDRE on the promoter of genes (in specific target cells), which are destined to be up- or downregulated. The heterodimer–DNA complex then recruits necessary coactivator proteins, TATA, TBP, and TFIIB, and other proteins to generate a competent transcriptional complex capable of modulating mRNA production.

and B). The removal of the flexible insertion domain in the VDR LBD produced a more soluble protein that was more amenable to crystallization. The VDR LBD protein structure is very similar to the LBD of the five other x-ray crystallographic nuclear receptor structures that had been determined before VDR in 2000 (Weatherman *et al.*, 1999). All nuclear steroid hormone receptors consist of a three-stranded β-sheet and 12 α-helices that are arranged to create a three-layer sandwich that completely encompasses the ligand [1α,25(OH)$_2$D$_3$ in the case of the VDR] in a hydrophobic core (see Fig. 10). The x-ray structures of all six nuclear hormone receptors are so similar that their ribbon diagrams are virtually superimposable, indicating a remarkable spatial conservation of the secondary and tertiary structures (Weatherman *et al.*, 1999). In addition, the AF-2 domain of the C-terminal helix 12 (domain F; residues 404–427), contributes to the hormone-binding pocket .

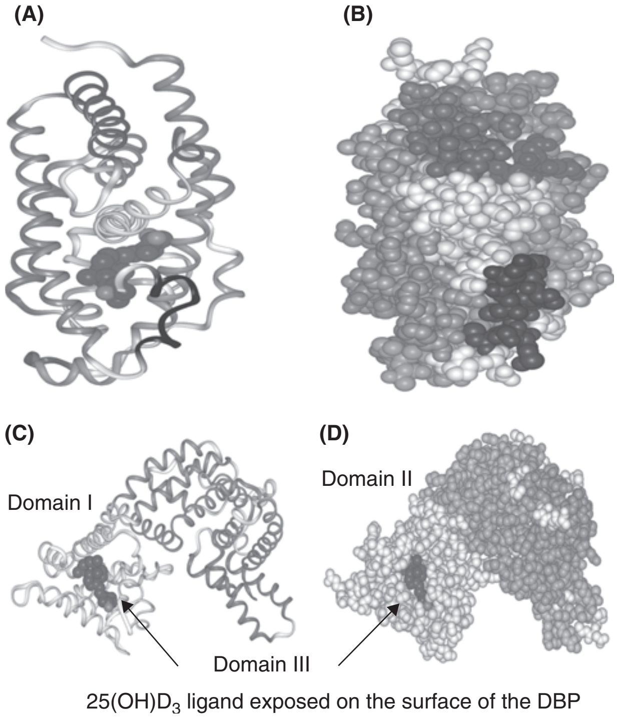

FIGURE 10 Comparison of the three dimensional structure of the vitamin D receptor (VDR) for the steroid hormone, 1α,25(OH)$_2$D$_3$ (**A** and **B**) with the three-dimensional structure of the vitamin D-binding protein (DBP) for 25(OH)D$_3$ (**C** and **D**). (**A**) Three-dimensional ribbon structure of the VDR ligand-binding domain for amino acid residues 118–425 (Δ165–215) as determined via x-ray crystallography (Rochel *et al.*, 2000). Also the presence of the bound ligand, 1α,25(OH)$_2$D$_3$, is shown; its structure and shape are presented in more detail in Fig 2F. The white regions represent loops and other flexible regions of the molecule. The ligand, 1α,25(OH)$_2$D$_3$, has its atoms indicated. (**B**) Illustration of the Corey–Pauling–Koltun (CPK) space-filling model of the VDR LBD. The position of helix-12 in the "closed" position effectively sequesters the ligand from the external environment of the protein, indicated by the absence of visible carbon and oxygen atoms from 1α,25(OH)$_2$D$_3$ in this view. (**C**) Three-dimensional structure of DBP for residues 1–458 as determined via x-ray crystallography (Verboven *et al.*, 2002). Illustration of the three domains (I, II, and III,) of the DBP in a ribbon structure representation. The atoms of the ligand, 25(OH)D$_3$ are black. The x-ray structure of DBP was determined separately with two different ligands. These ligands were 25(OH)D$_3$ and 22-(*m*-hydroxyphenyl)-23,24,25,26,27-pentanor vitamin D$_3$ (analog JY); both x-ray structures contained the same conformer shape of the bound ligands. The structure and shape of 25(OH)D$_3$ is presented in more detail in Figure 8C. (**D**) The ligand-binding domain of the DBP is a crevice located on the surface of domain I. The figure illustrates the CPK space-filling structure of DBP, with white regions indicating flexible regions of the molecule. Virtually the entire top face of the 25(OH) is exposed to the external environment.

Vitamin D-Binding Protein (DBP)

The vitamin D-binding protein (DBP), also historically referred to as group-specific component of serum or Gc-globulin, was initially identified by its polymorphic migration pattern on serum electrophoresis. Although its function was quite unknown, its polymorphic properties allowed DBP (Gc) to play a significant role in human population genetics. In 1975 human Gc protein was found to specifically bind radioactive vitamin D$_3$ and 25(OH)-vitamin D$_3$ thus identifying one of its biological functions (Daiger *et al.*, 1975, 1977; Constans *et al.*, 1983).

The vitamin D-binding protein (DBP) is the serum protein that serves as the transporter and reservoir for the principal vitamin D metabolites throughout the vitamin D endocrine system.(Bouillon *et al.*, 1981a; Haddad, 1995). These include 25(OH)D$_3$, the major circulating metabolite ($K_D \sim 6 \times 10^{-9}$ M) (Haddad *et al.*, 1976; Song *et al.*, 1999) and the steroid hormone 1α,25(OH)2D$_3$ ($K_D \sim 6 \times 10^{-8}$ M). DBP is similar in function to the corticosteroid-binding globulin (CBG), which carries glucocorticoids, and the steroid hormone-binding globulin (SHBG), which transports estrogens or androgen.

DBP is a slightly acidic (pI = 5.2) monomeric glycoprotein of 53 kDa that is synthesized and secreted by the liver as a major plasma constituent. DBP can be up to 5% glycosolated and is known to be one of the most polymorphic proteins, with three common allelic variants and more than 124 rare variants known (Song *et al.*, 1999). DBP's plasma concentration (4–8 μM) is approximately 20-fold higher than that of the total circulating vitamin D metabolites (~10^{-7} M). DBP binds 88% of the total serum 25(OH)D$_3$ and 85% of serum 1,25(OH)$_2$D$_3$, yet only 5% of the total circulating DBP actually carries vitamin D metabolites (Bouillon *et al.*, 1977). The concentration of the "free" hormone may be important in determining the biological activity of the 1α,25(OH)$_2$D$_3$ steroid hormone (Bouillon *et al.*, 1981b; Bikle *et al.*, 1985; Greenberg *et al.*, 1986). In addition to the vitamin D metabolite-binding properties of DBP, the protein has been shown to function as a high-affinity plasma actin–monomer scavenger functioning in concert with the protein gelsolin to prevent arterial congestion (Haddad *et al.*, 1990). There are stoichiometric, 1:1, molar amounts of DBP and actin in their high-affinity heterodimer; the actin/DBP K_D was determined in 2002 (Swamy *et al.*, 2002; Otterbein *et al.*, 2002). This information is not considered in detail in this presentation.

DBP has been proposed to be involved in the transport of fatty acids (Bouillon *et al.*, 1992); the DBP K_D for binding fatty acids is ~10^{-6} M. Also, DBP has been implicated in playing a role in complement C5a-mediated chemotaxis (DiMartino *et al.*, 2001)and has been found to be associated with immunoglobulin surface receptors on lymphocytes, monocytes, and neutrophils (DiMartino *et al.*, 1999).

DBP (~53 kDa) is a member of the albumin multigene family of proteins that also contains albumin (human serum albumin or HSA), α-fetoprotein (AFP), and afamin (AFM) (Gibbs *et al.*, 1987a, 1987b; Nishio *et al.*, 1996). AFP (~70 kDa) has an analogous function to albumin in the fetus and is measured clinically to diagnose or monitor fetal distress or fetal abnormalities, some liver disorders, and some cancers; however, AFP has no known function in adults. Albumin is the major protein component in human plasma and binds a number of relatively water-insoluble endogenous compounds, including fatty acids, bilirubin, and bile acids. DBP originally was referred to as the group-specific component (Gc), which was initially studied electrophoretically as a polymorphic marker in the α-globulin region of human serum. See the reviews in Haddad (1995).

The x-ray crystal structure of DBP has been determined; see Figure 10C and D (Verboven *et al.*, 2002, 2003).

The known multifunctionality of DBP (both vitamin D metabolite and actin binding) separates it from the other members of its family and other steroid transport proteins like retinal-binding protein (RBP) and thyroid-binding globulin (TBG). However, two proteins that bind and transport sterols, sex hormone-binding globulin (SHBG) and uteroglobulin (UG), have been implicated in physiological functions other than steroid transport. SHBG, which binds sex steroids in blood, triggers cAMP-dependent signaling through binding to specific cell surface receptors in prostate (Nakhla *et al.*, 1994) and breast cancer cells (Porto *et al.*, 1995).

The three-domain schematic plan of DBP is shown in Figure 6B where it is compared with the domain structure of the VDR (see Fig. 6A). Domains I, II, and III have been postulated to have evolved from a progenitor that arose from the triple repeat of a 192-amino-acid sequence (Gibbs *et al.*, 1987a); however, domain III is significantly truncated at the C terminus. The position of the vitamin D metabolite and actin-binding domains are specified in domains I and portions of domains I, II, and III, respectively.

The x-ray crystallographic structures of the human DBP with a bound ligand of 25(OH)-D_3 has been recently determined (Verboven *et al.*, 2002) (see Fig. 10C and D). The N-terminal region of DBP, helix 1 to helix 6 of domain I, forms the ligand-binding domain (LBD) where 25(OH)D_3 and other vitamin D metabolites bind. When bound to DBP, vitamin D sterols, including 1α,25(OH)$_2$D$_3$, remain highly exposed to the external environment, which is not the case for internally sequestered ligands bound to the other plasma transport proteins, e.g., steroid hormone-binding globulin (SHBG), retinol-binding protein (RBP), uteroglobulin (UG), and thyroid-binding globulin (TBG) and as well as the VDR (see Fig. 10A and B). Virtually the whole α-face, top view, of the 25(OH)D_3 molecule is exposed to the external environment when bound to the DBP, but the protein's affinity for 25(OH)D_3 still remains high (~6×10^{-9} M) presumably because of the relative strength of the protein–ligand interactions on the α-face of 25(OH)D_3.

Table V summarizes the important similarities and differences in the structure of the two key proteins of the vitamin D endocrine system, the VDR LBD and DBP. Even though there is no amino acid or structural similarity between the two proteins, each has independently evolved to create a unique but highly effective ligand-binding domain that can tightly bind its optimal vitamin D secosteroid ligand: for DBP, 25(OH)D_3, $K_D \sim 6 \times 10^{-9}$ M; and for the VDR, 1α,25(OH)$_2$D$_3$, $K_D \sim 1 \times 10^{-9}$ M. However, the location of the two ligand-binding domains is vastly different for the two proteins; for the VDR the ligand is sequestered inside the protein (see Fig. 10A and B), whereas for DBP the ligand is held in a surface crevice (see Fig. 10C and D) such that one face of the ligand is exposed to the solvent environment. Thus, there is a much greater freedom of ligand structure tolerated by the DBP ligand compared with the VDR; compare optimal DBP ligands with VDR ligand.

Genetics and the Vitamin D Endocrine System

Mutations in the VDR$_{nuc}$

Table VI summarizes the 6 natural and 26 experimental mutations in the LBD of the VDR$_{nuc}$ that have identified amino acids critical for normal LBD function. There are also at least 14 other natural mutations in the zinc finger DNA-binding domain C of the VDR$_{nuc}$ [data not presented but reviewed in Haussler *et al.* (1997b)].

Hereditary vitamin D-resistant rickets (HVDRR), also known as vitamin D-dependent rickets, type II (VDDRII) is a rare genetic disease. Genetic analysis has shown that it is autosomal recessive. Less than 30 kindreds have been reported. The combination of symptoms, i.e., defective bone mineralization, decreased intestinal calcium absorption, hypocalcemia, and increased serum levels of 1α,25(OH)$_2$D$_3$, suggest end-organ resistance to the action of 1α,25(OH)$_2$D$_3$. Patients do not respond to doses of vitamin D, 25(OH)D_3, or 1α,25(OH)$_2$D$_3$.

The unresponsiveness to 1α,25(OH)$_2$D$_3$ associated with the HVDRR has been demonstrated to arise from

TABLE V Comparison of DBP and VDR Protein Crystal Structures and the Ligands for Genomic and Rapid Responses and for DBP

Property	DBP	VDR$_{nuc}$	VDR$_{mem}$
Molecular mass (kDa)	58	51	51
Number of amino acid residues of protein	458	427	427
Number of residues in x-ray structure	458	158	NA
Location of LBD on the protein	Surface cleft	Interior pocket	Interior pocket
Ligand–protein contacts	Unique[a]	Unique[a]	Unique
Ligand A-ring conformation	α-chair	β-chair	β-chair
1α-OH	–	Equatorial	Equatorial or axial
3β-OH	Equatorial	Axial	Axial or equatorial
C5–C6–C7–C8 torsion angle	+149°	+211°	
General ligand shape	Twisted 6-s-*trans*	Bowl-shaped 6-s-*trans* with A-ring 30° above the plane of the CD ring	Planar 6-s-*cis* for 1α,25 (OH)$_2$-lumisterol
A-ring position	31° below C/D ring	30° above C/D ring	Planar
C17–C20–C22–C23 torsion angle	−70°	−156°	Not defined
Side-chain orientation	"Curled down"	Extended	Probably extended
Distance from C-19 to oxygen on C-25	3.8Å	6.9Å	
Overall ligand shape	Hook	Bowl	Relatively planar
Optimal ligand(s)	25(OH)D$_3$	6-s-*trans*-1α,25(OH)$_2$D$_3$	6-s-*cis*-1α,25(OH)$_2$D$_3$ 1α,25(OH)$_2$-lumisterol
K$_D$ for 1α,25(OH)$_2$D$_3$	5×10^{-7} M	1–5×10^{-9} M	1–5×10^{-9} M
General reference citations	Verboven *et al.* (2002, 2003)	Norman *et al.* (1999); Rochel *et al.* (2000); Mizwicki *et al.* (2004)	Mizwicki *et al.* (2003b, 2007)

[a] *The descriptor unique is used to indicate that the amino acid residues of the protein involved with the stabilizing hydrogen bond contact points with the respective ligands, 1α,25(OH)$_2$D$_3$ for VDR and 25(OH)D$_3$ for DBP, are totally different.*

[b] NA = *not available.*

defects in the gene coding for the VDR$_{nuc}$. Two types of abnormalities have been defined by binding studies: receptor-negative and receptor-positive phenotypes (see Table VI). The nucleotide sequence change identified in the receptor-negative phenotype is involved a mutation that introduces a premature stop codon in the message. The resulting truncated protein is not able to bind ligand. The receptor-positive phenotype arises from one of several missense mutations localized within the zinc finger domains of the DNA-binding domain. Several of these mutant receptors have been demonstrated to be defective in their ability to bind to DNA-cellulose and to be unable to mediate 1α,25(OH)$_2$D$_3$-stimulated gene transcription *in vitro* (Sone *et al.*, 1989; Ritchie *et al.*, 1989; Kristjansson *et al.*, 1993; Rut *et al.*, 1994).

Knockout of the VDR$_{nuc}$

Two different groups independently (Yoshizawa *et al.*, 1997; Li *et al.*, 1997) engineered an animal model of hereditary vitamin D-resistant rickets (HVDRR) by targeted disruption of DNA encoding the first and the second zinc finger of the DNA-binding domain of the VDR, respectively. The resultant animals were phenotypically normal at birth. No defects in development and growth were observed before weaning, irrespective of reduced

TABLE VI Genetic Analysis of the Nuclear Receptor for $1\alpha,25(OH)_2D$: Site of Mutation in the VDR and Functional Consequence

VDR domain	Mutation	Functional consequence	Reference
DNA-binding domain	R30Stop	Premature termination: no DNA binding, no ligand binding.	Mechica *et al.* (1997)
	R73Stop		Wiese *et al.* (1993)
	R88Stop		Mechica *et al.* (1997)
		Point mutation intron 4 results in premature stop codon	Hawa *et al.* (1996)
	R30G	Mutations occurring at highly conserved amino acid residues within the first and second zinc fingers. Mutation interferes with the ability of the receptor to interact normally with DNA.	Sone *et al.* (1989)
	G33D		Hughes *et al.* (1988)
	H35Q		Haussler *et al.* (1997a)
	K42I		Rut *et al.* (1994)
	K43E		Rut *et al.* (1994)
	F44I		Rut *et al.* (1994)
	G46D		Lin *et al.* (1996)
	R50Q		Saijo *et al.* (1991)
	R70D		Sone *et al.* (1989)
	R73Q		Hughes *et al.* (1988)
	R80Q		Malloy *et al.* (1994)
	K91N/E92Q		Hsieh *et al.* (1995)
Hinge region	148Stop	Premature termination—no ligand binding.	Wiese *et al.* (1993)
	Q152Stop		Kristjansson *et al.* (1993)
Ligand-binding domain	Y295Stop	Premature termination—no ligand binding.	Ritchie *et al.* (1989)
	C190W	HVDRR	Malloy *et al.* (1994)
	S208G	Phosphorylation that modulates transcription.	Jurutka *et al.* (1996)
	S208A	No enhancement of transcription.	
	F244G	Impaired transactivation; no RXR dimers.	Whitfield *et al.* (1995)
	K246G	Impaired transactivation.	Whitfield *et al.* (1995)
	L254G	Impaired transactivation; no RXR dimers.	Whitfield *et al.* (1995)
	Q259G		
	L262G		
	R274L	HVDRR	Malloy *et al.* (1994)
	C288G	Impaired ligand binding.	Nakajima *et al.* (1996)
	H305Q	Decreased binding (slight); decreased transactivation.	Malloy *et al.* (1997)
	I314S	Impaired transactivation and RXR dimerization.	Whitfield *et al.* (1996)
	C337G	Impaired ligand binding	Nakajima *et al.* (1996)
	C369G	Impaired transactivation and RXR dimerization	Whitfield *et al.* (1995)
	R391C		Whitfield *et al.* (1996)

expression of vitamin D target genes. After weaning (3 weeks after birth), however, the VDR null mutant mice showed marked growth retardation. No overt abnormalities, however, were found in the heterozygotes even at 6 months. Unexpectedly, all of the VDR null mutant mice developed alopecia (absence of hair) and had few whiskers by 7 weeks. Further, the serum levels of calcium and phosphate were reduced at 4 weeks, with markedly elevated serum alkaline phosphatase activity present in the null mutant mice, whereas in older VDR-deficient mice, these abnormalities became more prominent. These observations in the VDR null mutant mice are similar to those in a human vitamin D-dependant rickets type II disease, in which mutations in the VDR gene have been identified in several families, although this disease is not lethal.

In the VDR null mutant mice at 3 weeks, the serum levels of $1\alpha,25(OH)_2D_3$, $24R,25(OH)_2D_3$, and $25(OH)D_3$ were the same as those in the heterozygous and wild-type mice. However, a marked 10-fold increase in serum $1\alpha,25(OH)_2D_3$ and a clear reduction (to almost undetectable levels) in serum $24R,25(OH)_2D_3$ developed in the VDR null mutant mice at 4 weeks and persisted at 7 weeks. Immunoreactive PTH levels were also raised sharply after weaning and the size of the parathyroid glands in the 70-day-old VDR-ablated mice was increased more than 10-fold. These observations establish that the VDR is essential for regulation of the 1α- and 24R-hydroxylases by $1\alpha,25(OH)_2D_3$ after weaning, again supporting the idea that VDR plays a critical role only after weaning.

Severe bone malformation was induced by the inactivation of the VDR after weaning. Radiographic analysis of VDR null mutant mice at 7 weeks revealed growth retardation with loss of bone density. A 40% reduction in bone mineral density was observed in the homozygote mutant mice. In gross appearance and on x-ray analysis of tibia and fibula, typical features of advanced rickets were observed, including widening of epiphyseal growth plates, thinning of the cortex, fraying, cupping, and widening of the metaphysis. In marked contrast, in the VDR-ablated mice in whom normal mineral ion homeostasis had been preserved by feeding of a high-calcium, high-lactose diet, none of these bone parameters were significantly different from those in wild-type litter mates raised under identical conditions. In particular, the morphology and width of the growth plate were indistinguishable from those in wild-type controls, demonstrating that a calcium/phosphorus/lactose-enriched diet started at 16 days of age in the VDR null mice permits the development of both normal morphology in the growth cartilage and adjacent metaphysis and normal biomechanical competence of cortical bone. Thus, the remarkable conclusion is that there is no clear contribution of the VDR to normal bone development, skeletal growth, maturation, and remodeling. The major calcium homeostatic contribution of the VDR$_{\text{nuc}}$ is its role in intestinal calcium absorption.

The male and female VDR null mutant mice were infertile. Uterine hypoplasia and impaired folliculogenesis were observed in the female, and decreased sperm count and motility with histological abnormality of the testis were observed in the male. The aromatase activities in these mice were low in the ovary, testis, and epididymis. These results indicated that vitamin D is essential for full gonadal function in both sexes (Kinuta *et al.*, 2000).

Knockout of the 25(OH)D$_3$-24-hydroxylase

24R,25(OH)$_2$D$_3$ is the second major dihydroxylated metabolite of vitamin D$_3$ that is found in significant concentrations in the serum of humans (Nguyen *et al.*, 1979; Jongen *et al.*, 1989; Castro-Errecaborde *et al.*, 1991), rats (Jarnagin *et al.*, 1985), and chicks (Goff *et al.*, 1995). Although the production of 24R,25(OH)$_2$D$_3$ by the kidney is tightly regulated (Henry *et al.*, 1984), the biological importance of this compound is still the subject of uncertainty and question (Norman *et al.* 1982a, 1982b). Although several possible biological roles and sites of action have been suggested for 24R,25(OH)$_2$D$_3$, including regulation of parathyroid hormone release from the parathyroid gland (Canterbury *et al.*, 1978; Norman *et al.*, 1982a), most studies concerning this vitamin D metabolite have focused on its possible actions on bone biology (Nakamura *et al.*, 1992; Norman *et al.*, 1993a; Seo *et al.*, 1997a). The possible existence of a nuclear or cytosolic binding protein for 24R,25(OH)$_2$D$_3$ was reported in the chick parathyroid gland (Merke *et al.*, 1981), the long bone of rat epiphysis (Corvol *et al.*, 1980), and chick tibial fracture-healing callus (Seo *et al.*, 1996a). However, there has been no general confirmation of these early findings. Also, several more recent reports have described specific actions and accumulation of 24R,25(OH)$_2$D$_3$ in cartilage (Corvol *et al.*, 1980; Seo *et al.*, 1996b), and the bone fracture-healing callus tissue (Lidor *et al.*, 1987; Seo *et al.* 1997a, 1997b).

A strain of mice deficient for the 25(OH)D-24-hydroxylase enzyme have been generated (St.Arnaud *et al.*, 1997) through homologous recombination in embryonic stem cells in order to address the physiological functions of 24R,25(OH)$_2$D$_3$. The targeted mutation effectively deleted the heme-binding domain of the cytochrome P450 enzyme, ensuring that the mutated allele could not produce a functional protein. The analysis of the phenotype of the knockout animals revealed fascinating and previously unrecognized roles for 24R,25(OH)$_2$D$_3$. About half of the mutant homozygote mice born from heterozygote females died before weaning. Bone development of those survivors was abnormal in homozygous mutants born of homozygous females. Histological analyses of the bones from these mice revealed an accumulation of unmineralized matrix at sites of intramembranous ossification, in particular, the calvaria and exocortical surface of long bones. However, the growth plates from these mutant animals appeared normal, suggesting that 24R,25(OH)$_2$D$_3$ is not a major regulator of chondrocyte maturation *in vivo*.

EVIDENCE FOR A MEMBRANE RECEPTOR (VDR$_{\text{MEM}}$) FOR 1α,25(OH)$_2$D$_3$

The impact of 1α,25(OH)$_2$D$_3$-mediated RR on the VDR from 1994 to the present have been summarized in Table II. The concept of the existence of a membrane receptor for 1α,25(OH)$_2$D$_3$ has its origins in the study of the process of 1α,25(OH)$_2$D$_3$-mediated response of transcaltachia, or the rapid hormonal stimulation of intestinal Ca^{2+} absorption in the perfused chick intestine (Nemere *et al.*, 1984b) The most potent agonist is the natural hormone 1α,25(OH)$_2$D$_3$, which can stimulate the transport of calcium across the intestine within 4–5 minutes of its injection in the celiac artery that perfuses the duodenum (Nemere *et al.*, 1984b).

Some have questioned what is the true physiological relevance of 1α,25(OH)$_2$D$_3$-mediated rapid response because no phenotype or disease has yet been described. One hypothesis advanced by Gniadecki (1998b) is that 1α,25(OH)$_2$D$_3$-initiated rapid responses generate signal transduction pathways, which has the end result of altering gene transcription. Certainly the ability of the VDR$_{\text{nuc}}$ operating in the nucleus to activate/suppress gene transcription of appropriate genes is well established. However the process of genomic signaling lacks two important characteristics. (1) Rapidity—It can take several hours to achieve the transition to a new transcriptional steady state, which requires the integrated change at the transcription level of a gene followed by translational generation of the product protein in adequate amounts and, if necessary, post-translational modifications of the protein. When the production of multiple proteins is required for the desired biological response, the time may be increased even further. (2) Modulation—The fine tuning and expansion of the initiating signal to change gene transcription. Although the primary stimulus for a biological response is principally dependent upon the number of activated liganded VDR_{nuc} heterodimer + coactivator complex bound to the promoter, it may be possible for other signal transduction pathways (MAP kinase activation) to modulate the final outcome. One report clearly demonstrates, via gene array analysis, how activation of MAP kinase altered the expression of 383 genes (Roberts *et al.*, 2000).

The current view of this laboratory is that transcaltachia and many other $1\alpha,25(OH)_2D_3$-mediated RR are initiated by a membrane-localized receptor that binds $1\alpha,25(OH)_2D_3$ (Huhtakangas *et al.*, 2004) Recent work in our laboratory has focused on isolation of the intestinal, kidney, or lung membrane fractions which contain caveolae. Caveolae are flask-shaped membrane invaginations that are enriched in sphingolipids and cholesterol that are commonly found in both caveolae and/or lipid rafts (Razani *et al.*, 2002; Lucius *et al.*, 2003; Liscovitch *et al.*, 2004). The caveolae-enriched membrane fraction (CMF) is isolated from chick or rat intestine, kidney, or lung tissue by Percoll buoyant density centrifugation. We find that under both *in vivo* and *in vitro* conditions there is a saturable binding of high-specific-activity tritiated $1\alpha,25(OH)_2D_3$ in the CMF. This binding activity is steroid specific for 1α-hydroxylated analogs, and for $1\alpha,25(OH)_2D_3$, it has a K_D equal to 1.4 nM (see Table V) which is identical to the nuclear VDR K_D for $1\alpha,25(OH)_2D_3$ (Huhtakangas *et al.*, 2004) . Further, by confocal microscopy we showed that green fluorescent labeled antibodies to the classical nuclear VDR colocalized with red fluorescent labeled antibodies to caveolin-1 in caveolae present in the plasma membrane of ROS-17.2,8 osteoblast cells (Huhtakangas *et al.* 2004). Both my laboratory (Huhtakangas *et al.*, 2004) and that of Lieberherr and Garabedian (Nguyen *et al.*, 2004) have separately concluded that the rapid response effects of $1\alpha,25(OH)_2D_3$ require a functional VDR. We found that in VDR knockout mice (VDR KO) that $1\alpha,25(OH)_2D_3$ was unable to stimulate the opening of voltage-gated Ca^{2+} and Cl^- channels (Huhtakangas *et al.*, 2004; Bula *et al.*, 2005) and that there was a loss of 70–90% in the ability of $[^3H]$-$1\alpha,25(OH)_2D_3$ to bind to isolated caveolae membrane fractions obtained from VDR KO intestine, lung, and kidney tissues (Huhtakangas *et al.*, 2004). The residual binding of $[^3H]$-$1\alpha,25(OH)_2D_3$ present in VDR KO tissues was found to be attributable to the presence of a truncated form of the VDR present in the strain of VDR KO mice employed in our studies (Bula *et al.*, 2005). Lieberherr and Garabedian found that, in fibroblasts obtained from a patient with the genetic disease of vitamin D-resistant rickets (VDRR), which results from a single-point mutation in the VDR (Lys45Glu), there was a loss of $1\alpha,25(OH)_2D_3$ RR (Nguyen *et al.*, 2004).

Thus, this laboratory's view is that the classical VDR normally associated with the cell nucleus and gene transcription can also be resident near to or associated with caveolae present in the plasma membrane. It has also been reported that the estrogen receptor (ER) (Pedram *et al.*, 2006a, 2006b) and androgen receptor (AR) are associated with caveolae. For the nuclear estrogen, androgen, and progesterone receptors, recent evidence implicates that palmitoylation of nuclear receptor allows them to associate and become attached the plasma membrane caveolae (Guo *et al.*, 2006).

It is apparent that for the VDR there is a perceived problem, because a 6-s-*trans* shape of $1\alpha,25(OH)_2D_3$ is obligatory for genomic responses, whereas a 6-s-*cis* shape is required for RR. This then poses the conundrum as to how a receptor with one ligand-binding domain (LBD) can bind ligands of quite different shapes to generate two quite different biological outcomes? Yet, the x-ray structure of the VDR only revealed one ligand-binding domain, that which bound the 6-s-*trans* shape (see Fig. 10A).

One possible solution to the conundrum was obtained from molecular modeling of the VDR (Mizwicki *et al.*, 2004). Using the atomic coordinates of the x-ray structures of the VDR and computer modeling, we have been able to identify the presence of a putative alternative LBD in the VDR that can accommodate via computer "docking" either the appropriate natural hormone or analogs that are known to be agonists only for RR (see Fig. 11A) (Mizwicki *et al.*, 2004). Figure 11A illustrates for the VDR the classical nuclear ligand pocket (red) and the proposed alternative ligand pocket (blue). Each ligand pocket is envisioned to have separate portals. The entrance to the nuclear pocket depends on helix-12 being in an "open" configuration so that the hormone/ligand can enter into the LBD pocket and gain access to integral H-bonding residues, thereby fully occupying the genomic pocket. Next, helix-12 moves to "close" the portal. One surprising discovery is that the alternative pocket and the genomic pocket partially overlap. Each of these pockets uses the same hydrogen-bonding partners of the VDR to stabilize the A rings of the bound ligands; however, the remaining interactions of the C/D ring and side chain of the ligand in the genomic or alternative pockets are entirely unique.

My laboratory believes that the resolution of the conundrum stated earlier is to propose a receptor ensemble model that can describe how a classic steroid (nuclear) receptor could accommodate differently shaped ligands so as to result in the initiation of either rapid or genomic responses (Fig. 11B). This model posits that unbound receptor macromolecules exist in the cytoplasm as multiple, equilibrating receptor conformations that follow the laws associated with standard statistical distributions (Mizwicki *et al.*, 2004). Thus, a steroid hormone would sample the existing population of receptor conformations available and form a receptor–hormone complex with the receptor species that formed the best complementary fit between the two molecules; this

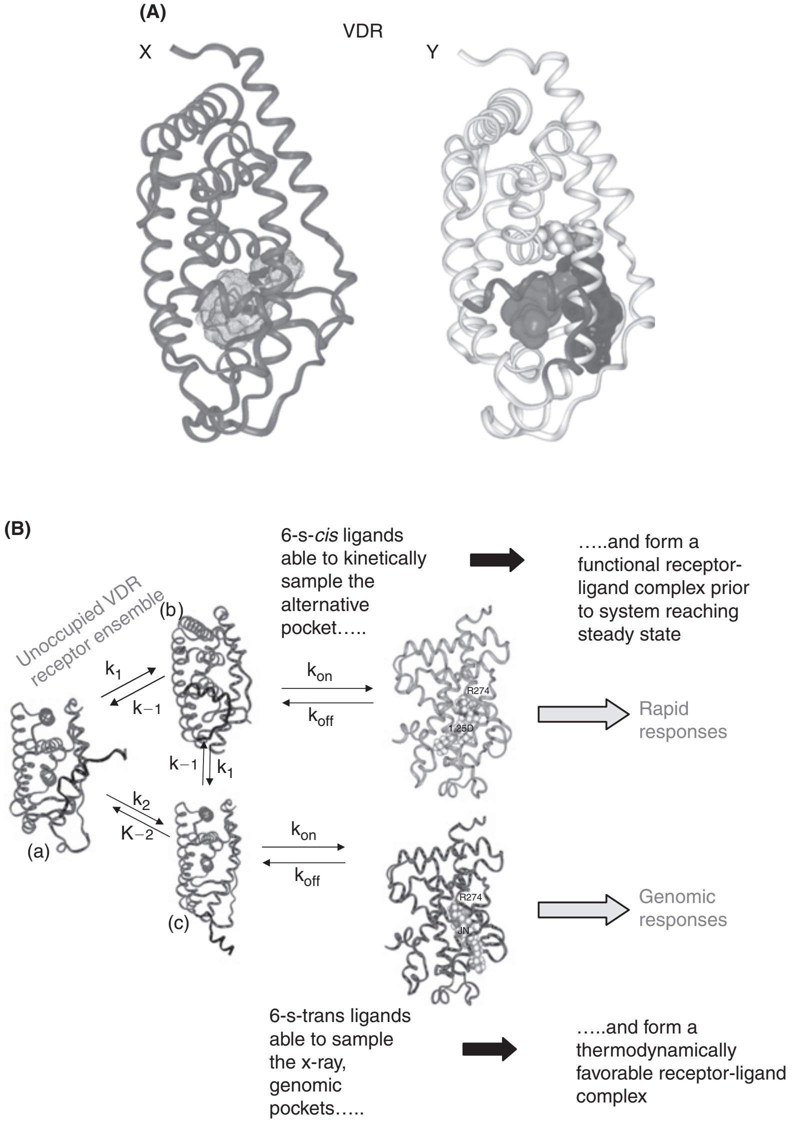
(A)
VDR
X
Y
(B)
Unoccupied VDR receptor ensemble
(b)
(a)
(c)
k1
k−1
k−1
k1
k2
K−2
kon
koff
kon
koff
R274
1,25D
R274
JN
6-s-cis ligands able to kinetically sample the alternative pocket…..
…..and form a functional receptor-ligand complex prior to system reaching steady state
Rapid responses
Genomic responses
6-s-trans ligands able to sample the x-ray, genomic pockets…..
…..and form a thermodynamically favorable receptor-ligand complex

FIGURE 11 Structural details of the VDR that support a conformational ensemble model. **(A)** Details of the ligand-binding domain(s) in the ribbon structures of the nuclear VDR. (*X*) Differences in the volume and shape of the interior of the VDR ligand-binding domain (light blue) with the structure of the $1\alpha,25(OH)_2D_3$ shown in wire frame as revealed by x-ray crystallography of the protein + ligand. (*Y*) Illustration for the VDR of the location of the classic genomic pocket (red: $1,25(OH)_2$-vitamin D_3; a genomic agonist) and a putative alternative pocket (blue: $1,25(OH)_2$-lumisterol; a nongenomic 6-s-*cis*-locked agonist) as studied by molecular modeling; helix-12 (H12) is brown and shown in the closed conformation. The proposed alternative pocket portal lies between the C terminus of H1 and N terminus of H3. An intriguing aspect of the two ligand binding pocket model, is that the A ring of ligands bound to either the genomic pocket or alternative pocket use the same hydrogen-bonding partners (R274, S237, S278) to stabilize their 1α- and 3β-hydroxyls; The conserved helix 5 R274 is shown in CPK; thus the VDR can not bind two ligands simultaneously. Details of the modeling are provided in (Norman *et al*., 2004; Mizwicki *et al*., 2004). **(B)** Schematic model of a receptor ensemble model to describe how a classic steroid receptor could accommodate differently shaped ligands that result in the initiation of either rapid responses or genomic responses. The ensemble model proposes that there is a population of three different unoccupied receptor species (*a*, *b*, and *c*) illustrated by different positions of helix-12 (H12;-brown) that are in rapid equilibrium with one another; each receptor conformer species may preferentially bind differently shaped ligands (Bursavich *et al*., 2002). In this example, transient occupancy of the alternative pocket by a ligand could/would lead to initiation of RR whereas occupancy of the classical pocket by a ligand would lead to activation of genomic responses; both of these pockets are illustrated for the VDR in Figure 2A. The alternative pocket is accessible in all three H12 conformers (*a*, *b*, and *c*). Occupancy of this alternative pocket is only favored prior to achieving the steady-state equilibrium for the receptor's natural ligands given the increased accessibility coupled with weaker van der Waals interactions compared with genomic pocket occupancy. Receptor conformer (*c*) is the only conformer able to accept ligands that can bind to the classical ligand-binding site leading to genomic responses because, in conformers a and b, H12 blocks ligand accessibility to the conserved H5 Arg residue located at the innermost part of the classic ligand-binding pocket. An interesting consequence of the receptor ensemble concept may be that association of the nuclear receptor with caveolae or cell membranes may change the ligand-binding preferences to favor the alternative pocket. Ligand occupancy of this genomic pocket is favored under steady-state conditions because of the increase in thermodynamic stability derived from hydrophobic interactions. It is possible that drug analogs, especially those related to the natural hormone(s), may have differential fractional occupancies of the two pockets, thus affecting the efficiency of the cellular signaling pathways mediated by either pocket.

would shift the equilibrium among the receptor species so as to favor the energetically most stable hormone-bound receptor conformation. It should be noted that 1α,25$(OH)_2D_3$ is able to change its conformation much more quickly than the receptor protein, so essentially the whole ensemble of 1α,25$(OH)_2D_3$ conformations can sample each of the individual protein ensemble conformations. Implicit in this model is that the receptor "sampling" by the ligand involves gaining entrance and exploring the interior surface of the LBD to determine whether a complementary "fit" can be achieved. A related model to describe ligand/receptor-induced separation of rapid from genomic responses was included in the comprehensive analysis of nongenotropic, sex-nonspecific signaling by the ER and AR (Kousteni *et al.*, 2001). Although this model has not yet been fully validated, it is possible to identify experiments involving site-directed mutagenesis of the VDR that may give further insight.

SUMMARY

In contrast to classical steroid hormones, like estradiol, which have only one shape, it is apparent that 1α,25$(OH)_2D_3$ can generate at least three functionally different shapes that effectively accommodate the ligand-binding domain requirements of the VDR_{nuc}, the caveolae-associated VDR_{mem}, and DBP proteins. These different shapes are described in Figure 2E to G and in Table V.

Figure 12 is a summary figure that integrates the signal transduction pathways that are used by the nuclear VDR and the membrane-associated VDR with 1α,25$(OH)_2D_3$ functioning as a conformationally flexible agonist. This model emphasizes the complexity of overlapping and interconnecting signal transduction pathways. Although there is a solid and secure foundation describing 1α,25$(OH)_2D_3$-mediated genomic actions, 1α,25$(OH)_2D_3$-mediated RRs are still in their developmental phase. One important challenge will be to identify and fully biochemically characterize the membrane-associated VDR. An equally daunting task will be to define in the various cell systems that display RR, the identification of the specific signal transduction pathways that contribute to mediation of a specific rapid biological response. A further important question is to determine whether the membrane VDR can communicate with the nucleus of the cell to modulate gene transcription. In data not shown, but summarized in Figure 11, there are

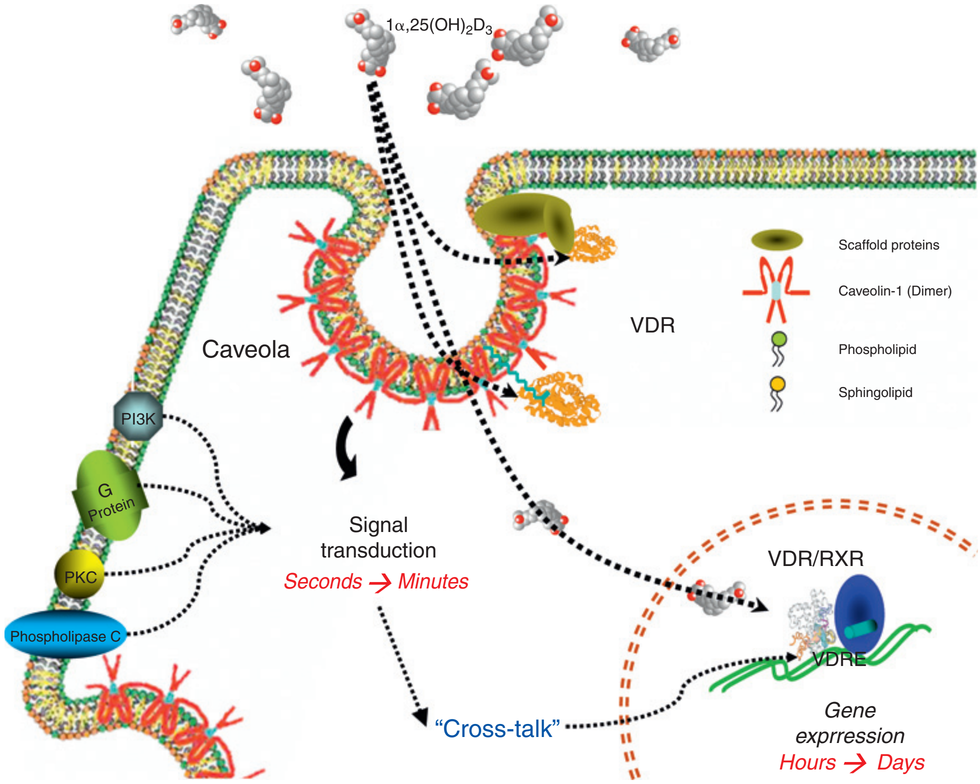

FIGURE 12 Schematic model describing how the conformationally flexible 1α,25$(OH)_2D_3$ can interact with the VDR localized in the cell nucleus to generate genomic responses or in caveolae of the plasma membrane to generate RR. Binding of 1α,25$(OH)_2D_3$ to the caveolae-associated VDR may result in the activation of one or more second messenger systems, including phospholipase C, protein kinase C, G protein-coupled receptors, or phosphatidylinositol-3′-kinase (PI3K). There are a number of possible outcomes including opening of the voltage-gated calcium or chloride channels or generation of the indicated second messengers. Some of these second messengers, in particular, RAF/MAP kinase, may engage in "crosstalk" with the nuclear VDR to modulate gene expression (Norman *et al*., 2000). (See plate section)

at least five different systems (pancreas β-cell, adipocytes, vascular smooth muscle, intestine, and osteoblasts) where molecular biological evidence has been obtained for the process of crosstalk from $1\alpha,25(OH)_2D_3$ rapid responses to changes in gene expression in the cell nucleus. In these studies, 6-s-*cis*-locked analogs have been shown to modulate genomic responses at relatively early time intervals (2–4 hours). This supports the generality of $1\alpha,25(OH)_2D_3$-mediated rapid responses in a wide variety of physiological systems. It adds further impetus to the need to continue studies at the molecular level to define and characterize the molecular physiology of $1\alpha,25(OH)_2D_3$-mediated responses.

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Chapter 36

Vitamin D Gene Regulation

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VITAMIN D METABOLISM

Vitamin D is a principal factor required for the development and maintenance of bone as well as for maintaining normal calcium and phosphorus homeostasis. In addition, evidence has indicated the involvement of vitamin D in a number of diverse cellular processes, including effects on differentiation and cell proliferation, on hormone secretion, and on the immune system (DeLuca, 2004; Christakos *et al*., 2003). For vitamin D to affect mineral metabolism as well as numerous other systems, it must first be metabolized to its active form. Vitamin D, which is taken in the diet or is synthesized in the skin from 7-dehydrocholesterol in a reaction catalyzed by ultraviolet irradiation, is transported in the blood by the vitamin D-binding protein to the liver. In the liver, vitamin D is hydroxylated at C-25, resulting in the formation of 25-hydroxyvitamin D_3 [25(OH)D_3]. 25-Hydroxyvitamin D proceeds to the kidney via the serum vitamin D-binding protein. Megalin, a member of the LDL receptor superfamily, plays an essential role in the renal uptake of 25(OH)D_3 (Nykjaer *et al.,* 1999). In the proximal convoluted and straight tubules of the kidney nephron, 25(OH)D_3 is hydroxylated at the position of carbon 1 of the A ring, resulting in the formation of the hormonally active form of vitamin D, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]. The kidney can also produce 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$]. 24-Hydroxylase [24 (OH)ase] has been reported to be capable of hydroxylating the 24 position of both 25(OH)D_3 and 1,25$(OH)_2D_3$ (Omdahl *et al*., 2003) (see Fig. 1). Because the K_m value of 24(OH)ase for 1,25$(OH)_2D_3$ is 1/5 to 1/30 of the K_m value for 25(OH)D_3 (Tomon *et al.,* 1990), it has been suggested that the preferred substrate for 24(OH)ase *in vivo* may be 1,25$(OH)_2D_3$ rather than 25(OH)D_3 (Shinki *et al.,* 1992). Studies using mice with a targeted inactivating mutation of the 24(OH)ase gene [24(OH)ase null-mutant mice] have provided the first direct *in vivo* evidence for a role for 24(OH)ase in the catabolism of 1,25$(OH)_2D_3$ (St-Arnaud *et al.,* 2000). Both chronic and acute treatment with 1,25$(OH)_2D_3$ resulted in an inability of 24(OH)ase-deficient mice to clear 1,25$(OH)_2D_3$ from their bloodstream. Impaired bone formation at specific sites (calvaria, mandible, clavicle, and periosteum of long bones) was also noted in the deficient mice. 24,25-Dihydroxyvitamin D_3 supplementation failed to correct most of the bone abnormalities. Because crossing 24(OH)ase-deficient mice to vitamin D receptor (VDR)-ablated mice totally rescued the bone phenotype, the authors suggested that elevated 1,25$(OH)_2D_3$ levels acting through VDR at specific sites and not the absence of 24,25$(OH)_2D_3$, were responsible for the abnormalities observed in bone development. Whether 24,25$(OH)_2D_3$ is an active metabolite has been a matter of debate. However, most studies indicate that 24,25$(OH)_2D_3$ appears to be relatively inactive when compared with 1,25$(OH)_2D_3$.

The production of 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ is under stringent control. Calcium and phosphorus deprivation

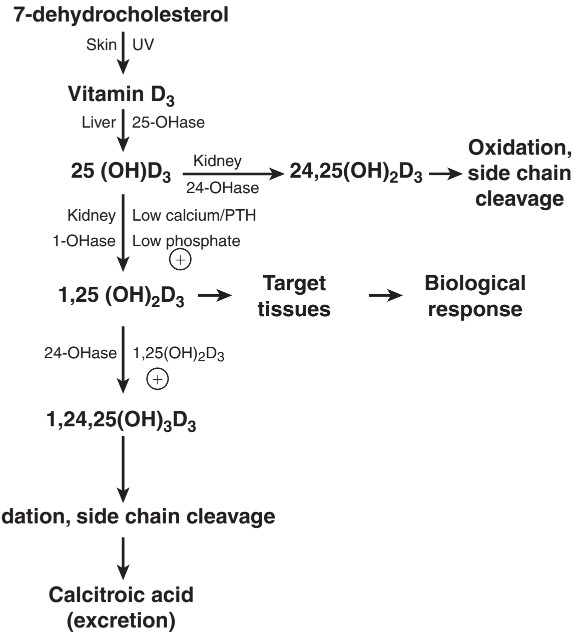

FIGURE 1 The metabolic pathway for vitamin D.

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results in enhanced production of $1,25(OH)_2D_3$ (Omdahl *et al.*, 2003). Elevated PTH resulting from calcium deprivation may be the primary signal mediating the calcium regulation of $1,25(OH)_2D_3$ synthesis (Boyle *et al.*, 1971; Henry, 1985; Murayama *et al.*, 1999). PTH has been reported to stimulate 25-hydroxyvitaminD_3 1α hydroyxlase (1α (OH)ase) by acting on the promoter of the 1α (OH)ase gene (Brenza *et al.*, 1998). 1,25-dihydroxyvitamin D_3 also regulates its own production by inhibiting 1-hydroxylase (Brenza and DeLuca, 2000; Murayama *et al.*, 1999; Murayama *et al.*, 2004). The synthesis of $24,25(OH)_2D_3$ has been reported to be reciprocally regulated when compared with the synthesis of $1,25(OH)_2D_3$ [stimulated by $1,25(OH)_2D_3$, and inhibited by low calcium and PTH] (Omdahl *et al.*, 2003). In addition to PTH, phosphorus, calcium, and $1,25(OH)_2D_3$, it has been reported that sex hormones can influence production of the renal vitamin D hydroxylases. Estrogens alone or when combined with androgens have been reported to stimulate $1,25(OH)_2D_3$ production, and estradiol has been reported to suppress $24,25(OH)_2D_3$ synthesis in avian species (Pike *et al.*, 1978; Tanaka *et al.*, 1976). It is not clear, however, whether a similar relationship exists between sex steroids and vitamin D hydroxylases in mammalian species (Baski and Kenny, 1978). It is of interest that calcitonin has been reported to stimulate the expression of 1α (OH)ase under normocalcemic conditions (Shinki *et al.*, 1999). Because calcitonin is elevated in the serum of the fetus, it has been suggested that calcitonin may have a role (in addition to its known role in shrinking osteoclasts under high calcium conditions) in regulating 1α (OH)ase during development. FGF23, a recently discovered phosphaturic factor, has also been identified as a physiological regulator of vitamin D metabolism, which reduces 1α (OH)ase mRNA (Shimada *et al.*, 2004). Low dietary phosphate intake results in decreased FGF23 levels and a corresponding increase in 1α (OH)ase activity (Perwad *et al.*, 2005). It has been reported that FGF23 requires the klotho protein (a multifunctional protein involved in phosphate and calcium homeostasis and in aging that binds to FGF receptors) to activate FGF signaling (Kuro-o, 2006). Further studies are needed to determine the exact mechanisms by which FGF23 acts to affect vitamin D metabolism. Although the proximal tubule of the kidney, placenta, and macrophages are sites of synthesis of 1α (OH)ase, it has been suggested that 1α (OH)ase is present in other cell types and that local production of $1,25(OH)_2D_3$ could have a role in the function of extra renal tissues. 1α (OH)ase null mutant mice have recently been generated (Panda *et al.*, 2001). It is of interest that besides a phenotype identical to the human vitamin D-dependent rickets type I phenotype, immune and reproductive dysfunction has been noted in these mice (Panda *et al.*, 2001). Further studies are needed to test the hypothesis of a physiological role of 1α (OH)ase in extra renal tissues that has been a matter of debate.

Serum $1,25(OH)_2D_3$ levels and the capacity of the kidney to hydroxylate $25(OH)D_3$ to $1,25(OH)_2D_3$ have been reported to decline with age (Armbrecht *et al.*, 1980). In addition, an increase in renal 24-hydroxylase gene expression and an increase in the clearance of $1,25(OH)_2D_3$ with aging have been reported (Matkovits and Christakos, 1995; Wada *et al.*, 1992). These findings have implications concerning the etiology of osteoporosis and suggest that the combined effect of a decline in the ability of the kidney to synthesize $1,25(OH)_2D_3$ and an increase in the renal metabolism of $1,25(OH)_2D_3$ may contribute to age-related bone loss. Whether there is an interrelationship between the decline of sex steroids with age and age-related changes in the 1- and 24-hydroxylase enzymes remains to be determined.

ROLE OF $1,25(OH)_2D_3$ IN CLASSICAL TARGET TISSUES

Bone

Exactly how $1,25(OH)_2D_3$ affects mineral homeostasis is a subject of continuing investigation. It has been suggested that the antirachitic action of $1,25(OH)_2D_3$ is indirect and the result of increased intestinal absorption of calcium and phosphorus by $1,25(OH)_2D_3$, thus resulting in their increased availability for incorporation into bone (Underwood and DeLuca, 1984; Weinstein *et al.*, 1984). Studies using VDR-ablated mice (VDR knockout mice) also suggest that a principal role of the vitamin D receptor in skeletal homeostasis is its role in intestinal calcium absorption (Li *et al.*, 1997; Yoshizawa *et al.*, 1997; Amling *et al.*, 1999). VDR knockout mice were found to be phenotypically normal at birth, but developed hypocalcemia, hyperparathyroidism, and alopecia within the first month of life. Rickets and osteomalacia were seen by day 35. When VDR knockout mice were fed a calcium/phosphorus/lactose-enriched diet, serum-ionized calcium levels were normalized, the development of hyperparathyroidism was prevented, and the animals did not develop rickets or osteomalacia, although alopecia was still observed. Thus, it was suggested that skeletal consequences of VDR ablation are due primarily to impaired intestinal calcium absorption. *In vitro* studies, however, have shown that $1,25(OH)_2D_3$ can resorb bone (Raisz *et al.*, 1972). Although $1,25(OH)_2D_3$ stimulates the formation of bone-resorbing osteoclasts, receptors for $1,25(OH)_2D_3$ are not present in osteoclasts but rather in osteoprogenitor cells, osteoblast precursors, and mature osteoblasts. Stimulation of osteoclast formation by $1,25(OH)_2D_3$ requires cell-to-cell contact between osteoblastic cells and osteoclast precursors and involves upregulation by $1,25(OH)_2D_3$ in osteoblastic cells of osteoprotegerin ligand or RANKL (osteoclast differentiating factor; Takeda *et al.*, 1999; Yasuda *et al.*, 1998a, 1998b). RANKL is induced by $1,25(OH)_2D_3$ (Kim *et al.*, 2007) as well as by PTH, interleukin 11, and prostaglandin E_2 in osteoblasts/stromal cells. RANKL is a member of the membrane-associated tumor necrosis factor ligand family that enhances osteoclast formation by mediating direct interactions between osteoblast/stromal cells and osteoclast precursor cells. Osteoclastogenesis inhibitory factor/osteoprotegerin, a member of the tumor necrosis factor receptor family, is a soluble decoy receptor for RANKL that antagonizes RANKL function, thus blocking osteoclastogenesis. Osteoprotegerin is downregulated by 1,25(OH)$_2$D$_3$ (Yasuda *et al.*, 1998a).

In addition to increasing the availability of calcium and phosphorus for incorporation into bone and stimulating osteoclast formation, 1,25(OH)$_2$D$_3$ also has a direct effect on osteoblast-related functions. For example, 1,25(OH)$_2$D$_3$ has been reported to stimulate the production of osteocalcin (OC) (Price and Baukol, 1980) and osteopontin (OPN) (Prince and Butler, 1987) in osteoblastic cells. The exact function of osteocalcin, a noncollagenous protein associated with the mineralized matrix, is not known. However, increased synthesis of osteocalcin has been positively correlated with new bone formation. Concerning osteopontin, also a major noncollagenous bone protein, it has been suggested that this secreted, glycosylated phosphoprotein is important for resorption of the bone matrix (the OPN-$\alpha_v\beta_3$ integrin interaction has been reported to be important for the adherence of the osteoclast to bone), as well as for mineralization (Denhardt and Guo, 1993). OPN-deficient mice are resistant to mineral loss and bone resorption upon estrogen deprivation and have impaired activation of osteoclasts, suggesting the requirement for OPN in bone resorption (Asou *et al.*, 2001; Yoshitake *et al.*, 1999). Runx2, identified as a transcriptional regulator of osteoblast differentiation, is involved in regulation of the expression of OPN and OC and has also been reported to be regulated by 1,25(OH)$_2$D$_3$ (Javed *et al.*, 1999; Shen and Christakos, 2005; Centrella *et al.*, 2004). In addition, analogs of 1,25(OH)$_2$D$_3$ have been developed that exhibit selective anabolic actions in osteoblasts and result in enhanced bone formation (Shevde *et al.*, 2002), suggesting that selective bone analogs of 1,25(OH)$_2$D$_3$ may be therapeutically beneficial for the treatment of bone loss disorders. Studies using transgenic mice overexpressing VDR in osteoblastic cells have noted increased bone formation, further indicating direct effects of 1,25(OH)$_2$D$_3$ on bone (Gardiner *et al.*, 2000). Besides analogs of 1,25(OH)$_2$D$_3$ that have direct effects on bone and enhance bone formation, it has also been suggested that vitamin D may reduce falls and therefore fractures by improving neuromuscular function (Dhesi *et al.*, 2004). However, further studies are needed to determine whether the vitamin D endocrine system has direct effects on neuromuscular function or whether the observed effects are secondary to a primary effect on maintenance of calcium homeostasis.

Intestine

In addition to its effect on bone, another extensively studied action of 1,25(OH)$_2$D$_3$ is the stimulation of intestinal calcium absorption (Wasserman and Fullmer, 1995). Although the exact mechanisms involved in this process have still not been defined, it has been suggested that 1,25(OH)$_2$D$_3$ affects intracellular calcium diffusion by inducing the calcium-binding protein, known as calbindin. One of the most pronounced effects of 1,25(OH)$_2$D$_3$ is increased synthesis of calbindin (for reviews see Christakos, 1995, and Christakos *et al.*, 1989). Two major subclasses of calbindin have been described: a protein of a molecular weight of ~9,000 (calbindin-D$_{9k}$) and a protein with a molecular weight of ~28,000 (calbindin-D$_{28k}$). Calbindin-D$_{9k}$ has two calcium-binding domains, has been observed only in mammals, and is present in highest concentration in mammalian intestine. Calbindin-D$_{28k}$, unlike calbindin-D$_{9k}$, is highly conserved in evolution and has four functional high-affinity, calcium-binding sites. Calbindin-D$_{28k}$ is present in highest concentrations in avian intestine and in avian and mammalian kidney, brain, and pancreas. There is no amino acid sequence homology between calbindin-D$_{9k}$ and calbindin-D$_{28k}$. In VDR-ablated mice, which demonstrate impaired intestinal calcium absorption, calbindin-D$_{9k}$ mRNA is reduced dramatically (Li *et al.*, 1998). However, studies using analogs of 1,25(OH)$_2$D$_3$ suggest that there need not be a direct correlation between calbindin induction and stimulation of intestinal calcium transport. Intestinal calbindin-D$_{9k}$ mRNA but not intestinal calcium transport has been reported to be induced by 1,25(OH)$_2$D$_3$ 24-homologues (Krisinger *et al.*,1991). In addition, 1,25,28-trihydroxyvitamin D$_2$ has been found to have no effect on intestinal calcium absorption but to result in a significant induction in rat intestinal calbindin-D$_{9k}$ mRNA and protein (Wang *et al.*, 1993). These findings, as well as recent studies that indicated normal serum calcium levels in calbindin-D$_{9k}$ null mutant mice regardless of age or gender (Kutazova *et al.*, 2006; Benn *et al.*, 2008), provide evidence that calbindin alone is not responsible for the 1,25(OH)$_2$D$_3$-mediated intestinal transport of calcium. Besides acting as a facilitator of intestinal calcium diffusion, it is also possible that calbindin in the intestine may act as an intracellular buffer to prevent toxic levels of calcium from accumulating in the intestinal cell during 1,25(OH)$_2$D$_3$-dependent transcellular calcium transport (Christakos *et al.*, 1989).

1,25(OH)$_2$D$_3$ also affects calcium extrusion from the intestinal cell, which involves calcium transport against a concentration gradient. The intestinal plasma membrane calcium pump (PMCA-1) and PMCA-1 mRNA are stimulated by 1,25(OH)$_2$D$_3$, suggesting that the intestinal calcium absorptive process may involve a direct effect of 1,25(OH)$_2$D$_3$ on calcium pump expression (Cai *et al.*, 1993; Wasserman *et al.*, 1992; Zelinski *et al.*, 1991).

In addition to the role of 1,25(OH)$_2$D$_3$ on transcellular movement of calcium and on the extrusion of calcium from the intestinal cell, it has been known that calcium transfer into the intestinal cell can be increased by 1,25(OH)$_2$D$_3$. However, the existence of a calcium channel responsible for this process had been controversial. In 1999 a calcium selective channel, which is potentially important in the control of intestinal calcium absorption, TRPV6, was cloned from rat duodenum (Peng *et al.,* 1999). Calmodulin binds to TRPV6 and regulates its activity and the association of S100A10-annexin 2 complex has been reported to be required for the targeting and retention of TRPV6 to the plasma membrane (Lambers *et al.,* 2004; van de Graaf *et al.,* 2003). TRPV6 is induced by 1,25$(OH)_2D_3$ in intestine and in VDR knockout mice TRPV6 was found to be more markedly decreased than calbindin-D_{9k} (Song *et al.,* 2003; Van Cromphaut *et al.,* 2001). It has been suggested that TRPV6 may play a key role in vitamin D-dependent calcium entry into the enterocyte and that TRPV6 may be the rate-limiting step in vitamin D-dependent intestinal calcium absorption (Van Cromphaut *et al.,* 2001). TRPV6 null mutant mice have recently been generated (Bianco *et al.,* 2007). Although these mice have normal serum calcium levels, they have increased serum PTH and 1,25$(OH)_2D_3$ levels as well as a 9.3% decrease in femoral bone density, suggesting a disturbance in calcium homeostasis (Bianco *et al.,* 2007). Further studies using these mice as well as TRPV6/calbindin-D_{9k} double knockout mice should result in new insight in our understanding of the process of vitamin D-dependent intestinal calcium absorption.

Kidney

In addition to bone and intestine, a third target tissue involved in the regulation by 1,25$(OH)_2D_3$ of mineral homeostasis is the kidney. Micropuncture data, as well as studies using a mouse distal convoluted tubule cell line, have indicated that vitamin D metabolites can enhance the stimulatory effect of PTH on calcium transport in the distal nephron (Friedman and Gesek, 1993; Winaver *et al.,* 1980). 1,25$(OH)_2D_3$ has been reported to increase PTH receptor mRNA and binding activity in distal tubule cells, providing a mechanism whereby 1,25$(OH)_2D_3$ enhances the action of PTH (Sneddon *et al.,* 1998). In the mouse, both vitamin D-dependent calcium-binding proteins (calbindin-D_{9k} and calbindin-D_{28k}) have been reported to be localized in the distal nephron (distal convoluted tubule, connecting tubule, and cortical collecting tubule) (Rhoten *et al.,* 1985). Kinetic analysis has suggested that the two proteins affect renal calcium reabsorption by different mechanisms. Calbindin-D_{28k} stimulates the high-affinity system in the distal luminal membrane (Bouhtiauy *et al.,* 1994a), whereas calbindin-D_{9k} was found to enhance the ATP-dependent calcium transport of the basolateral membrane (Bouhtiauy *et al.,* 1994b). These findings provide evidence for a role for calbindins in vitamin D-dependent calcium transport processes in the kidney. Similar to studies in intestine, an apical calcium channel, TRPV5, was also identified in the distal convoluted tubule and distal connecting tubules (Hoenderop *et al.,* 1999; Vennekens *et al.,* 2000). TRPV5 shows 73.4% sequence homology with TRPV6 and is also induced by 1,25$(OH)_2D_3$ (Song *et al.,* 2003). The S100A10-annexin 2 complex was found to play a role in routing TRPV5 to the plasma membrane as well as TRPV6 (van de Graaf *et al.,* 2003). The klotho protein activates TRPV5 by hydrolyzing TRPV5 N-linked oligosaccharides (Chang *et al.,* 2005). In addition, calbindin-D_{28k} was reported to associate directly with TRPV5 and to control TRPV5-mediated calcium influx (Lambers *et al.,* 2006). Mice lacking TRPV5 display diminished calcium reabsorption in the distal tubule, hypercalciuria, and disturbances in bone structure, supporting the suggested role for TRPV5 in renal calcium handling (Hoenderop *et al.,* 2003). Thus, 1,25$(OH)_2D_3$ may affect calcium transport in the distal tubule by enhancing the action of PTH and by inducing TRPV5 and the calbindins. In addition to the calbindins and TRPV5, the plasma membrane calcium pump has also been localized immunocytochemically exclusively to the distal tubule and to the collecting duct (Borke *et al.,* 1988). However, the interrelationship between the renal calcium pump and 1,25$(OH)_2D_3$ is not clear at this time. In addition to the suggested role of 1,25$(OH)_2D_3$ in the tubular reabsorption of calcium, another important effect of 1,25$(OH)_2D_3$ in the kidney is inhibition of the 25$(OH)D_3$ 1α-hydroxylase enzyme and stimulation of the 24-hydroxylase enzyme. Both the 1α-hydroxylase and 24(OH)ase genes have been cloned, and studies indicate that they are regulated through liganded VDR (Takeyama *et al.,* 1997; Shinki *et al.,* 1997; Murayama *et al.,* 1999; Murayama *et al.,* 2004; Kerry *et al.,* 1996; Zierold *et al.,* 1995). In addition to effects on calcium transport in the distal nephron and modulation of the 25$(OH)D_3$ hydroxylases, effects of 1,25$(OH)_2D_3$ on phosphate reabsorption in the proximal tubule have also been suggested. Vitamin D has been reported to increase or decrease renal phosphate reabsorption depending on the parathyroid status and on experimental conditions. Mutations in the PHEX gene, a phosphate-regulating gene, are responsible for X-linked hypophosphatemia. 1,25$(OH)_2D_3$ has been reported to downregulate PHEX gene expression, suggesting a role for 1,25$(OH)_2D_3$ in renal phosphate transport (Hines *et al.,* 2004). Although progress has been made, further studies are needed to provide new insight concerning the renal effects of 1,25$(OH)_2D_3$ that have not been well understood.

Parathyroid Glands

In the regulation of mineral homeostasis, the parathyroid glands are also an important target of 1,25$(OH)_2D_3$ action. 1,25-Dihydroxyvitamin D_3 inhibits the secretion and synthesis of PTH. A direct action of 1,25$(OH)_2D_3$ on the preproparathyroid hormone gene has been reported (Demay *et al.,* 1992; Mackey *et al.,* 1996). 1,25$(OH)_2D_3$ also functions to prevent the proliferation of the parathyroid gland and thus helps to maintain normal parathyroid status (Martin and Gonzalez, 2004).

NONCLASSICAL ACTIONS OF 1,25$(OH)_2D_3$

Effects of 1,25$(OH)_2D_3$ on Differentiation and Proliferation

In addition to affecting tissues involved in mineral homeostasis, 1,25$(OH)_2D_3$ has been reported to affect numerous other systems. Because 1,25$(OH)_2D_3$ receptors have been identified in tissues not involved in calcium homeostasis, it has been suggested that the actions of 1,25$(OH)_2D_3$ in these nonclassical target tissues are mediated, at least in part, by genomic mechanisms. One of the best characterized actions of 1,25$(OH)_2D_3$ in a number of different normal and malignant cells is the ability of 1,25$(OH)_2D_3$ to inhibit proliferation and to stimulate differentiation. The effect of 1,25$(OH)_2D_3$ on the inhibition of proliferation and the stimulation of differentiation is of interest because it has been related to the treatment of skin lesions found in psoriasis with 1,25$(OH)_2D_3$ or analogs of 1,25$(OH)_2D_3$. In addition to keratinocytes, 1,25$(OH)_2D_3$ has been reported to inhibit the proliferation and induce the differentiation of leukemia cells (Suda, 1989) and to inhibit the proliferation of a number of malignant cells, including colon, breast, and prostate cancer cells (Gonzalez-Sancho *et al*., 2006; Lowe *et al.,* 2003; Krishnan *et al.,* 2003; Wang *et al.,* 2000). 1,25$(OH)_2D_3$ is known to induce expression of p21 and p27, proteins that play an important role in regulation of growth (Inoue *et al.,* 1999; Liu *et al*., 1996). A very active area of current investigation is the development of analogs of 1,25$(OH)_2D_3$, which can inhibit cell growth and promote differentiation but do not affect serum calcium and the testing of their therapeutic potential in the treatment of leukemia and other malignancies.

Effects of 1,25$(OH)_2D_3$ on Hormone Secretion

Studies using VDR knockout mice have indicated that vitamin D is essential for full gonadal function in both sexes (Kinuta *et al.,* 2000). Although vitamin D plays a role in estrogen biosynthesis partially by maintaining calcium homeostasis, direct regulation by 1,25$(OH)_2D_3$ of the aromatase gene was suggested (Kinuta *et al.,* 2000). 1,25-Dihydroxyvitamin D_3 treatment can also enhance the secretion of insulin from the pancreas (Chertow *et al.,* 1983; Clark *et al.,* 1981; Norman *et al.,* 1980), the first nonclassical target tissue in which 1,25$(OH)_2D_3$ receptors were identified (Christakos and Norman, 1979). Although normalization of insulin secretion by vitamin D has been observed in vitamin-deficient rats, it has been argued that this effect of vitamin D may be secondary to a primary effect of vitamin D on serum calcium or to other beneficial effects of vitamin D on growth and nutrition. However, this is still a matter of debate. It is indeed possible that 1,25$(OH)_2D_3$ may act together with calcium to control insulin secretion. One of the earliest indications that the β cell may be a target for 1,25$(OH)_2D_3$ came from immunocytochemical studies that localized calbindin-D_{28k} to the islet (Morrissey *et al.,* 1975). Studies using pancreatic islets from calbindin-D_{28k} null-mutant mice and β cell lines stably transfected and overexpressing calbindin have provided evidence for a role for calbindin in the modulation of depolarization-stimulated insulin release and suggest that calbindin can control the rate of insulin release by regulating intracellular calcium and by modulating calcium influx via L type calcium channels (Sooy *et al.,* 1999; Lee *et al.,* 2006).

Effects of 1,25$(OH)_2D_3$ on the Immune System

1,25$(OH)_2D_3$ has been reported to inhibit IFN-γ, IL-2, granulocyte/macrophage colony-stimulating factor (GM-CSF) and TNF-α. For IL-2, IFN-γ, and GM-CSF the mechanism of 1,25$(OH)_2D_3$ mediated inhibition involves VDR-mediated inhibition of gene transcription (Alroy *et al.,* 1995; Cippitelli and Santoni, 1998; Towers *et al.,* 1999). 1,25$(OH)_2D_3$ has also been reported to upregulate IL-4 (Cantorna *et al.,* 1998). Studies have indicated that 1,25$(OH)_2D_3$ can also inhibit the differentiation and survival of dendritic cells, resulting in impaired alloreactive T-cell activation (Penna and Adorini, 2000; Griffin *et al.,* 2001). Due to its immunosuppressive actions, it has been suggested that 1,25$(OH)_2D_3$ or analogs of 1,25$(OH)_2D_3$ may prevent the induction of certain autoimmune disorders and may have beneficial effects when given in combination with immunosuppressive drugs such as cyclosporin A.

Very recent studies have indicated that 1,25$(OH)_2D_3$ is a direct inducer of antimicrobial peptide gene expression, resulting in killing of *Mycobacterium* tuberculosis. These findings indicate a novel action of 1,25$(OH)_2D_3$ in the regulation of innate immunity and suggest that decreased ability to produce 1,25$(OH)_2D_3$ may contribute to increased tuberculosis susceptibility (Liu *et al.,* 2006).

TRANSCRIPTIONAL REGULATION BY 1,25$(OH)_2D_3$

Vitamin D-Regulated Genes

1,25$(OH)_2D_3$, similar to other steroid hormones, is known to act by binding stereospecifically to a high-affinity, low-capacity nuclear receptor (VDR) resulting in the concentration of the 1,25$(OH)_2D_3$ receptor complex in the nucleus and the activation or repression of target genes. To date, more than 50 vitamin D-dependent genes have been identified in different target tissues in a number of species (see Segaert and Bouillon, 1998, for lists of vitamin D-dependent genes). However, only a limited number of vitamin D responsive elements (VDREs) have been defined. In general, the

VDRE consensus consists of two direct imperfect repeats of the hexanucleotide sequence GGGTGA separated by three nucleotide pairs (Table I).

Calbindin-D_{9K}, Calbindin-D_{28K}

As mentioned previously, one of the most pronounced effects of $1,25(OH)_2D_3$ is increased synthesis of calbindin. Although sequence elements in the mouse calbindin-D_{28k} promoter (–200/–169) and in the rat calbindin-D_{9k} promoter (–489/–445) that respond to $1,25(OH)_2D_3$ have been identified, the response observed using the calbindin-D_{9k} or the calbindin-D_{28k} responsive sequences is modest (Darwish and DeLuca, 1992; Gill and Christakos, 1993). This modest response reflects previous *in vivo* findings that indicated that $1,25(OH)_2D_3$ induces the expression of the calbindin-D_{9k} or the calbindin-D_{28k} gene by a small, rapid transcriptional stimulation followed by a large accumulation of calbindin mRNA long after $1,25(OH)_2D_3$ treatment (Christakos *et al.,* 1989). These findings suggest that the large induction of calbindin mRNA by $1,25(OH)_2D_3$ may be due primarily to post-transcriptional mechanisms. More recent studies noted the requirement of the homeodomain protein Cdx2, a transcription factor active only in intestinal epithelium, for calbindin-D_{9k} expression and that cooperation between the proximal calbindin-D_{9k} promoter and a distal element located in an open chromatin structure (–3600/–3400) is needed for vitamin D responsiveness (Colnot *et al.,* 1998). These studies suggest that the mechanism of action of $1,25(OH)_2D_3$ on calbindin regulation is more complicated than the conventional hormone-receptor transcriptional activation model. Calbindin-D_{9k} and calbindin-D_{28k} are regulated by a number of hormones in addition to $1,25(OH)_2D_3$. Calbindin-D_{9k} in rat uterus and calbindin-D_{28k} in mouse uterus, oviduct, and ovary have been reported to be regulated by estradiol (see Christakos, 1995, and Christakos *et al.,* 1989, for review). 1,25-Dihydroxyvitamin D_3 has no effect on calbindins in these female reproductive tissues. Regulation of calbindin-D_{28k} by retinoic acid has also been reported (Wang and Christakos, 1995). In addition, neurotrophin 3, brain-derived neurotrophic factor, fibroblast growth factor, and tumor necrosis factor have all been observed to increase the expression of calbindin-D_{28k} in the brain, suggesting regulation of calbindin by signal transduction, as well as by steroids (see Christakos, 1995, for review). Thus, it has become evident that calbindin is no longer considered a calcium-binding protein whose synthesis is dependent solely on vitamin D. Calbindin is present in a number of different tissues, may have multiple functions, and can be regulated by different ligands as well as by signal transduction.

TABLE I Vitamin D Responsive Elements Present in Vitamin-Regulated Genes

Rat 24-hydroxylase	AGGTGA gtg AGGGCG (–151/–137)
	CGCACC cgc TGAACC (–259/–245)
Human 24-hydroxylase	AGGTGA gcg AGGGCG (–171/–143)
	ACTTCA ccg GGTGTG (–293/–273)
Mouse osteopontin	GGTTCA cga GGTTCA (–757/–743)
Human osteocalcin	GGGTGA acg GGGGCA (–499/–485)
Rat osteocalcin	GGGTGA atg AGGACA (–455/–441)
Mouse calbindin-D_{28k}	GGGGAT gtg AGGAGA (–198/–182)
Mouse calbindin-D_{9k}	GGGTGT cgg AAGCCC (–488/–474)
Human TRPV 6	AGGTCT tgg GGTTCA (–2,170/–2,156)
	GGGGTA gtg AGGTCA (–4,287/–4,273)

Osteocalcin, Osteopontin

Studies concerning the regulation by vitamin D of two other calcium-binding proteins, osteocalcin (OC) and osteopontin (OP), which are secreted by the osteoblasts, have been important to our understanding of transcriptional activation by $1,25(OH)_2D_3$. VDREs in both the human and the rat OC promoter (Demay *et al.,* 1990; Kerner *et al.,* 1989) and in the mouse OP promoter (Noda *et al.,* 1990; Shen and Christakos, 2005) have been well characterized. Runx2 is important for basal and vitamin D responsive transcription of the OC gene (Javed *et al.,*1999; Lian *et al.,* 2001). Recent studies indicated cooperative effects among Runx2, VDR, and Hes-1 (a downstream factor in the notch signaling pathway) in the transcriptional regulation of OPN (Shen and Christakos, 2005).

24-Hydroxylase

Vitamin D responsive elements have also been identified in the rat 24-hydroxylase [24(OH)ase] gene (Kerry *et al.,* 1996; Ohyama *et al.,* 1996; Zierold *et al.,*1995). The 24(OH)ase gene is the first vitamin D-dependent gene reported to be controlled by two independent VDREs (at –259/–245 and at –151/–137). It has been suggested that the proximal VDRE is more responsive to $1,25(OH)_2D_3$ than the distal VDRE (Ohyama *et al.,* 1996). The most pronounced effects of $1,25(OH)_2D_3$ in intestine and kidney are increased synthesis of 24(OH)ase and calbindin (Matkovits and Christakos, 1995). However, unlike calbindin, which is only modestly transcriptionally responsive to $1,25(OH)_2D_3$, 24(OH)ase is strongly responsive to $1,25(OH)_2D_3$ at the level of transcription.

PARATHYROID HORMONE AND 1α HYDROXYLASE

The first demonstration of a negative VDRE was by Demay *et al.* (1992), who indicated that sequences in the human parathyroid hormone (PTH) gene (–125/–101) mediate transcriptional repression by 1,25(OH)$_2$D$_3$. Only a single copy motif (AGGTCA) is identified within this region, and vitamin D receptor binding to this element does not require the retinoid X receptor (RXR) (Mackey *et al.*, 1996). The response is tissue specific because the 25-bp oligonucleotide was reported to mediate transcriptional repression in GH4C1 cells but not in ROS 17/2.8 cells.

A negative VDRE has also been demonstrated in the human 1α(OH)ase promoter (–537/–514). No direct binding of VDR or VDR/RXR was detected. A helix-loop-helix factor designated as vitamin D interacting repressor (VDIR) was identified as a direct sequence-specific activator of the 1α(OH)ase negative VDRE (Murayama *et al.*, 2004).

Interleukin-2 and Granulocyte/Macrophage Colony-Stimulating Factor

Mechanisms involved in mediating the effects of 1,25(OH)$_2$D$_3$ in systems other than those involved in maintaining mineral homeostasis have only recently begun to be explored. A decrease in the proliferation of T lymphocytes in the presence of 1,25(OH)$_2$D$_3$ is correlated with a decrease in interleukin-2 (IL-2) mRNA and GM-CSF mRNA. Transcriptional repression of these genes contributes to the overall immunosuppressive effects of 1,25(OH)$_2$D$_3$ (Alroy *et al.*, 1995; Towers *et al.*, 1999). Mechanisms involved in the repression by 1,25(OH)$_2$D$_3$ of IL-2 and GM-CSF have been provided (Alroy *et al.*, 1995; Towers *et al.*, 1999). VDR can block the positive transcription factors NFATp and Jun/Fos, which bind to a composite site containing a consensus NFAT1-binding site. These findings provide novel insight concerning how 1,25(OH)$_2$D$_3$ can act as an immunosuppressive agent and may provide a general mechanistic basis for how steroid receptors elicit immunosuppressive responses.

Transient Receptor Potential Vanilloid Type 6

Transient receptor potential vanilloid type 6 (TPRV6), the calcium channel in intestinal epithelial cell membranes, is regulated at the transcriptional level by 1,25(OH)$_2$D$_3$ (Meyer *et al.*, 2006). The human TPRV6 promoter was found to contain multiple VDR/RXR binding sites (at –1.2, –2.1, –3.5, –4.3, and –5.5 kb). Only the elements at –2.1 and –4.3 kb were 1,25(OH)$_2$D$_3$ responsive (Table 1). The unexpected feature of this study is the unusual distant location of the VDREs relative to the transcription start site, revealing new insight into how 1,25(OH)$_2$D$_3$ acts and suggesting a chromatin looping mechanism whereby these regulatory regions can be brought into close proximity with the gene's natural promoter.

FACTORS INVOLVED IN VITAMIN D-MEDIATED TRANSCRIPTIONAL REGULATION

Vitamin D Receptor (VDR)

VDR Regulation

Due to the importance of VDR in the molecular mechanism of vitamin D action, the regulation of VDR has been a focus of a number of studies. Upregulation of VDR by 1,25(OH)$_2$D$_3$ (known as homologous upregulation) has been shown in several different systems, including pig kidney LLCPK-1 cells (Costa *et al.*, 1985), HL-60 leukemia cells (Lee *et al.*, 1989), osteoblastic cells (Arbour *et al.*, 1993), calvarial tissue (Zella *et al.*, 2006), parathyroid gland (Naveh-Many *et al.*, 1990), and kidney (Healy *et al.*, 2003, 2005a). In kidney the induction of VDR and VDR mRNA by 1,25(OH)$_2$D$_3$ is entirely dependent on levels of dietary calcium sufficient to maintain normal serum calcium (Healy *et al.*, 2003, 2005a). *In vivo* studies have shown that neither calcium nor 1,25(OH)$_2$D$_3$ have any significant effect on intestinal VDR or VDR mRNA expression, indicating tissue-specific regulation of VDR (Huang *et al.*, 1989; Healy *et al.*, 2005a). Although homologous upregulation of the VDR has been reported to be due to increased stability of the occupied receptor (Lee *et al.*, 1989; Wiese *et al.*, 1992; Arbour *et al.*, 1993), recent studies showed that 1,25(OH)$_2$D$_3$ can induce VDR, at least in bone, by an induction in transcriptional activity mediated by enhancers located within two introns of the mouse VDR gene (Zella *et al.*, 2006). VDR has also been reported to be regulated by a number of other factors, including PTH, activation of protein kinase A and protein kinase C. *In vivo* PTH decreases renal VDR expression, suggesting that PTH may be the trigger responsible for the hypocalcemia-mediated suppression of renal VDR (Healy *et al.*, 2005b). Treatment of NIHT3 mouse fibroblasts with forskolin (Krishnan and Feldman, 1992) and treatment of mouse osteoblasts (MC3T3-E1 cells) or rat osteosarcoma cells (UMR-106–01) with forskolin or PTH (Krishnan *et al.*, 1995) result in an induction in VDR abundance. Treatment of these cells with the phorbol ester, phorbol myristate acetate (PMA), whose actions are mediated by protein kinase C, results in a downregulation of VDR (Krishnan and Feldman, 1991; Krishnan *et al.*, 1995). Up- or downregulation of VDR in NIHT3 cells by forskolin or PMA, respectively, results in a corresponding induction or attenuation of reporter activity in cells transfected with the human OC VDRE fused to a reporter gene (Krishnan and Feldman, 1992). Treatment of UMR cells with PTH enhances the 1,25(OH)$_2$D$_3$-mediated induction of 24(OH)ase mRNA (Krishnan *et al.*, 1995; Huening *et al.*, 2002). Thus the functional response corresponds to the change in VDR. However, opposite findings concerning the effect of activation of protein kinase A or protein kinase C on VDR have been reported by others

(Reinhardt and Horst, 1990, 1994; Barletta *et al.,* 2004), suggesting that proliferation state, cell type, and stage of differentiation affect the interaction between $1,25(OH)_2D_3$ and signal transduction pathways. In general, these studies suggest cooperativity between signal transduction pathways and $1,25(OH)_2D_3$. Effects of second messenger systems may be on the VDR. However, effects on the promoter of the target gene or on other transcription factors that may be interacting with the VDR also need to be considered (increased interaction between VDR and coactivators such as DRIP205 has been suggested as an additional mechanism that can couple extracellular signals to vitamin D action [Barletta *et al.*, 2002; Liu *et al.*, 2005]. Studies related to the regulation of VDR will be facilitated by further analysis of the hVDR gene that spans more than 60 kb, consists of at least 14 exons, and is directed by at least 2 distinct promoters (Miyamoto *et al.,* 1997).

Phosphorylation of the VDR

Although the VDR, similar to other steroid receptors, is phosphorylated, the exact functional role of phosphorylation of the VDR remains to be further elucidated. It has been suggested that phosphorylation may play a role in the binding of the receptor to DNA or in the interaction of VDR with other transcription factors. Phosphorylation of VDR was shown to involve serine residues. Serine-208 in the ligand-binding domain has been identified as a site of phosphorylation that accounts for at least 60% of the phosphorylation of the receptor (Hilliard *et al.,* 1994; Jurutka *et al.,* 1993). Casein kinase II has been reported to mediate VDR phosphorylation at serine-208 (Jurutka *et al.,* 1996). Although transcriptional activation by $1,25(OH)_2D_3$ is not dependent on serine-208 phosphorylation, studies have shown that VDR phosphorylation by casein kinase II at serine-208 can potentiate transcriptional activation (Jurutka *et al.,* 1996).

VDR Receptor Homodimerization vs. Heterodimerization

VDR functions as a heterodimer with RXR for activation of gene transcription. However, a few studies indicated that purified VDR can bind as a homodimer to certain VDREs but that RXR was required for binding to other VDREs (Freedman *et al.,* 1994; Nishikawa *et al.,* 1994). The physiological relevance of VDR homodimers has been questioned. Cheskis and Freedman (1994) reported that VDR exists as a monomer in solution and homodimerization occurs upon binding to the OPN VDRE. 1,25-Dihydroxyvitamin D_3 was reported to destabilize homodimerization, resulting in VDR/RXR heterodimer formation. Thompson *et al.* (1998) also reported that formation of the VDRE-complexed VDR-RXR heterodimer is strikingly dependent on the presence of $1,25(OH)_2D_3$. These studies suggest that although the VDR homodimer can exist in solution, the heterodimer is the functional transactivating species. 9-*cis*-Retinoic acid has been reported to decrease heterodimer formation by driving the equilibrium from the VDR heterodimer to the RXR homodimer or to the interaction of RXR with other receptors (Cheskis and Freedman, 1994; MacDonald *et al.,* 1993). Further studies using VDR mutants also suggest the importance of heterodimerization, as none of the mutants without the capability to form heterodimers showed $1,25(OH)_2D_3$-dependent transcriptional activation (Nakajima *et al.,* 1994).

Regions of VDR within the ligand-binding domain (LBD) that may be crucial for heterodimerization have been suggested. Mutagenesis studies have indicated that the regions in the C-terminal between amino acids 317 and 395 and between amino acids 244 and 263 corresponding to portions of helices 7–10 and 3–4, respectively, are important for ligand dependent heterodimerization (Nakajima *et al.,* 1994; Jin *et al.,* 1996; Rosen *et al.,* 1993; Whitfield *et al.,* 1995). The LBD of VDR also has been shown to be involved in protein-protein interaction of VDR with other cofactors. A more complete understanding of the three-dimensional contacts between VDR and RXR and between VDR and other accessory factors will be obtained now that the crystal structure of the VDR ligand domain bound to its ligand has been published (Rochel *et al.,* 2000).

Interaction of VDR with Transcription Machinery

The mechanisms involved in VDR-mediated transcription following binding of the VDR-RXR heterodimer to DNA are only now beginning to be defined. Initiation of basal transcription involves binding of TFIID, which is composed of the TATA box-binding protein and associated TAFs, to the TATA element. After the binding of TFIID to the TATA element, other factors, including TFIIA, TFIIB, RNA polymerase II, TFIIE, TFIIF, and TFIIH, are recruited and associated with the complex. Studies have suggested that VDR can interact physically and functionally with TFIIB (Blanco *et al.,* 1995; Masuyama *et al.,* 1997). The interaction of TFIIB is with unliganded VDR, and the $1,25(OH)_2D_3$ ligand disrupts the VDR-TFIIB complex (Masuyama *et al.,* 1997). These findings suggest that VDR prerecruits TFIIB and, in the presence of ligand, TFIIB is released for assembly into the preinitiation complex to facilitate activated transcription.

Several TAFs have been suggested to be involved in VDR-mediated transcriptional activity. The TFIID subunit $TAF_{II}135$ potentiates the transcriptional activity of VDR (Mengus *et al.,* 1997) and $TAF_{II}28$ (Mengus *et al.,* 2000) and $TAF_{II}55$ (Lavigne *et al.,* 1999) interact with VDR at α helices H3 to H5 and at α helix 8. Determinants for interaction with $TAF_{II}28$ or $TAF_{II}135$ are not identical. A mutation in the H3-H5 region that determines interaction with $TAF_{II}28$ was reported to abolish VDR-mediated transactivation (Mengus *et al.,* 2000).

SRC/p160 Coactivators

Over the past few years a number of proteins known as p160 coactivators (based on one of the first identified members, the 160-kDa protein, steroid receptor coactivator-1; SRC-1) that bind to steroid receptors and enhance their activity have been identified. Three related family members, based on homologies, include SRC-1(NcoA1 or p160), GRIP-1(TIF-2, NcoA-2, or SRC-2) and ACTR (pCIP, RAC3, or SRC-3) (for reviews, see McKenna *et al.,* 1999; Smith and O'Malley, 2004; Fig. 2). They interact with the AF2 domain of steroid receptors, including VDR (C-terminal helix 12 contains the core AF2) in a ligand-dependent manner. Studies indicate that helix 3 of the VDR is also important for interaction with p160 coactivators (Jimenez-Lara and Aranda 1999; Kraichely *et al.,* 1999). These coactivators share a series of LxxLL (or NR box) motifs and possess histone acetylase (HAT) activity. This modification of histones is thought to destabilize the interaction between DNA and the histone core, liberating DNA for transcription. These coactivators can also form complexes with CBP (CREB-binding protein). CBP also has HAT activity. Thus the SRC/p160 family of coactivators can recruit CBP to the nuclear receptor, resulting in a multisubunit complex.

Vitamin D Receptor Interacting Proteins (DRIP) Complex

VDR-mediated transcription is also mediated by a coactivator complex, DRIP (Rachez *et al.,* 1998, 1999; Fig. 2). These proteins are also called TRAP and ARC, depending on the transcription factor initially identified as the target (TR or androgen receptor, respectively), but are now thought to have broader target specificity due to their close identity. This complex does not have HAT activity but rather functions, at least in part, through recruitment of RNA polymerase II (Rachez *et al.,* 1999). Results of chromatin immunoprecipitation assays have indicated that the CBP/SRC coactivator complex may be needed first for chromatin remodeling followed by the recruitment of the transcription machinery by the DRIP/TRAP/ARC complex (sequential model).

OTHER FACTORS MODULATING VDR-MEDIATED TRANSCRIPTION

Various additional factors have been reported to modulate VDR-mediated transcription. A binding site for the

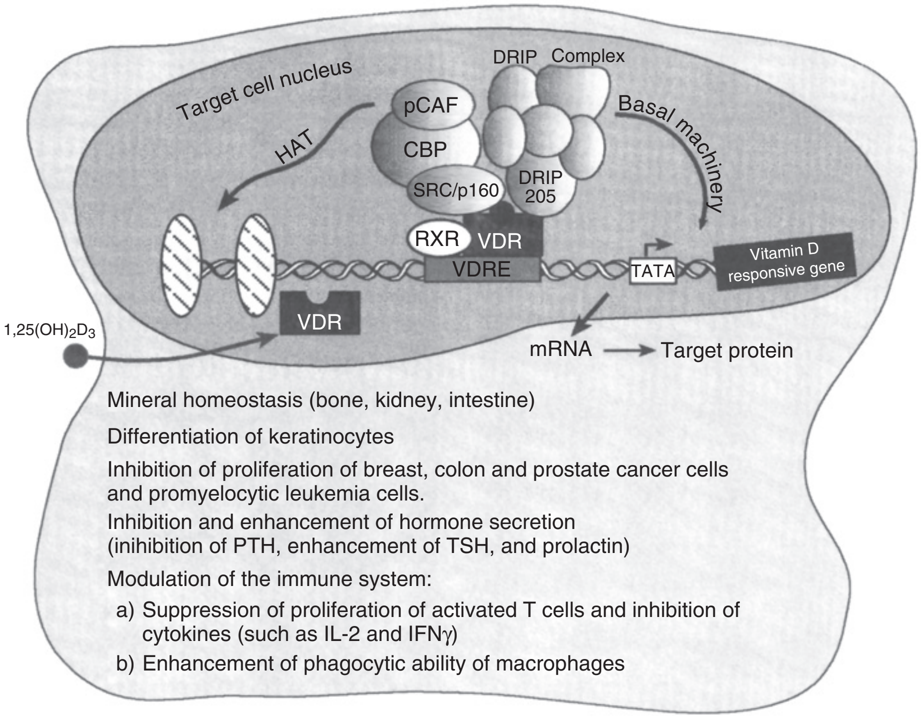
Target cell nucleus
DRIP Complex
pCAF
CBP
SRC/p160
DRIP 205
HAT
Basal machinery
RXR
VDR
VDRE
TATA
Vitamin D responsive gene
VDR
$1,25(OH)_2D_3$
mRNA
Target protein
Mineral homeostasis (bone, kidney, intestine)
Differentiation of keratinocytes
Inhibition of proliferation of breast, colon and prostate cancer cells and promyelocytic leukemia cells.
Inhibition and enhancement of hormone secretion (inihibition of PTH, enhancement of TSH, and prolactin)
Modulation of the immune system:
a) Suppression of proliferation of activated T cells and inhibition of cytokines (such as IL-2 and IFNγ)
b) Enhancement of phagocytic ability of macrophages

FIGURE 2 Genomic mechanism of action of $1,25(OH)_2D_3$ in target cells. 1,25-Dihydroxyvitamin enters the target cell and interacts with the nuclear VDR, which heterodimerizes with RXR. After interaction with the VDRE, transcriptional activation or repression proceeds through the interaction of VDR with coactivators and with the transcription machinery. Two models for the genomic action of $1,25(OH)_2D_3$ are shown. The histone acetyltransferase (HAT) activity-containing complex of SRC/p160 and CBP may be recruited first by VDR in response to the ligand. This would lead to DNA accessibility that would allow as a second step the binding of the DRIP complex (DRIP 205 subunit binds directly to VDR) and recruitment of RNA polymerase II (sequential model). Alternatively, there could be simultaneous chromatin remodeling and basal machinery recruitment (cooperative model). 1,25-Dihydroxyvitamin D_3 is known to affect mineral homeostasis, to differentiate keratinocytes, to inhibit the proliferation of cancer cells, to affect hormone secretion, and to modulate the immune system. Adapted with permission from Christakos *et al.* (1996).

Ras-activated Ets transcription factor was identified downstream from the proximal VDRE in the rat 24(OH)ase promoter, and this site was found to be critical for 1,25$(OH)_2D_3$ mediated 24(OH)ase transcription (Dwivedi *et al.,* 2000). The multifunctional transcriptional regulator YY1 represses 1,25$(OH)_2D_3$-induced transcription of 24(OH)ase and OC (Raval-Pandya *et al.*, 2001; Guo *et al.*, 1997). CCAAT enhancer binding protein (C/EBP) β and δ are induced by 1,25$(OH)_2D_3$ in osteoblasts and can enhance 24(OH)ase and OC transcription (Dhawan *et al.,* 2005; Gutierrez *et al.,* 2002). In the regulation of OC transcription there is synergism between C/EBP β and Runx2 mediated by the interaction between Runx2 and C/EBP β (Gutierrez *et al.,* 2002). C/EBP β has also been reported to be involved in the immune-mediated regulation of 1α-hydroxylase in monocytes and macrophages (Esteban *et al.,* 2004; Stoffels *et al.,* 2007).

SWI/SNF, complexes that remodel chromatin using the energy of ATP hydrolysis, are also involved in VDR-mediated transcription. Functional cooperation between C/EBP β and SWI/SNF has been reported in the regulation of 1,25$(OH)_2D_3$-induced 24(OH)ase and OC transcription (Christakos *et al.,* 2006; Villagra *et al.,* 2006).

Preliminary results have indicated that cooperativity between histone methyltransferases and p160 coactivators may also play a fundamental role in VDR-mediated transcriptional activation (Christakos *et al.*, 2007).

FUTURE DIRECTIONS

New target genes, novel vitamin D responsive elements, and new factors involved in vitamin D-mediated transcription will undoubtedly be identified in numerous different systems, which are currently known to be affected by 1,25$(OH)_2D_3$. Sequences divergent from the current consensus VDRE or at distant locations from the transcriptional start site may be observed, which should expand our understanding of 1,25$(OH)_2D_3$-mediated genomic responses. In addition to transcriptional regulation, it is likely that post-transcriptional mechanisms will be an important mechanism of control of a number of newly identified target genes. In addition to studies concerning the mechanisms involved in mediating the genomic actions of 1,25$(OH)_2D_3$, further studies related to the physiological significance of target proteins using null mutant mice are needed in the future. Finally, in the next few years, with the elucidation of the crystal structure of VDR, we will obtain an increased understanding of the structure of VDR in the presence and absence of ligand and/or protein partners. Based on the structural information, synthetic analogs of 1,25$(OH)_2D_3$ may be designed that would selectively modulate specific 1,25$(OH)_2D_3$ responses. Thus, new insight into the multiple roles of 1,25$(OH)_2D_3$ will be obtained and selective modulation of 1,25$(OH)_2D_3$ responses in bone and other target tissues may indeed be possible.

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Chapter 37

Photobiology and Noncalcemic Actions of Vitamin D

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PHOTOBIOLOGY OF VITAMIN D

Photosynthesis of Provitamin D_3 and Its Conversion to Vitamin D_3

When human skin is exposed to sunlight, a photo-chemical process occurs that is essential for the maintenance of calcium homeostasis and a healthy skeleton. During exposure to sunlight, the ultraviolet B (UV-B; 290–315 nm) portion of the solar spectrum is responsible for photolyzing 7-dehydrocholesterol (the precursor cholesterol; provitamin D_3) to previtamin D_3(Holick, 1994). Once formed, previtamin D_3 undergoes an internal isomerization of its three double bonds to form a more thermodynamically stable, 5,6-cis-triene and is transformed in vitamin D_3 (Fig. 1). For warm-blooded animals, such as humans, this process would, under normal circumstances, take approximately 24 hr for 50% of previtamin D_3 to convert to vitamin D_3. However, in cold-blooded animals, this process could take several days (Holick *et al.,* 1980). It has now been found that there is membrane enhancement for the conversion of previtamin D_3 to vitamin D_3 in both cold-blooded and warm-blooded animals, including humans (Holick *et al.,* 1995). 7-Dehydrocholesterol is found principally in the cell membrane. Within the membrane, the hydrophobic side chain of 7-dehydrocholesterol is aligned with the hydrophobic chains of the fatty acids and cholesterol, thereby restraining the confirmation of previtamin D_3 when it is formed (Tian *et al.*, 1993) (Fig. 2). Thus, when 7-dehydrocholesterol is exposed to sunlight, 7-dehydrocholesterol is photolyzed to the s-*cis,* s-*cis* conformer of previtamin D_3. In an organic solvent, the s-cis, s-*cis*-previtamin D_3 conformer is thermodynamically unstable and immediately isomerizes to the s-*cis,* s-*trans* form. Because only the s-*cis,* s-*cis* conformer is able to isomerize to vitamin D_3, the entrapment of previtamin D_3 in its s-*cis,* s-*cis* form within the plasma membrane promotes a more than 10-fold increase in its rate of isomerization to vitamin D_3 when compared to the same reaction in an organic solvent (Tian *et al.,* 1994; Holick *et al.,* 1995). This process guarantees that the precious previtamin D_3 that is made in the skin is converted efficiently to vitamin D_3. In addition, as vitamin D_3 is being formed from previtamin D_3, its conformational change probably permits it to selectively exit from the membrane into the extracellular space.

Factors That Regulate Photosynthesis of Previtamin D_3 in Skin

Sunlight-Mediated Photolysis

It is well known that intense prolonged exposure to sunlight will not cause vitamin D intoxication. The reason for this is that during the initial exposure to sunlight, 7-dehydrocholesterol is converted to previtamin D_3. However, because previtamin D_3 is photolabile, when exposed to sunlight it is converted to lumisterol and tachysterol, which are thought to be biologically inert on calcium metabolism (Holick *et al.,* 1981) (Fig. 1). Once previtamin D_3 is isomerized to vitamin D_3, vitamin D_3 is also extremely photosensitive and is isomerized rapidly by sunlight to supersterol 1, suprasterol 2, and 5,6-transvitamin D_3 (Fig. 1), which are also thought to be either biologically inert or have less activity on calcium metabolism than vitamin D_3 (Webb *et al.,* 1989).

Melanin, Sunscreens, Clothing, Glass, and Plastics

Melanin is a natural sunscreen that effectively absorbs ultraviolet B radiation, thereby competing with 7-dehydrocholesterol for these photons. As a result, increased skin pigmentation requires longer exposure to sunlight to produce the same amount of previtamin D_3 as in a lighter-skinned individual (Clemens *et al.,* 1982).

Sunscreen use is highly recommended, especially for individuals who are prone to sunburning. Sunscreens like

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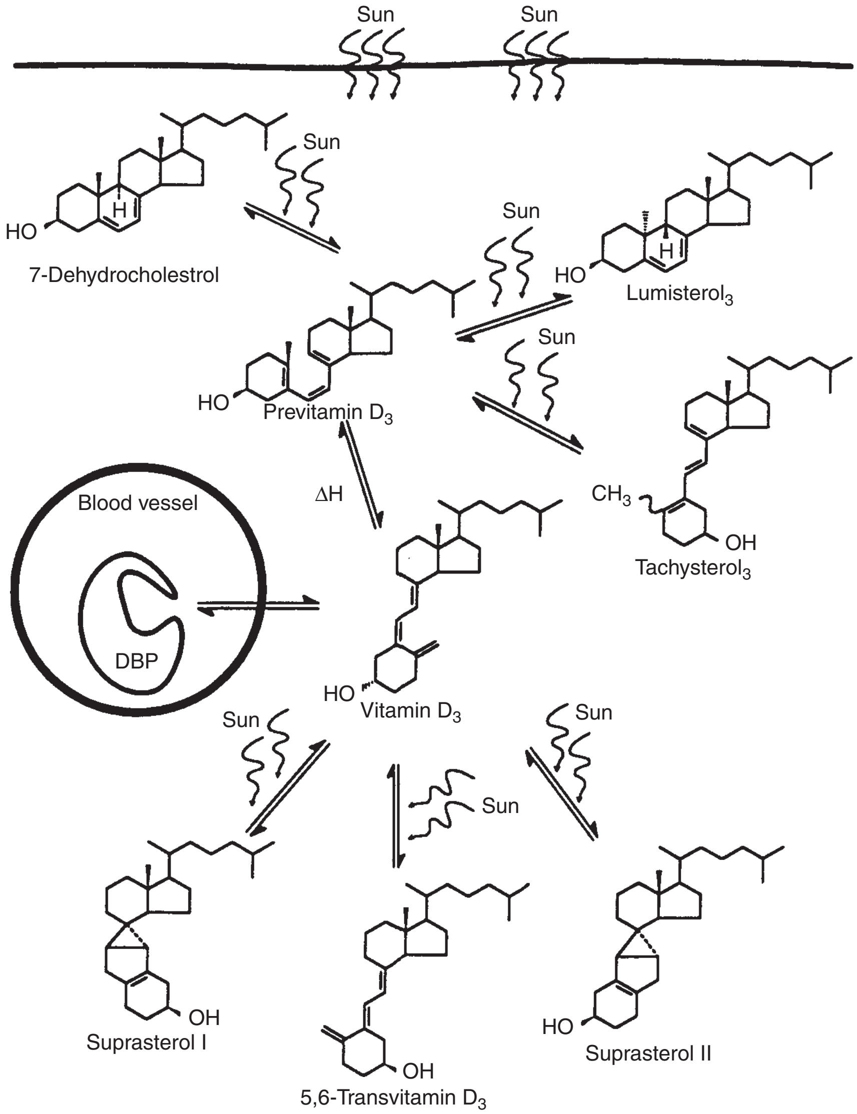

FIGURE 1 Photochemical events in the skin that lead to the production of vitamin D_3 and the regulation of vitamin D_3 in the skin. Reproduced with permission from Holick (1994).

melanin absorb ultraviolet B radiation. Therefore, the topical application of a sunscreen will substantially diminish or completely prevent the cutaneous production of previtamin D_3. When young adults were covered with a sunscreen preparation with a sun protection factor of 8 (SPF 8) followed by a whole body exposure to one minimal erythemal dose of simulated sunlight, they were unable to elevate their circulating concentrations of vitamin D above baseline values (Matsuoka *et al.,* 1987) (Fig. 3). Similarly, clothing absorbs most ultraviolet radiation and therefore prevents the cutaneous production of vitamin D_3 (Matsuoka *et al.,* 1994). Chronic use of a sunscreen will diminish circulating concentrations of 25-hydroxyvitamin D_3 as a measure of vitamin D status (Matsuoka *et al.,* 1998). In addition to sunscreens, exposure of the skin to sunlight that has passed through windowpane glass or Plexiglas will not permit any significant synthesis of vitamin D_3 in the skin because most glass and plastics absorb ultraviolet B radiation efficiently (Holick, 1994).

Aging

Aging influences a variety of metabolic processes. Therefore, it is not surprising that aging also markedly decreases the free concentrations of 7-dehydrocholesterol in the epidermis (MacLaughlin and Holick, 1985). When healthy young and elderly volunteers were exposed to the same amount of simulated sunlight, the circulating concentrations of vitamin D in the young volunteers (aged 22–30 years) increased to a maximum of 30 ng/ml within 24 hr after exposure, whereas

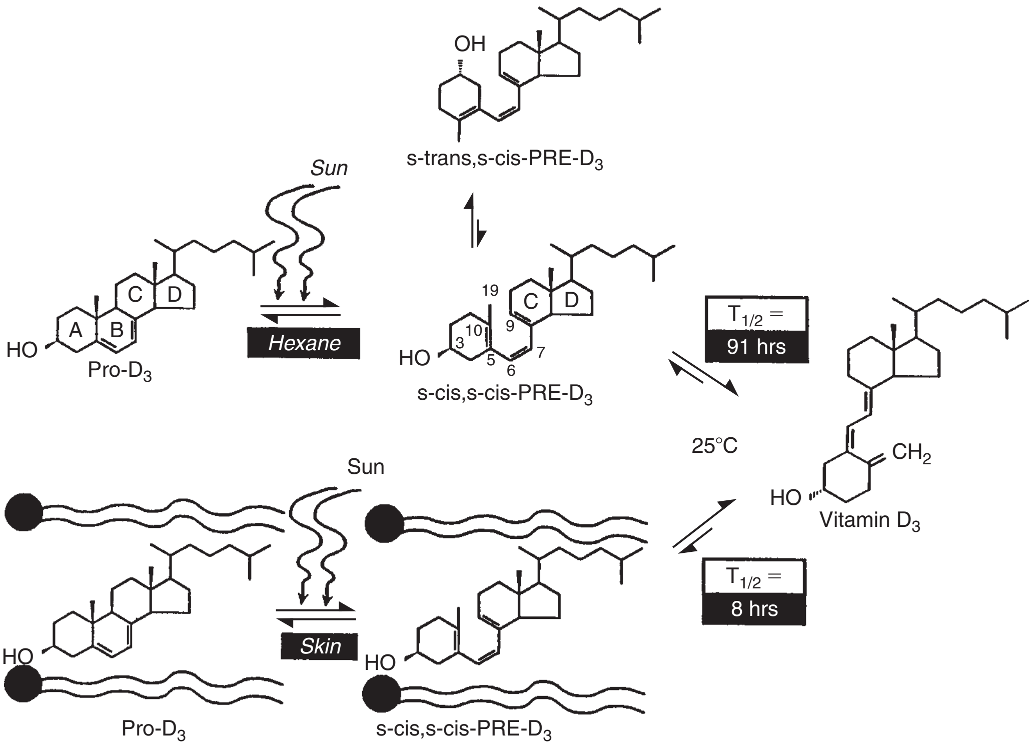

FIGURE 2 Photolysis of provitamin D_3 (pro-D_3) into previtamin D_3 (pre-D_3) and its thermal isomerization to vitamin D_3 in hexane and in skin. In hexane, pro-D_3 is photolyzed to s-*cis*,s-*cis*-pre-D_3. Once formed, this energetically unstable conformation undergoes a conformational change to the s-*trans*,s-*cis*-pre-D_3. Only the s-*cis*,s-*cis*-pre-D_3 can undergo thermal isomerization to vitamin D_3. The s-*cis*,s-*cis* conformer of pre-D_3 is stabilized in the phospholipid bilayer by hydrophilic interactions between the 3β–hydroxyl group and the polar head of the lipids, as well as by van der Waals' interactions between the steroid ring and side chain structure and the hydrophobic tail of the membrane lipids. This "entrapment" significantly decreases its conversion to the s-*trans*,s-*cis* conformer, thereby facilitating the thermal isomerization of s-*cis*,s-*cis*-pre-D_3 to vitamin D_3. Reproduced with permission from Holick *et al.* (1995).

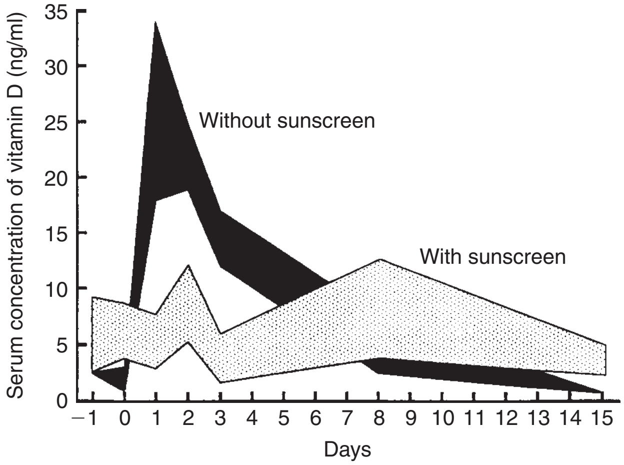

FIGURE 3 Circulating concentrations of vitamin D in healthy volunteers who applied an oil that contained a sunscreen with SPF 8 or no sunscreen over their entire bodies after a single exposure to one minimal erythemal dose of simulated sunlight. Adapted from Matsuoka *et al.*, 1987. Reproduced with permission from Holick (1994).

the older subjects (aged 62–80 years) were only able to achieve a maximum concentration of 8 ng/ml (Holick *et al.*, 1989) (Fig. 4).

Season, Latitude, and Time of Day

Season, latitude, and time of day can greatly influence the cutaneous production of vitamin D_3. As the zenith angle of the sun becomes more oblique, the ultraviolet B photons have to pass through the stratospheric ozone layer at a more oblique angle. This results in the ozone layer absorbing an increasing number of ultraviolet B photons. This can have a dramatic effect on the cutaneous production of previtamin D_3 (Webb *et al.*, 1988) (Fig. 5). In Boston, exposure to sunlight between the months of March and October is capable of producing previtamin D_3 in the skin. However, between the months of November and February, little if any cutaneous vitamin D_3 production can occur no matter how long one stays outdoors. The time of day also

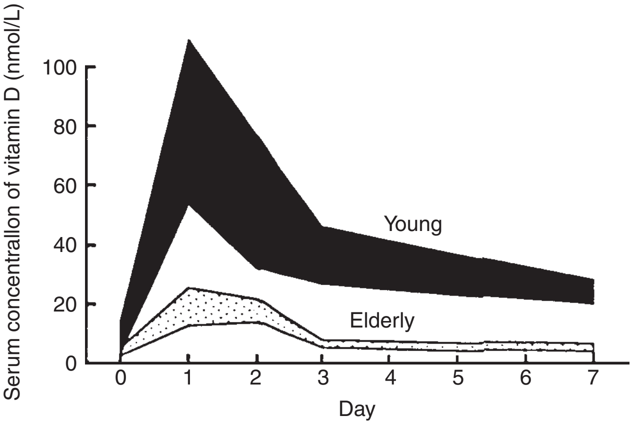

FIGURE 4 Circulating concentrations of vitamin D in response to a whole body exposure to one minimal erythemal dose in healthy young and elderly subjects. Adapted from Holick *et al*. (1989). Reproduced with permission from Holick (1994).

greatly influences the cutaneous production of vitamin D_3 (Fig. 6). During the summer in Boston, exposure of the skin to sunlight from 07:00 to as late as 17:00 hr eastern standard time (EST) resulted in previtamin D_3 production in human skin. However, in the spring and autumn, previtamin D_3 synthesis began at approximately 09:00 EST and ceased at approximately 15:00 EST.

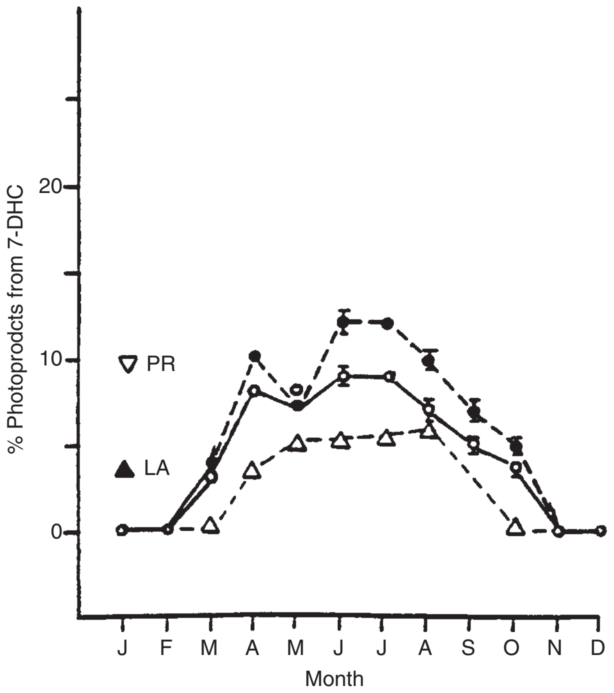

FIGURE 5 Photosynthesis of previtamin D_3 lumisterol, and tachysterol (photoproducts) after exposure of 7-dehydrocholesterol to sunlight in Boston (42° N) for 1 (○) and 3 (●) hours, Edmonton, Canada (52°N) after 1 hour (△) each month for 1 year, Los Angeles (34°N) (▲) and Puerto Rico (18°) in January (▽). Adapted from Webb *et al.* (1988).

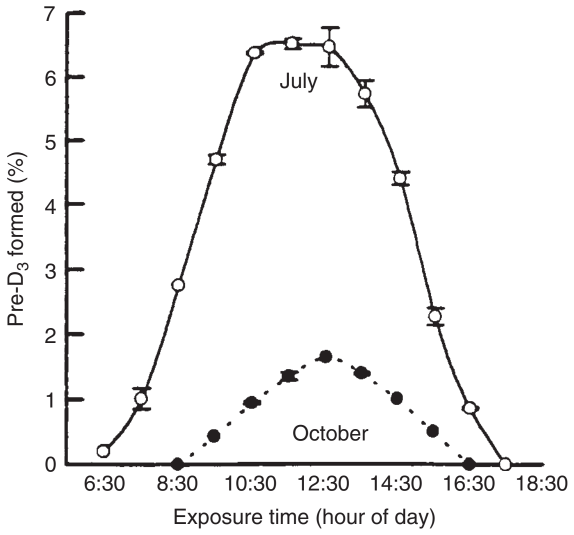

FIGURE 6 Photosynthesis of previtamin D_3 at various times on cloudless days in Boston in October and July. Adapted from Lu *et al.* (1992). Reproduced with permission from Holick (1994).

PERSPECTIVE ON UTILIZATION OF SUNLIGHT FOR VITAMIN D

It is not well appreciated that casual exposure to sunlight provides most of us with our vitamin D requirement. With the exception of cod liver oil, fatty fish, and other fish liver oils, there are very few foods that have naturally occurring vitamin D. Although some foods are fortified with vitamin D, most notably milk, a recent survey of the vitamin D content in milk suggests that more than 50% of milk samples in the United States contained less than 80% of the vitamin D content stated on the label and approximately 15% contained no detectable vitamin D (Tanner *et al.,* 1988; Holick *et al.,* 1992; Chen *et al.,* 1993). Some orange juices are fortified with 100 IU of vitamin D_3 along with 300 mg calcium/8 oz.

The alarming increase in the incidence of skin cancer that has been directly related to an increased exposure to sunlight has prompted widespread use of sunscreening agents for preventing the damaging effects of sunlight on the skin. Because children and young adults will not routinely cover all sun-exposed areas with a sunscreen all of the time, there is no need for concern about the topical use of sunscreens in causing vitamin D deficiency in this population. However, elderly people, who have decreased capacity to produce vitamin D_3 in their skin, are concerned about developing wrinkles and skin cancer and will religiously topically apply a sunscreen on all sun-exposed areas before going outdoors. This can result in vitamin D insufficiency or overt vitamin D deficiency (Matsuoka *et al.,* 1988). People over 65 years of age who are not taking a vitamin D supplement can satisfy their bodies' vitamin D requirement by exposing their hands, arms, and face to suberythemal doses of sunlight (usually 5 to 15 min, depending on location and time of day) two to three times a week. For those who wish to stay outdoors for longer than the initial suberythemal exposure, it is recommended that they apply a sunscreen with a sun protection factor of equal to or greater than 15 to sun-exposed areas. Therefore, the intelligent use of sunlight to promote the cutaneous synthesis of vitamin D_3 and the topical use of sunscreening agents after the initial exposure prevent the damaging effects due to excessive chronic exposure to sunlight while providing it beneficial effect: vitamin D_3 (Holick, 1994).

VITAMIN D METABOLISM

Once vitamin D is made in the skin or ingested in the diet, it is bound to the vitamin D-binding protein (DBP). It travels to the liver where it is metabolized to 25-hydroxyvitamin D [25(OH)D]. Once formed, it leaves the liver bound to the DBP and is filtered in the gomerulus into the ultrafiltrate. 25(OH)D-DBP is reabsorbed from the ultrafiltrate by the tubules by the endocytic receptor megalin (Nykjaer *et al.,* 1999). This endocytotic process is required to preserve 25(OH)D and to deliver it to the renal tubular cells for cytochrome P_{450} 25(OH)D-1 α-hydroxylase (1α-OHase). This mitochondrial enzyme hydroxylates 25(OH)D on carbon 1 to form the biologically active form of vitamin D: 1,25-dihydroxyvitamin D [1,25(OH)$_2$D](Holick, 1999; Holick 2007) (Fig. 7).

Originally, it was believed that the kidney was the sole source of 1α-OHase. This was based on the observation that anephric rats could not metabolize 25(OH)D_3 to 1,25(OH)$_2D_3$ (DeLuca, 1988; Holick, 1989b). This was also confirmed by many observations that low and undetectable concentrations of 1,25(OH)$_2D_3$ are present in patients who have no kidneys or no kidney function (Holick, 1989b). There is, however, compelling evidence that a wide variety of tissues also possess 1α-OHase activity. The first tissue demonstrated to have 1α-OHase was the skin (Bikle *et al.,* 1986). It has now been demonstrated that normal prostate and prostate cancer cells express 1α-OHase activity (Schwartz *et al.,* 1998). Using *in situ* hybridization and antibodies to 1α-OHase, it was found that 1α-OHase was present in the basal keratinocytes, hair follicles, lymph nodes, parasympathetic ganglion, pancreas, islet cells, adrenal medulla, brain (cerebellum and cerebral cortex), and placenta (Zehnder *et al.,* 2001).

Although the exact function of the extrarenal 1α-OHase is not well understood, it appears that this enzyme may be important for producing 1,25(OH)$_2D_3$ locally to act as a cellular growth modulator (Holick, 2007) (Fig. 8). To determine the effect of this enzyme on cellular growth and differentiation, we made a plasmid construct containing the 1α-OHase gene that was tagged with the green fluorescent protein gene (Flanagan *et al.,* 1999). A prostate cell line LnCaP that has a vitamin D receptor (VDR) but no 1α-OHase activity was transfected with the 1α-OHase plasmid. It was observed that cells expressed in their mitochondria a protein that had green fluorescence. These cells also had the capability of converting 25(OH)D_3 to 1,25(OH)$_2D_3$, whereas cells transfected with on empty vector were unable to produce any 1,25(OH)$_2D_3$. Cells transfected with the 1α-OHase construct were exposed to 25(OH)D_3, as were cells transfected with the empty vector construct. Cells transfected with 1α-OHase gene had decreased proliferative activity in the presence of 10^{-8} and 10^{-7} *M* 25(OH)D_3 whereas there was no effect in cells transfected with the empty vector (Flanagan *et al.,* 1999; Whitlatch *et al.,* 2002). These results suggest that 1,25(OH)$_2D_3$ may be produced locally in a wide variety of cells and that the function of 1,25(OH)$_2D_3$ is to regulate cell growth (Holick, 2001). Indeed, there have been numerous reports of the expression and activity of 1-OHase in normal and maligant human colon tissue (Tangpricha 2002), (Cross *et al.,* 2001), lung (Mawrer *et al.,* 1994), pancrease among other tissues (Chen and Holick, 2003; Holick 2007). This could be the explanation for why people who live at higher latitudes, and therefore make less vitamin D_3, are more likely to die of colon, breast, prostate, and ovarian cancer (Garland *et al.,* 1989, 1991; Ahonen *et al.,* 2000; Schwartz *et al.,* 1998; Gorham *et al.,* 2005; Grant and Garland 2005). It may be that higher circulating concentrations of 25(OH)D are required in order for the extrarenal 1α-OHase to maximally function to produce 1,25(OH)$_2D_3$ locally to regulate cell growth and prevent metastatic activity of cells that become cancerous (Holick, 1999; Holick 2001; Holick 2006; Holick 2007).

NONCALCEMIC ACTIONS OF 1,25-DIHYDROXYVITAMIN D_3

Nuclear Localization of ^{3}H-1,25(OH)$_2D_3$ in Noncalcemic Tissues

In 1979, Stumpf and colleagues reported that autoradiographic analysis of frozen sections of tissues from vitamin D-deficient rats that received an intravenous injection of [^{3}H-]1,25(OH)$_2D_3$ showed nuclear localization of [^{3}H-] 1,25(OH)$_2D_3$ in a multitude of tissues that were not associated with calcium metabolism, including pituitary gland, thymus, gonads, stomach, breast, pancreas, and skin. Since this initial observation, a variety of investigators have reported that these tissues, as well as transformed cells and cancer cells, possess a vitamin D receptor (VDR) (Table I) (Eisman *et al.,* 1981; Colston *et al.,* 1981; Abe *et al.,* 1981; Tanaka *et al.,* 1982; Simpson *et al.,* 1985; Holick, 1995).

Noncalcemic Functions of 1,25-Dihydroxyvitamin D_3

Cancer Cells

Initially, when normal tissues and cells such as the skin and immune cells were found to have receptors for 1,25(OH)$_2$D, it was thought that this was either an artifact or was of little

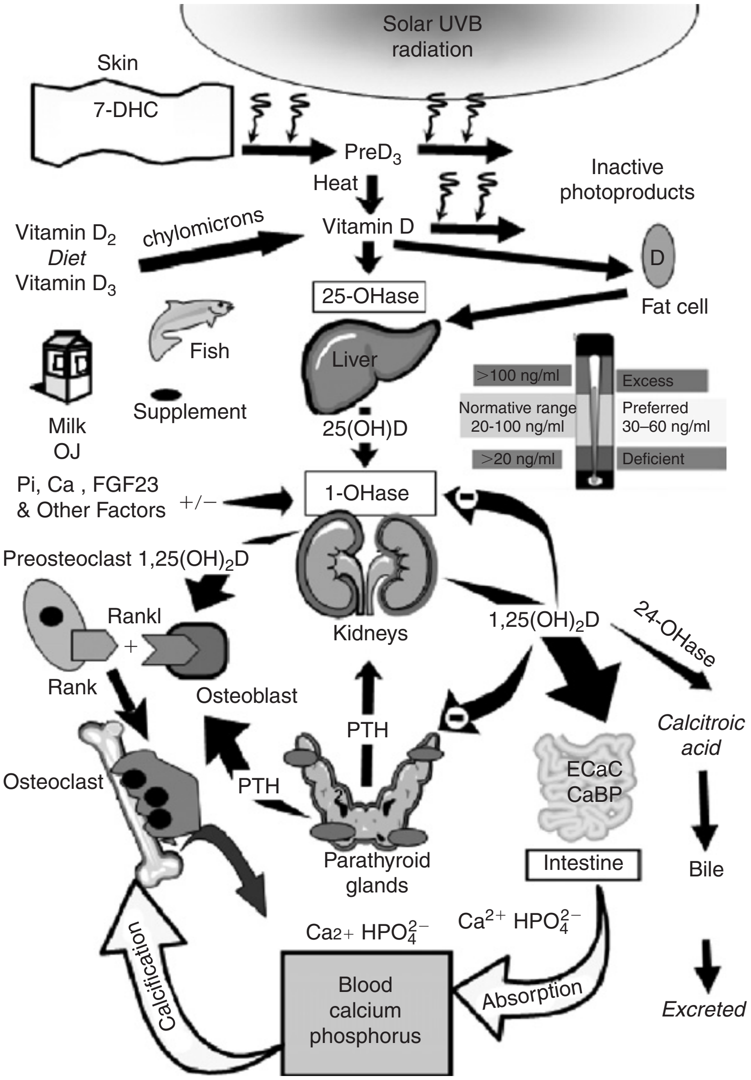
Solar UVB radiation
Skin
7-DHC
PreD$_3$
Heat
Inactive photoproducts
Vitamin D$_2$
Diet
Vitamin D$_3$
chylomicrons
Vitamin D
D
Fat cell
25-OHase
Fish
Milk
OJ
Supplement
Liver
25(OH)D
>100 ng/ml
Excess
Normative range 20-100 ng/ml
Preferred 30–60 ng/ml
>20 ng/ml
Deficient
Pi, Ca , FGF23 & Other Factors +/−
1-OHase
Preosteoclast 1,25(OH)$_2$D
Rankl
+
Rank
Osteoblast
Kidneys
1,25(OH)$_2$D
24-OHase
Calcitroic acid
PTH
PTH
Osteoclast
ECaC
CaBP
Intestine
Bile
Parathyroid glands
Ca2+ HPO$_4^{2-}$
Ca^{2+} HPO$_4^{2-}$
Calcification
Blood calcium phosphorus
Absorption
Excreted

FIGURE 7 Schematic representation of the synthesis and metabolism of vitamin D for regulating calcium, phosphorus, and bone metabolism. During exposure to sunlight 7-dehydrocholesterol (7-DHC) in the skin is converted to previtamin D_3 (preD_3). PreD_3 immediately converts by a heat-dependent process to vitamin D_3. Excessive exposure to sunlight degrades previtamin D_3 and vitamin D_3 into inactive photoproducts. Vitamin D_2 and vitamin D_3 from dietary sources are incorporated into chylomicrons, transported by the lymphatic system into the venous circulation. Vitamin D (D represents D_2 or D_3) made in the skin or ingested in the diet can be stored in and then released from fat cells. Vitamin D in the circulation is bound to the vitamin D binding protein that transports it to the liver where vitamin D is converted by the vitamin D-25-hydroxylase (25-OHase) to 25-hydroxyvitamin D [25(OH)D]. This is the major circulating form of vitamin D that is used by clinicians to measure vitamin D status (although most reference laboratories report the normal range to be 20–100 ng/ml, the preferred healthful range is 30–60 ng/ml). 25(OH)D is biologically inactive and must be converted in the kidneys by the 25-hydroxyvitamin D-1α-hydroxylase (1-OHase) to its biologically active form 1,25-dihydroxyvitamin D [1,25(OH)$_2$D]. Serum phosphorus, calcium, fibroblast growth factor (FGF-23) and other factors can either increase (+) or decrease (−) the renal production of 1,25(OH)$_2$D. 1,25(OH)$_2$D feedback regulates its own synthesis and decreases the synthesis and secretion of parathyroid hormone (PTH) in the parathyroid glands. 1,25(OH)$_2$D increases the expression of the 25-hydroxyvitamin D-24-hydroxylase (24-OHase) to catabolize 1,25(OH)$_2$D and 25(OH)D to the water soluble biologically inactive calcitroic acid that is excreted in the bile. 1,25(OH)$_2$D enhances intestinal calcium absorption in the small intestine by stimulating the expression of the epithelial calcium channel (ECaC; also known as transient receptor potential cation channel subfamily V member 6; TRPV6) and the calbindin 9 K (calcium-binding protein; CaBP). 1,25(OH)$_2$D is recognized by its receptor in osteoblasts causing an increase in the expression of receptor activator of NFκB ligand (RANKL). Its receptor RANK on the preosteoclast binds RANKL, which induces the preosteoclast to become a mature osteoclast. The mature osteoclast removes calcium and phosphorus from the bone to maintain blood calcium and phosphorus levels. Adequate calcium and phosphorus levels promote the mineralization of the skeleton and maintain neuromuscular function. Holick copyright 2007, with permission.

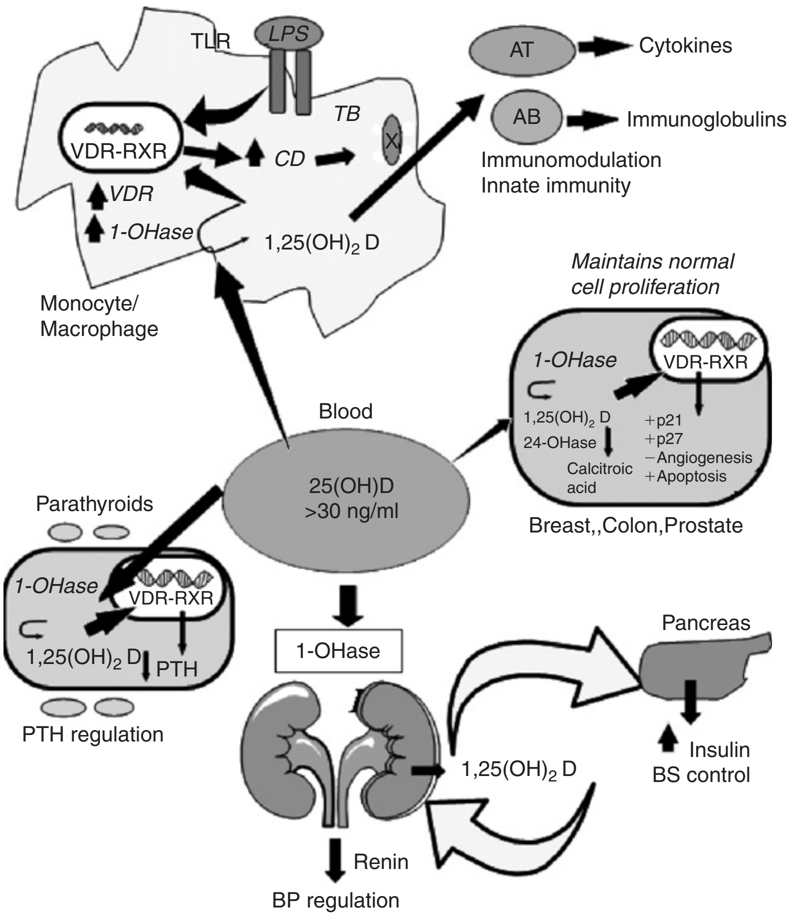
TLR
LPS
TB
VDR-RXR
CD
X
VDR
1-OHase
1,25(OH)$_2$ D
Monocyte/
Macrophage
AT
Cytokines
AB
Immunoglobulins
Immunomodulation
Innate immunity
Maintains normal
cell proliferation
1-OHase
VDR-RXR
1,25(OH)$_2$ D
24-OHase
Calcitroic
acid
+p21
+p27
−Angiogenesis
+Apoptosis
Breast,,Colon,Prostate
Blood
25(OH)D
>30 ng/ml
Parathyroids
1-OHase
VDR-RXR
1,25(OH)$_2$ D
PTH
PTH regulation
1-OHase
Pancreas
1,25(OH)$_2$ D
Insulin
BS control
Renin
BP regulation

FIGURE 8 Metabolism of 25-hydroxyvitamin D [25(OH)D] to 1,25 dihydroxyvitamin D 1,25(OH)$_2$D for nonskeletal functions. When a monocyte/macrophage is stimulated through its toll-like receptor 2/1 (TLR2/1) by an infective agent such as Mycobacterium tuberculosis (TB) or its lipopolysaccharide (LPS), the signal upregulates the expression of vitamin D receptor (VDR) and the 25-hydroxyvitamin D-1-hydroxylase (1-OHase). 25(OH)D levels >30 ng/ml provides adequate substrate for the 1-OHase to convert it to 1,25(OH)2D. 1,25(OH)$_2$D returns to the nucleus where it increases the expression of cathelicidin (CD), which is a peptide capable of promoting innate immunity and inducing the destruction of infective agents such as TB. It is also likely that the 1,25(OH)$_2$D produced in the monocytes/macrophage is released to act locally on activated T (AT) and activated B (AB) lymphocytes, which regulate cytokine and immunoglobulin synthesis, respectively. When 25(OH)D levels are ≈30 ng/ml, it reduces risk of many common cancers. It is believed that the local production of 1,25(OH)$_2$D in the breast, colon, prostate, and other cells regulates a variety of genes that control proliferation including p21 and p27 as well as genes that inhibit angiogenesis and induced apoptosis. Once 1,25(OH)$_2$D completes the task of maintaining normal cellular proliferation and differentiation, it induces the 25-hydroxyvitamin D-24-hydroxylase (24-OHase). The 24-OHase enhances the metabolism of 1,25(OH)$_2$D to calcitroic acid, which is biologically inert. Thus, the local production of 1,25(OH)$_2$D does not enter the circulation and has no influence on calcium metabolism. The parathyroid glands have 1-OHase activity and the local production of 1,25(OH)$_2$D inhibits the expression and synthesis of PTH. The production of 1,25(OH)$_2$D in the kidney enters the circulation and is able to downregulate renin production in the kidney and to stimulate insulin secretion in the β-islet cells of the pancreas. Holick copyright 2007, with permission.

physiologic significance. In 1981, Eisman and co-workers reported that 80% of 54 breast cancer tissues possessed VDR activity. During the same year, Abe *et al.* (1981) and Feldman *et al.* (1982) reported that a mouse myeloid leukemic cell line (M-1) and melanoma cells, respectively, possessed a VDR (VDR+). Abe *et al.* (1981) showed a dose-dependent induction of differentiation of these myeloid leukemic cells by 1,25(OH)$_2$D$_3$, and Colston *et al.* (1981) found that 1,25(OH)$_2$D$_3$ inhibited melanoma cell proliferation. Cultured human promyleocytic leukemic cells (HL-60), which were VDR+ , responded in a similar fashion (Tanaka *et al.,* 1982; Suda *et al.,* 1984). 1,25(OH)$_2$D$_3$ was found to decrease cellular proliferative activity, reduce c-myc-mRNA, and induce the expression of monocyte-specific cell surface antigen 63D3 (Tanaka *et al.,* 1982). Of great interest was the *in vivo* observation that when M-1 leukemic mice were treated with 1,25(OH)$_2$D$_3$ or 1α-hydroxyvitamin D$_3$ (1α(OH)D$_3$), their survival was enhanced substantially compared to the control group (Honma *et al.,* 1982). This suggested the possibility of using 1,25(OH)$_2$D$_3$ or one of its analogs as an antiproliferative agent for the treatment of some leukemias and other malignant disorders.

TABLE I Vitamin D Receptor Activity

Calcemic tissues	
Small intestine	
Bone	
Kidney	
Noncalcemic tissues	
Pituitary	Epidermis
Prostate	Melanocytes
Gonads	Hair follicles
Thymus	Dermis
Parathyroids	Monocytes
Pancreas	Lymphocytes
Breast	Myocytes
Stomach	Cardiac muscle
Placenta	Brain

Immune System

In the early 1980s, with the revelation that many tissues possessed a VDR, it was of great interest to determine whether cells of the immune system also possessed a VDR. Initial studies showed that resting T lymphocytes from the circulation did not possess VDR activity. However, upon stimulation with phytohemoglutinin or concanavalin A (Con A), these cells were induced to produce a VDR (Bhalla *et al.,* 1983; Tsoukas *et al.,* 1984). Once activated T lymphocytes developed VDR activity, they responded to $1,25(OH)_2D_3$ in a variety of ways, including decreased interleukin (IL)-2, interferon-γ, and GM-CSF production (Tsoukas *et al.,* 1984; Bhalla *et al.,* 1986; Binderup, 1992; Vanhan *et al.,* 1989). Like resting T lymphocytes, resting B lymphocytes do not possess a VDR. When B cells were stimulated, a VDR was induced, which resulted in decreased DNA synthesis and immunoglobulin production in response to $1,25(OH)_2D_3$ (Lemire *et al.,* 1984; Provvedini *et al.,* 1986b). (Moller *et al.,* 1991) Circulating monocytes also possessed a VDR. In transformed and normal monocytes, $1,25(OH)_2D_3$ induced phagocytic activity in a time-and dose-dependent manner, increased OKII binding, augmented IL-1 production, enhanced lysosomal activity, and increased expression of cell surface antigens, including Fc and C_3 (Gray and Cohen, 1985; Amento, 1987; Suda *et al.,* 1984). When normal human monocytes were incubated with $1,25(OH)_2D_3$, cells developed morphologic and enzymatic changes consistent with their differentiation into macrophages (Provvedini *et al.,* 1986a).

Therefore, it would appear that the immune system is potentially very sensitive to the modulating activities of $1,25(OH)_2D_3$. However, the exact physiological role of $1,25(OH)_2D_3$ on regulating the immune system is not well understood. An insight into the potential physiologic action of $1,25(OH)_2D_3$ on the immune system can best be seen in animals and patients with vitamin D deficiency and in patients with an inborn error in the metabolism of 25-OH-D to $1,25(OH)_2D$ or a defective VDR. Patients with vitamin D-deficient rickets have been noted to have recurrent infections, mainly of the respiratory tract (Lorente *et al.,* 1976). Vitamin D-deficient patients also have a depressed inflammatory and phagocytic response that is corrected by vitamin D replacement (Lorente *et al.,* 1976). A more subtle defect in the immune system is seen in patients with vitamin D receptor defects [vitamin D-dependent rickets type II (DDR II)]. Circulating mononuclear cells from these patients that had been stimulated previously with Con A did not respond to the same degree as normal monocytic cells to the antiproliferative activity of $1,25(OH)_2D_3$ (Koren *et al.,* 1985). Furthermore, $1,25(OH)_2D_3$ and 1α-OH-D_3 treatment restored deficient macrophage and lymphocyte activities in vitamin D-deficient rats, in patients with vitamin D resistance, and in renal failure patients (Weintraub *et al.,* 1989; Binderup, 1992; Kitajima, 1989; Tabata *et al.,* 1988).

As early as 1849, it was appreciated that cod liver oil was effective in treating tuberculosis. In the early 1900's, patients with TB were routinely sent to sunny areas or at higher altitudes to help in their treatment. How sunlight and vitamin D played a role in the prevention and treatment of TB was unclear until Liu *et al.,* 2006 reported that the local production of $1,25(OH)_2D_3$ plays a critical role in innate immunity. Exposure of monocytes/macrophages to either lipopolysaccharide or mycobacterium tuberculosis induced the toll-like receptors which in turn enhanced the nuclear expression of the VDR and the 1-OHase (Fig. 8). Further investigation revealed that the local increase of $1,25(OH)_2D_3$ in these monocytes/macrophage increased the expression of the bacteriocidal protein cathelicidin. To make certain that the observation was clinically relevant, they took serum from African-Americans who typically have a circulating level of 25(OH)D of < 20 ng/ml and infected monocytes with TB. The monocytes/macrophages were unable to significantly increase the production of cathelicidin. When the African-American serum was supplemented with $25(OH)D_3$ to a concentration that is typical for a Caucasian, i.e. ~ 26 ng/ml, the monocytes/macrophages enhance cathelicidin expression by 5 fold and they destroyed the TB. Based on this observation, it was suggested that raising blood levels of 25(OH)D to > 26 ng/ml is clinically important for innate immunity, and may help explain why African-Americans who are more prone to vitamin D deficiency are also more likely to be infected with TB and have more aggressive illness.

$1,25(OH)_2D_3$ has a variety of *in vitro* and *in vivo* effects on the immune system. However, the *in vitro* observations do not necessarily predict *in vivo* outcomes. This may be due to the multitude of effects $1,25(OH)_2D$ has on T and B

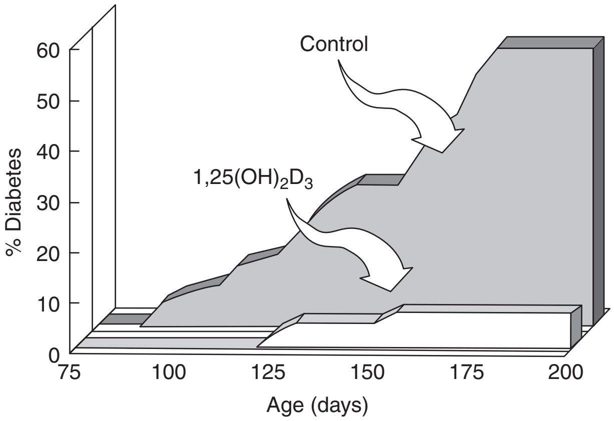

FIGURE 9 The effect of 1,25(OH)$_2$D$_3$ on reducing the incidence of diabetes mellitus type I in NOD mice. Adapted from Mathieu *et al*. (1994).

lymphocytes and monocytes. *In vivo,* the combination of these effects manifest themselves in numerous ways. In mice, 1,25(OH)$_2$D$_3$ substantially reduces the development of autoimmune thyroiditis (Fournier *et al.,* 1990), encephalomyelitis (Lemire and Archer, 1991), and multiple sclerosis (Hayes *et al.,* 1997). 1,25(OH)$_2$D$_3$ prolongs the survival of transplanted skin allografts in mice (Chiocchia *et al.,* 1991) and prevents the incidence of autoimmune diabetes in nonobese diabetic (NOD) mice (Mathieu *et al.,* 1994; Mathieu *et al.,* 1998) (Fig. 9). Whereas *in vitro* 1,25(OH)$_2$D$_3$ decreases immunoglobulin synthesis in B lymphocytes, *in vivo,* its precursor analog, 1α(OH)D$_3$, leads to an increase in primary antibody response (Komori *et al.,* 1985).

Many of these *in vivo* animal studies have been corroborated by retrospective and prospective epidemiologic studies. A birth cohort study involving 10,366 children conducted in Finland showed that during the first year of life, children who took the recommended supplemental dose of 2,000 IU/d of vitamin D had a rate ratio of 0.022 (range, 0.05–0.89) for type I diabetes mellitus compared to those who regularly received $<$2000 IU/d (Hypponen *et al.,* 2001). Stene *et al.,* 2003 reported a lower risk of type I diabetes in children of mothers who took cod liver oil during pregnancy. Vitamin D deficiency has been implicated in several other diseases including rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus, osteoarthritis and periodontal disease (Holick, 2006, 2007). Vitamin D intake was reported to be inversely associated with rheumatoid arthritis (Merlino *et al.*, 2004), and Munger *et al.*, 2004, found that higher intake of vitamin D $>$ 400 IU/d was associated with a lower risk of developing multiple sclerosis.

Skin

The original observation that [^{3}H]1,25(OH)$_2$D$_3$ localized in the nuclei of cells in the basal layer of the epidermis has now been extended to include nuclei of cells in the outer root sheath of the hair follicle and in the stratum granulosum and stratum spinosum of the epidermis (Stumpf *et al.,* 1979, 1984). The presence of and amounts of VDR in keratinocytes appear to be related to the proliferative and differentiation activity of the cells; more VDR activity is observed in preconfluent proliferating cells than in postconfluent cells (Pillai *et al.,* 1987). VDR immunoreactivity has been detected in nuclei of dermal papilla cells and outer root sheath keratinocytes of the hair follicle. During hair follicle proliferation, the VDR immunoreactivity was enhanced significantly in both cell types, suggesting a potential role of 1,25(OH)$_2$D$_3$ in regulating the hair cycle (Reichrath *et al.,* 1994). Although the physiologic function of 1,25(OH)$_2$D$_3$ in these skin cells is not well understood, in cultured human and murine keratinocytes, 1,25(OH)$_2$D$_3$ inhibited their proliferation in a dose-dependent fashion and caused them to terminally differentiate (Fig. 10) (Hosomi *et al.,* 1983; Smith *et al.,* 1986; Pillai *et al.,* 1987). Human skin fibroblasts also have VDR and respond to the hormone in a similar manner (Feldman *et al.,* 1982; Clemens *et al.,* 1983; Holick, 1995).

When cultured melanoma cells with VDR+ were incubated with 1,25(OH)$_2$D$_3$, this hormone inhibited their proliferation and induced them to differentiate (Colston *et al.,* 1981). These data suggest that melanocytes may also be a target cell for 1,25(OH)$_2$D$_3$. There is also immunohistochemical evidence for the presence of VDR in melanocytes from skin biopsies of patients with psoriasis (Milde *et al.,* 1991). However, there is no direct evidence that normal human melanocytes either possess a VDR or respond to 1,25(OH)$_2$D$_3$ (Mansur *et al.,* 1988).

Other Tissues

A wide variety of other cells and tissues from the brain to the gonads possess VDR (Clemens *et al.,* 1988). Cultured chick embryo skeletal myoblasts have receptor binding for 1,25(OH)$_2$D$_3$ (Boland *et al.,* 1985). Furthermore, when cultured VDR+ myoblast cells (G-8 and H9c2) were incubated with 1,25(OH)$_2$D$_3$, there was a dose-dependent decrease in cell proliferation and induction of terminal differentiation. When the cells became fused microtubules, VDR activity decreased (Simpson *et al.,* 1985). VDR is also present in rodent heart tissue, and when isolated cardiac muscle cells were exposed to 1,25(OH)$_2$D$_3$, the hormone increased calcium uptake in a time-and dose-dependent fashion (Weishaar and Simpson, 1989).

Ovaries and testes have VDR activity. Sertoli cells in culture increase rapid uptake of calcium when exposed to 1,25(OH)$_2$D$_3$ (Akerstrom and Walters, 1992). Of great interest was the observation that primary cultured prostate cells derived from normal, benign prostatic hyperplasia and prostate cancer tissues possess VDR (Skowronski *et al.,* 1995). Prostate cancer cell lines and primary cultures of stromal and epithelial cells derived from normal and

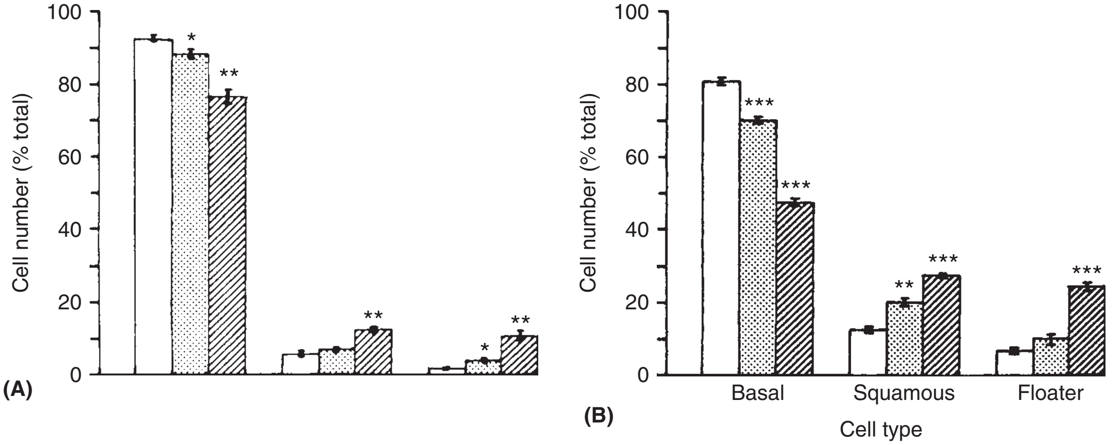

FIGURE 10 Effect of $1\alpha,25(OH)_2D_3$ on the morphologic differentiation of cultured human keratinocytes. The proportion of different keratinocyte cell types after 1 (**A**) or 2 (**B**) weeks of incubation with vehicle alone (open bar), $1\alpha,25(OH)_2D_3$ at 10^{-10}M (dotted bar), or $1\alpha,25(OH)_2D_3$ at 10^{-8}M (striped bar). Each bar represents the mean of triplicate determinations ± SEM. Student's *t* test was used to assess the level of significance ($*p < 0.05$; $**p < 0.001$). Reproduced with permission from Smith *et al.* (1986).

malignant prostate tissues respond in a dose-dependent fashion to the antiproliferative activity of $1,25(OH)_2D_3$.

β islet cells of the pancreas possess a VDR. There is some evidence that $1,25(OH)_2D$ may alter insulin secretion (Cade and Norman, 1986). Pittas *et al.*, 2006, reported a 33% reduction in risk of developing type 2 diabetes when 1200 mg of calcium and 800 IU vitamin D were taken daily. The parathyroid glands possess VDR, and there is strong evidence that $1,25(OH)_2D_3$ suppresses preproparathyroid hormone mRNA levels (Naveh-Many and Silver, 1990; Dusso *et al.*, 2005).

RELEVANCE OF VDR AND $1,25(OH)_2D_3$ IN NONCALCEMIC CELLS AND TISSUES

Physiologic Actions

It is remarkable that most cells and tissues in the human body possess VDR and are therefore potential target tissues for $1,25(OH)_2D_3$ (Table I). Although there is very strong evidence *in vitro* and *in vivo* that $1,25(OH)_2D_3$ can have a wide range of noncalcemic activities that have an impact on the function of the immune system, skin, gonads, prostate gland, brain, skeletal and smooth muscle, and pancreas, the true physiologic function of $1,25(OH)_2D_3$ is not well understood. To put this into perspective, patients who are vitamin D deficient or patients who suffer from DDR II and are therefore totally resistant to the action of $1,25(OH)_2D_3$ do not seem to have major deficits in the physiologic function of most of the tissues described. There is evidence that vitamin D deficiency causes muscle weakness (Bishoff-Ferrari *et al.*, 2005) and alters the immune system to make these patients more prone to some infections, and in the case of DDR II, the patients often suffer from alopecia (Demay, 1995; Holick, 1995). They, however, do not have a higher incidence of cancer such as leukemia, they do not suffer from diabetes mellitus, and their skin appears to be normal with no evidence of hyperproliferation, such as psoriasis or pigmentation disorders.

Pharmacologic Actions

The recognition in the early 1980s that $1,25(OH)_2D_3$ inhibited proliferation and induced differentiation of normal and tumor cells that possessed VDR was greeted with great excitement. The observation that mice with an M-1 cell leukemia had a marked prolongation in their survival when they received $1\alpha(OH)D_3$ or $1,25(OH)_2D_3$ suggested that the antiproliferative activity of $1,25(OH)_2D_3$ and its analogs could be used to treat a variety of cancers (Honma *et al.*, 1982). Eighteen patients with myelodysplasia (preleukemia) were treated with 2 μg of $1,25(OH)_2D_3$ for 12 weeks. A majority of the patients initially had a significant increase in their granulocyte, monocyte, and platelet counts, suggesting that $1,25(OH)_2D_3$ was inhibiting the proliferation and inducing terminal differentiation of the myelodysplastic cells. After 12 weeks of the study, however, there was no significant difference in the blood count for granulocytes, monocytes, and platelets compared to baseline and most patients progressed to acute myelocytic leukemia. In addition, most of the patients had developed hypercalcemia, limiting the amount of drug that could be used (Koeffler *et al.*, 1985). Three patients with myelofibrosis who received $1,25(OH)_2D_3$ (0.5 μg daily) had some improvement in their blood count indices after therapy (Arlet *et al.*, 1984).

Definition of vitamin D deficiency

There is no general consensus as to what the levels of 25(OH)D need to be to satisfy the body's vitamin D requirement. However, many experts believe that a 25(OH)D of >20 ng/ml is important to help maximally suppress PTH levels. Chapuy et al reported that PTH levels reach their nadir when 25(OH)D levels approach 30–40 ng/ml. This observation has been confirmed by both Thomas *et al.*, 1998 and Holick *et al.*, 2005. In addition, Heaney *et al.*, 2003 reported that women who had on average a serum 25(OH)D of 20 ng/ml and then raised their blood level to above 32 ng/ml were able to increase their efficiency of intestinal calcium absorption by 45–65%. Thus, based on this new information, most experts now agree that a preferred 25(OH)D level should be > 30 ng/ml. (Fig. 7)

The adequate intake recommendation by the Institute of Medicine in 1997 for vitamin D intake (Standing Committee, 1999) is now considered to totally inadequate for both children and adults. Most experts agree that 1,000 IU of vitamin D/d is necessary in the absence of any sun exposure to maintain 25(OH)D levels >30 ng/ml (Vieth *et al.*, 2007). Patients, especially the elderly who are vitamin D deficient, don't necessarily have elevated PTH levels which has been perplexing. However, it is now recognized that many of these patients are magnesium deficient, and since magnesium is important in the synthesis and secretion of PTH, this is the likely explanation for the lack of expected secondary hyperparathyroidism associated with a 25(OH)D level < 20 ng/ml.

Vitamin D deficiency is now routinely treated by giving adults 50,000 IU of vitamin D_2/week for 8 weeks (Holick 2007). To maintain a 25(OH)D > 30 ng/ml, 50,000 IU of vitamin D every two weeks is effective. Vitamin D_2 is as effective as vitamin D_3 in maintaining serum 25(OH)D levels and giving 50,000 IU of vitamin D_2 every two weeks will maintain 25(OH)D levels > 30 ng/ml (Holick 2007).

There is great fear in the medical community regarding the use of 50,000 IU of vitamin D_2 because of concern for vitamin D toxicity. However, Vieth *et al.*, 2004 reported that healthy males receiving 10,000 IU of vitamin D_3/d for up to five months demonstrated no untoward toxicity. Vitamin D intoxication is extremely rare and often occurs because of inadvertent or intentional ingestion of upwards of 50,000 IU of vitamin D/d for several months. Typically vitamin D intoxication is observed when 25(OH)D are >150 ng/ml. (Holick 2007).

This collective information has now been translated by reference laboratories who perform 25(OH)D levels either by a radioimmuno assay (RIA) or by liquid chromatography – tandem mass spectroscopy (LC-MS) to report levels of 25(OH)D of 20–100 ng/ml as the normative range. It should be noted that LC-MS assay is able to separate $25(OH)D_2$ from $25(OH)D_3$, and, thus, are reported separately. It is the total 25(OH)D that is the measure of vitamin D status and levels above 30 ng/ml by both RIA and LC-MS assay is what is now considered to be healthyl (Holick 2007).

CLINICAL UTILITY OF NONCALCEMIC ACTIONS OF $1,25(OH)_2D_3$ AND ITS ANALOGS

Use of $1,25(OH)_2D_3$ and Its Analogs for Treatment of Skin Diseases

Rationale for Their Use

In the mid-1980s, there was mounting evidence that epidermal skin cells were very sensitive to the antiproliferative activity of $1,25(OH)_2D_3$ (Hosomi *et al.*, 1983; Smith *et al.*, 1988). Because psoriasis is a nonmalignant hyperproliferative disorder of the epidermis, it was reasoned that if psoriatic skin cells possessed a VDR, then it might be possible to use $1,25(OH)_2D_3$ or one of its analogs to decrease psoriatic keratinocyte proliferation, thereby treating this disorder. Before initiating a clinical trial to evaluate the therapeutic efficacy of $1,25(OH)_2D_3$, MacLaughlin *et al.* (1985) obtained skin biopsies from six patients with psoriasis to determine whether cultured psoriatic fibroblasts responded to the antiproliferative activity of $1,25(OH)_2D_3$. It was found that psoriatic fibroblasts had a partial resistance to the antiproliferative activity of $1,25(OH)_2D_3$, and it was concluded that pharmacologic rather than physiologic amounts of $1,25(OH)_2D_3$ and its analogs could be used for the treatment of psoriasis (MacLaughlin *et al.*, 1985). At the same time, Morimoto *et al.* (1986) treated an osteoporosis patient with 1α-OH-D and observed that this patient, who also suffered from psoriasis, had significant improvement in her disease while on therapy. There are now numerous reports that topical $1,25(OH)_2D_3$, (Fig. 11) as well as topical application of analogs of $1,25(OH)_2D_3$, including 1,24-dihydroxyvitamin D_3 and calcipotriene (Dovonex; Bristol-Meyers Squibb, Buffalo, NY), is safe and effective for the treatment of psoriasis (Kato *et al.*, 1986; van de Kerkhof *et al.*, 1989; Kragballe *et al.*, 1989; Kragballe *et al.*, 1991; Staberg *et al.*, 1989; Langner *et al.*, 1992; Bourke *et al.*, 1993; Langner *et al.*, 1993; Holick, 1993; Bruce *et al.*, 1994; Perez *et al.*, 1996a, 2001).

There has been great concern that in light of the earlier studies using $1,25(OH)_2D_3$ for treating preleukemia that caused severe hypercalcemia (Koeffler *et al.*, 1985) that $1,25(OH)_2D_3$ would not be a safe medication for treating psoriasis. However, for the most part, these concerns have not proven to be correct (Holick, 1993; Perez, *et al.*, 1996a,b).

Several other analogs have been developed for the treatment of psoriasis. The most commonly used analog is calcipotriene (Dovonex). The strategy for developing this analog was to alter the side chain so that it would be metabolized rapidly and, therefore, less prone to developing hypercalciuria and hypercalcemia (Binderup and Bramm, 1988; Kragballe *et al.*, 1989; Kragballe, 1991). Indeed,

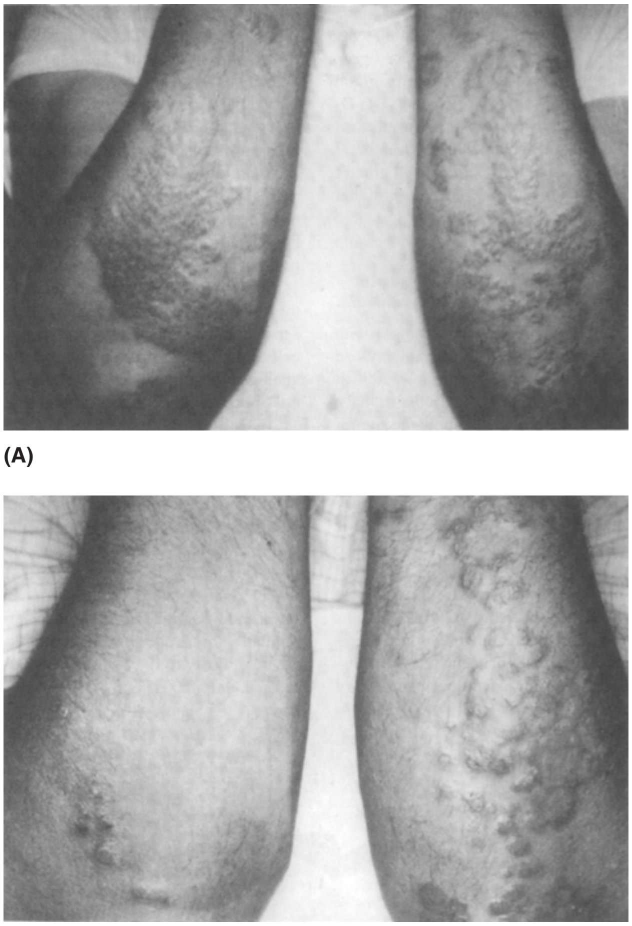

FIGURE 11 (**A**) Arms of a patient with a long history of plaque psoriasis before treatment with the topical form of 1,25-dihydroxyvitamin D_3. (**B**) The same patient who applied only petroleum jelly on the left forearm (at right) and petroleum jelly containing 15 μg/g of $1,25(OH)_2D_3$ on the right forearm (at left) for 3 months. Reproduced with permission from Holick (1994).

calcipotriene is metabolized and degraded rapidly (Binderup *et al.,* 1988; Sorensen *et al.,* 1990). Calcipotriene at 50 μg/g of ointment or cream is used worldwide for the treatment of psoriasis. Other analogs, including 1,24-hydroxyvitamin D_3 (Kato *et al.,* 1986) and hexafluoro-1,25-dihydroxyvitamin D_3 (Durakovic *et al.,* 2001), have been shown to be effective for treating psoriasis. However, calcipotriene can cause a dermatitis that occurs on very sensitive skin areas, such as the face and genital regions (Yip *et al.,* 1991), and very rarely hypercalcemia (Hoeck *et al.,* 1994).

Treatment of Psoriatic Arthritis with $1,25(OH)_2D_3$

It has been estimated that approximately 10% of patients with psoriasis suffer from psoriatic arthritis. In an open-label trial, we found that 10 patients with active psoriatic arthritis who received up to 2.5 μg of oral $1,25(OH)_2D_3$ each night had a statistically significant improvement in mean tender joint count and physician global assessment (Huckins *et al.,* 1990). Forty percent of patients had greater than 50% improvement in their disease and an additional 30% had greater than 25% improvement.

CONCLUSION

Casual exposure to sunlight provides most humans with their vitamin D requirement. Because vitamin D plays an essential role in the maintenance of a healthy skeleton, it is important that all vertebrates, including humans, have a steady supply of vitamin D. The skin is not only the site for the synthesis of this important calciotropic hormone, but is also a major target tissue for $1,25(OH)_2D_3$. The skin may also be a site for the metabolism of 25(OH)D to $1,25(OH)_2D$ (Bikle *et al.,* 1986). It is remarkable that $1,25(OH)_2D_3$ has so many potential biologic actions for health (Holick 1998; Holick 2007) (Fig. 12). As a result, $1,25(OH)_2D_3$ and its analogs have been developed for the treatment of a wide variety of clinical disorders. $1,25(OH)_2D_3$ and its analogs have been very effective in the treatment of hypocalcemic disorders and for the treatment of metabolic bone diseases associated with acquired and inherited disorders of 25(OH)D metabolism and VDR defects (Demay, 1995; Holick, 1999). $1,25(OH)_2D_3$ and its analogs have also been shown to be of value for the treatment of osteoporosis (Tilyard *et al.,* 1992). What has been most intriguing about $1,25(OH)_2D_3$ is its potent antiproliferative properties. One might assume that because $1,25(OH)_2D_3$ is such a potent antiproliferative agent that its chronic use for the treatment of a hyperproliferative disorder would ultimately result in an atrophy of the treated tissues. For example, for the treatment of psoriasis, would the chronic use of $1,25(OH)_2D_3$ and its analogs cause senescence of the skin similar to topical steroids? All of the experience has suggested that $1,25(OH)_2D_3$ will not cause any thinning of the skin, unlike topical steroids. This suggests that $1,25(OH)_2D_3$ is able to sense abnormal proliferative activity and return the activity of the cell to normal. It is for this reason that $1,25(OH)_2D_3$ and its analogs hold such promise for the treatment of a wide variety of proliferative disorders, most notably some cancers.

The observations that many nonrenal tissues, including the skin, colon, and prostate, have 1α-OHase activity opens a new chapter in the vitamin D story (Holick, 2007). Why $1,25(OH)_2D$ would be produced locally in the skin, prostate, colon, and so on remains unknown but may be important in the regulation of cell growth and cancer prevention (Fig. 8). The observation that the product of the Wilm's tumor gene modulates cellular proliferative activity of renal and hemopoetic cells and also regulates the expression of the VDR provides insight into the complexity of the function of $1,25(OH)_2D$ in cell growth (Mawer *et al.,* 2001).

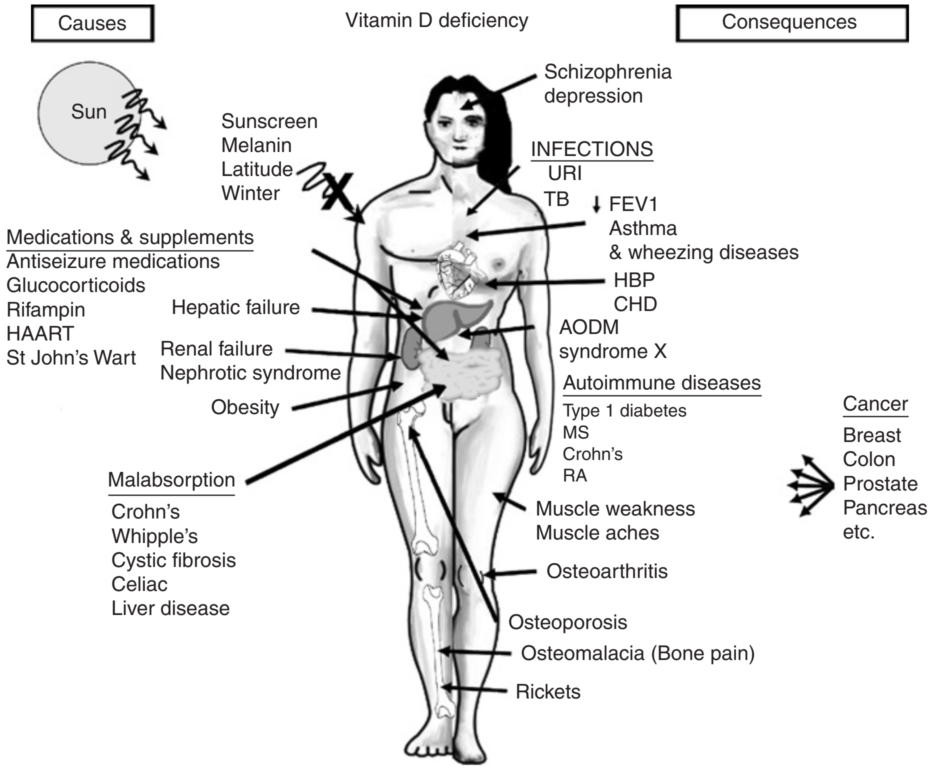
Causes
Vitamin D deficiency
Consequences
Sun
Sunscreen
Melanin
Latitude
Winter
Medications & supplements
Antiseizure medications
Glucocorticoids
Rifampin
HAART
St John's Wart
Hepatic failure
Renal failure
Nephrotic syndrome
Obesity
Malabsorption
Crohn's
Whipple's
Cystic fibrosis
Celiac
Liver disease
Schizophrenia
depression
INFECTIONS
URI
TB
↓ FEV1
Asthma
& wheezing diseases
HBP
CHD
AODM
syndrome X
Autoimmune diseases
Type 1 diabetes
MS
Crohn's
RA
Cancer
Breast
Colon
Prostate
Pancreas
etc.
Muscle weakness
Muscle aches
Osteoarthritis
Osteoporosis
Osteomalacia (Bone pain)
Rickets

FIGURE 12 A schematic representation of the major causes for vitamin D deficiency and potential health consequences. Holick copyright 2007, with permission.

$1,25(OH)_2D_3$ and its analog $1\alpha(OH)D_3$ have been used successfully during the past two decades for treating a variety of acquired and inborn errors in the metabolism of 25(OH)D to $1,25(OH)_2D$, as well as other hypocalcemic disorders (Demay, 1995; Holick, 1995). The revelation that $1,25(OH)_2D_3$ has noncalcemic activities, including regulating proliferation and differentiation of cells and altering the immune function, sparked great interest in developing selective analogs of $1,25(OH)_2D$ that had the desirable noncalcemic actions of $1,25(OH)_2D$ without the potential toxic side effect on calcium metabolism. There are a very large number of analogs that have been synthesized and well reviewed by Bouillon *et al.* (1995). At the present time, there are very few analogs of $1,25(OH)_2D_3$ that are available commercially and have limited calcemic activity.

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Chapter 38

Calcitonin Gene Family of Peptides

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INTRODUCTION

In 1961, Copp observed that protein extract derived from the ultimobranchial body of salmon had a hypocalcemic effect when administered to rodents (Copp and Cameron 1961; Copp *et al.*, 1962). He named this substance calcitonin (CT). In mammalian species, this activity was subsequently localized to the thyroid gland (Hirsch *et al.*, 1963). This hormone was then shown to be produced by parafollicular cells, called C cells (calcitonin-producing cells), of the thyroid gland (Hirsch *et al.*, 1963; Foster *et al.*, 1964). The active principle subsequently was shown to be a 32-amino-acid peptide, released in response to an increase in the plasma calcium concentration.

What these discoverers could not have envisioned was that they had identified the first of a complex series of ligands, receptors, and coreceptors that regulate a broad spectrum of physiological functions that include skeletal remodeling, blood pressure control, neural transmission in both the central and peripheral nervous system, cardiovascular development, and development of the lymphatic system. Although, in this chapter, we will focus primarily on the role of the calcitonin and calcitonin gene-related family of peptides in bone, we will also provide an overview of current progress associated with the entire family. As one assesses the whole of this family of peptides, receptors, and receptor-activity-modifying proteins, one comes to the inescapable conclusion that this receptor family has served as the "pinch hitter" of signaling systems throughout evolutionary history. This system, modified by evolutionary pressure, is present in a broad spectrum of species that encompasses the whole of life. It is expressed very early in development and has important roles in normal mammalian development. At the same time, current evidence suggests that it functions in a secondary role in a variety of developmental systems. This ligand–receptor system is rarely the primary driver in developmental biology, but it frequently plays a crucial role as a secondary driver of differentiation. Thus, deletion or overexpression of the components of this gene family in animal models is more likely to produce complex and nuanced phenotypes rather than the startling phenotypes associated with gene's development. We will attempt to highlight some examples in this chapter.

CALCITONIN GENE FAMILY

The nomenclature for the calcitonin gene family has evolved largely from work performed in humans and rodents. Although the human and rodent gene families have considerable homology, each has evolved with a different nomenclature. In this chapter we will focus on these two mammalian species, but it is important to recognize that this family of genes is present in some of the simplest forms of life where it has evolved very differently. For example, there are substantial differences between mammalian, fish, and bird gene families. Because the focus on this chapter is on bone biology, we will limit our discussion to human and rodent models that have been studied most intensively.

The human calcitonin/calcitonin gene-related peptide (CGRP) gene family, based on their nucleotide sequence homologies, is composed of five separate genes: calcitonin-I (CALC-I), -II, -III, -IV, and -V (Table I). With the exception of CALC-IV, which is on chromosome 12, all of these genes are located on chromosome 11. CALC-I gene consists of six exons, of which tissue-specific alternative RNA splicing yields CT (designated calcitonin gene-related peptide-alpha, CGRP-alpha, in rodents). CALC-II, considered to have arisen from gene duplication, encodes CGRP-beta (or CGRP-II); it is unclear which gene, CALC-I or CALC-II, appeared first (Wimalawansa, 1996). The CALC-III gene is not transcribed and is considered a pseudogene. CALC-IV and -V encode amylin (AMY) and the vasodilator peptide, adrenomedullin (AM), respectively (Fig. 1).

Several common features characterize the classic hormones of the calcitonin gene family (Fig. 2). Calcitonin, CGRP (alpha and beta), amylin, and AM all have a ring

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TABLE I The Calcitonin Gene Family and Peptide Products

Gene*	HUGO gene name	Mature peptide product(s)	Immature peptide products†	Chromosome
CALC-I	CALCA	Calcitonin (CT)	Pre-ProCT ProCT nProCT CCP-I (katacalcin, PDN-21)	11
		CGRP-alpha	CGRP-alpha precursor	
CALC-II	CALCB	CGRP-beta	CGRP-beta precursor	11
CALC-IV	IAPP	Amylin (AMY)	Proamylin	12
CALC-V	ADM	Adrenomedullin (AM)	Proadrenomedullin	11

HUGO, *Human Genome Organization*; CGRP, *calcitonin gene-related peptide*; Pre-ProCT, *preprocalcitonin*; ProCT, *procalcitonin*; CCP-I, *calcitonin carboxyl-terminal peptide*-I; IAPP, *islet amyloid polypeptide*.

*CALC-III *gene is not transcribed and is considered a pseudogene.*

†*Produced during proteolytic processing.*

structure formed by a disulfide bridge at the amino terminus and an amidated amino acid at the carboxyl terminus. The midregion of each hormone forms an α-helical structure. Additionally, as will be discussed later, CT gene family peptides exert their biological effects by binding to one of two receptors: calcitonin receptor (CTR) or calcitonin receptor-like receptor (CRLR) with or without a co-receptor (receptor-activity-modifying protein, RAMP) (Table II). The homology seen among these peptide ligands suggests that the genes in this family are derived from a primordial gene, which underwent duplication and sequence-divergent events.

The peptide products of CALC-I gene (i.e., CT or CGRP-alpha), those most relevant to bone biology, arise through alternative RNA splicing of the primary mRNA transcript that includes or excludes specific exons; this process is tissue specific (Amara *et al.*, 1982) (Fig. 1a). Although there is some overlap, CT mRNA is the major product in thyroid and other tissues, whereas CGRP-alpha mRNA is expressed mainly in neural cells in the brain, spinal cord, and various sympathetic ganglia. In the normal thyroidal C cell, 99% of the primary RNA transcript is processed via a CT-specific pathway; in neuronal tissue, 95% of the primary transcript is processed in a CGRP-specific manner (Lou and Gagel, 1998). The regulatory pathways that control alternative RNA processing are complex and involve multiple factors that promote inclusion (CT-specific pattern) or exclusion (CGRP-specific pattern) of exon IV containing the coding sequence for calcitonin. An enhancer element located downstream of exon IV was identified to facilitate polyadenylation and inclusion of the exon (Lou *et al.*, 1996). A number of factors have been identified to affect the CT-specific pathway (Tran and Roesser, 2003; Zhu *et al.*, 2003; Roesser 2004). Recently, Hu proteins, RNA-binding proteins involved in neuronal differentiation and maintenance, have been identified as the first neuron-specific regulators of the CT/GCRP system by blocking the activity of proteins (TIA-1/TIAR) required for the inclusion of exon IV (Zhu *et al.*, 2006). Additionally, Fox-1 and -2 proteins were recently reported to mediate the neuron-specific splicing pattern by repressing exon IV inclusion (Zhou *et al.*, 2007). The pattern that emerges is one in which inclusion or exclusion of exon IV from the final transcript appears to be a major regulatory event.

HUMAN CALCITONIN AND ITS SYNTHESIS

Biochemistry

Based on its primary structure, CT can be found in at least three categories of species: teleost/avian (i.e., salmon, eel, goldfish, and chicken), artiodactyl (i.e., porcine, bovine, and ovine), and primate/rodent (i.e., human and rat). In several different biological assays of capacity for lowering serum calcium and stimulating urinary cyclic adenosine monophosphate excretion (cAMP) after administration, CT can be ordered by species with regard to potency as follows: teleost/avian > artiodactyls > primate/rodent (Gennari *et al.*, 1990). However, the absolute potency of CT is dependent on the specific species studied. Calcitonin from teleost and avian species is produced in the ultimobranchial glands, which remain in these species as discrete organs. In contrast, mammals demonstrate the greatest concentration of calcitonin-producing cells (i.e., C cells) within the thyroid gland, especially in the upper one-third of each thyroid lobe; embryologically, the C cells migrate from the neural crest to the developing thyroid gland.

Mature human CT is a 32-amino-acid peptide hormone with a molecular mass of 3418 Da. A disulfide bridge connects the cysteine residues at positions 1 and 7 to form a 7-amino-acid ring structure at the amino terminus. At the

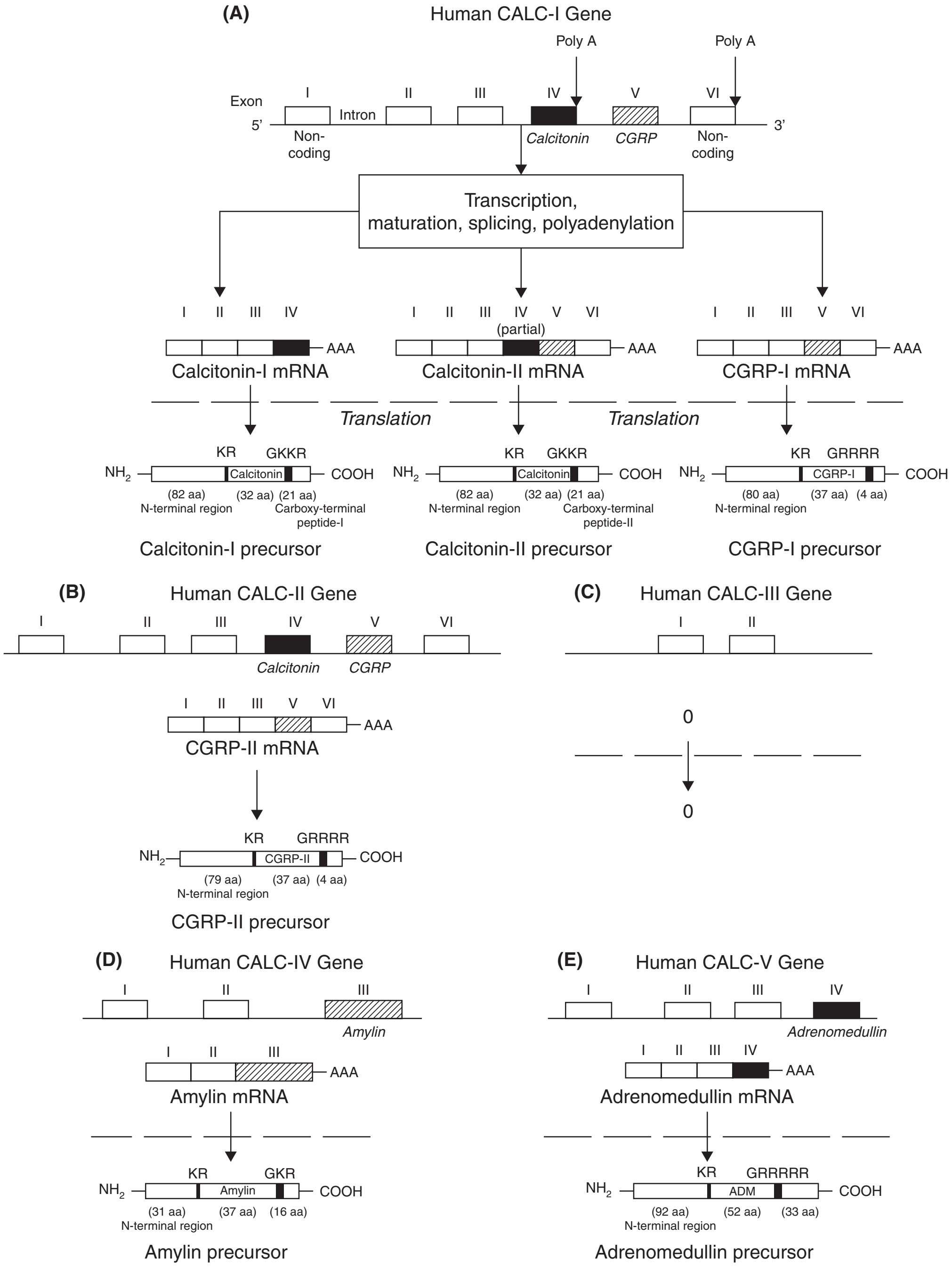
(A)
Human CALC-I Gene
Poly A
Poly A
Exon
I
II
III
IV
V
VI
5'
3'
Intron
Non-coding
Calcitonin
CGRP
Non-coding
Transcription, maturation, splicing, polyadenylation
I II III IV
AAA
Calcitonin-I mRNA
I II III IV V VI
(partial)
AAA
Calcitonin-II mRNA
I II III V VI
AAA
CGRP-I mRNA
Translation
Translation
KR
GKKR
NH2
Calcitonin
COOH
(82 aa)
N-terminal region
(32 aa)
(21 aa)
Carboxy-terminal peptide-I
Calcitonin-I precursor
KR
GKKR
NH2
Calcitonin
COOH
(82 aa)
N-terminal region
(32 aa)
(21 aa)
Carboxy-terminal peptide-II
Calcitonin-II precursor
KR
GRRRR
NH2
CGRP-I
COOH
(80 aa)
N-terminal region
(37 aa)
(4 aa)
CGRP-I precursor
(B)
Human CALC-II Gene
I II III IV V VI
Calcitonin
CGRP
I II III V VI
AAA
CGRP-II mRNA
KR
GRRRR
NH2
CGRP-II
COOH
(79 aa)
N-terminal region
(37 aa)
(4 aa)
CGRP-II precursor
(C)
Human CALC-III Gene
I II
0
0
(D)
Human CALC-IV Gene
I II III
Amylin
I II III
AAA
Amylin mRNA
KR
GKR
NH2
Amylin
COOH
(31 aa)
N-terminal region
(37 aa)
(16 aa)
Amylin precursor
(E)
Human CALC-V Gene
I II III IV
Adrenomedullin
I II III IV
AAA
Adrenomedullin mRNA
KR
GRRRRR
NH2
ADM
COOH
(92 aa)
N-terminal region
(52 aa)
(33 aa)
Adrenomedullin precursor

FIGURE 1 The human calcitonin (CT) gene family: organization of genes, mRNAs, and their hormone precursors. **(A)** The CALC-I primary transcript is processed by the inclusion or exclusion of exons through alternative RNA splicing into three different mRNAs: CT, CT-II, and CGRP-alpha (Jacobs *et al.,* 1981). Exons I to III are common to these three mRNAs. The combination of exons I, II, III, and IV codes for CT mRNA (with polyadenylation resulting in truncation of the mRNA immediately following exon IV). CT-II mRNA includes exons I, II, III, IV (partial), V, and VI. CGRP-alpha mRNA contains exons I, II, III, V, and VI. Each mRNA is translated into a specific precursor peptide. CT mRNA codes mainly for an N-terminal region, mature CT, and a specific C-terminal peptide (CCP-I, katacalcin, or PDN-21), constituting the CT-I precursor. The N-terminal region includes a signal peptide of 25 amino acids and an N-terminal peptide of 57 amino acids (i.e., NProCT or PAS-57). The C-terminal peptide region of CT-I precursor (CCP-I) differs from that of CT-II precursor (CCP-II) by its last 8 amino acids. CGRP-alpha mRNA codes for an N-terminal region, mature CGRP-alpha, and a cryptic peptide. **(B)** The CALC-II gene encodes only the precursor of the CGRP-beta peptide. The composition of six exons in this gene is similar to that of the CALC-I gene; however, examination of the exon 4-like region of CALC-II indicates that CT mRNA production is unlikely. The CGRP-beta peptide differs from CGRP-alpha by three amino acids (Steenbergh *et al.,* 1985). **(C)** The CALC-III gene contains only two exons, which have sequences homologous to exons 2 and 3 of CALC-I and -II genes. However, the CALC-III gene is not transcribed into RNA and, consequently, has no protein product. **(D)** The CALC-IV gene codes for a precursor to the amylin peptide. This gene, expressed predominantly in the islet cells of the pancreas, contains only three exons. The third exon codes for amylin. This 37-amino-acid peptide has marked homology with CGRP peptides. It has been suggested that CT and CGRP exons are derived from a primordial gene and that the different genes have arisen by duplication and sequence-divergent events. **(E)** The CALC-V gene is translated into adrenomedullin. This gene contains four exons. Adrenomedullin (AM) is coded by the fourth exon. The amino-terminal peptides, encoded by exons 2 and 3, also have some bioactivity. Modified from Becker (2001).

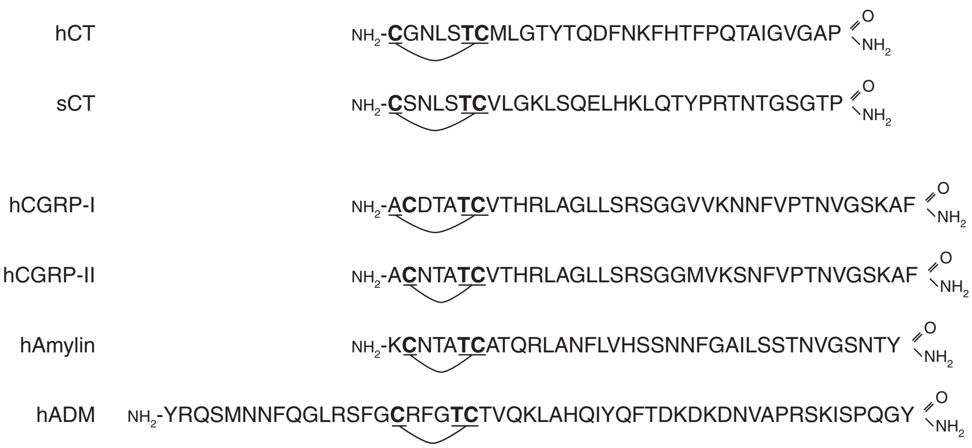
hCT
NH2-CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAP
O
NH2
sCT
NH2-CSNLSTCVLGKLSQELHKLQTYPRTNTGSGTP
O
NH2
hCGRP-I
NH2-ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF
O
NH2
hCGRP-II
NH2-ACNTATCVTHRLAGLLSRSGGMVKSNFVPTNVGSKAF
O
NH2
hAmylin
NH2-KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY
O
NH2
hADM
NH2-YRQSMNNFQGLRSFGCRFGTCTVQKLAHQIYQFTDKDKDNVAPRSKISPQGY
O
NH2

FIGURE 2 Amino acid sequences of human calcitonin (hCT), salmon CT (sCT), human CGRP-I (hCGRP-I), human CGRP-II (hCGRP-II), human amylin, and human adrenomedullin (hADM). Two structural features that are essential for full functional activity are conserved between the peptides: they contain two N-terminal cysteines that form a disulfide bridge resulting in an N-terminal loop and a C-terminal amide. Modified from Becker (2001).

TABLE II Affinity of Peptides of the Calcitonin Gene Family with Receptor Types Based on RAMP Expression

Receptor	RAMP	Peptide	Reference
Calcitonin receptor	–	Calcitonin	Poyner *et al.*, 2002
	RAMP-1	Amylin	Hay *et al.*, 2004
		CGRP	Leuthauser *et al.*, 2000
	RAMP-2	Amylin	Hay *et al.*, 2004
	RAMP-3	Amylin	Hay *et al.*, 2004
Calcitonin receptor-like receptor	RAMP-1	CGRP	Buhlmann *et al.*, 1999
		Intermedin	Roh, *et al.*, 2004
	RAMP-2	Adrenomedullin	Buhlmann *et al.*, 1999
		Intermedin	Roh *et al.*, 2004
	RAMP-3	Adrenomedullin	McLatchie *et al.*, 1998
		Intermedin	Roh *et al.*, 2004

RAMP, *receptor activity-modifying proteins*; CGRP, *calcitonin gene-related peptide*.

carboxyl terminus, there is an amidated proline. The polypeptide precursor of CT, preprocalcitonin (Pre-ProCT) (molecular mass, 15,466 Da), contains 141 amino acid residues (Le Moullec *et al.*, 1984). The leader sequence (signal peptide), composed of 25 amino acid residues, assists in the transport of the ribosomal precursor molecule into the cysternae of the rough endoplasmic reticulum. Early in post-translational processing, the leader sequence is cleaved from the Pre-ProCT precursor molecule by a signal peptidase. The resultant prohormone, procalcitonin (ProCT, also termed PAN-116), may be glycosylated. It consists of 116 amino acid residues (molecular mass, 12,795 Da), which are folded into their appropriate three-dimensional conformation. At the amino terminus portion of ProCT, there is a 57-amino-acid peptide called nProCT (also called PAS-57) (Fig. 3). The immature CT, which is placed centrally within ProCT, consists of 33 amino acid residues, including a carboxyl-terminal glycine. The final 21 amino acid residues comprise the CT carboxyl-terminal peptide-I (CCP) (also termed carboxyl-terminal flanking peptide-I, PDN-21, or katacalcin).

Regulation of Calcitonin Synthesis and Secretion

Synthesis

The mechanisms by which the large precursor, ProCT, is serially processed and its component peptides are sorted into nascent secretory vesicles involve a complex series of progressive modifications that eventuate in the final exocytosis of mature secretory products. Although the mechanisms for post-translational processing have not been fully clarified, much that has been learned from nProCT:

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Ala — Pro — Phe — Arg — Ser — Ala — Leu — Glu — Ser — Ser — Pro — Ala — Asp — Pro — Ala — Thr — Leu — Ser — Glu — Asp —

21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
Glu — Ala — Arg — Leu — Leu — Leu — Ala — Ala — Leu — Val — Gln — Asp — Tyr — Val — Gln — Met — Lys — Ala — Ser — Glu —

41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57
Leu — Glu — Gln — Glu — GLn — Glu — Arg — GLu — Gly — Ser — Ser — Leu — Asp — Ser — Pro — Arg — Ser

CCP-I:

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Aap — Met — Ser — Ser — Asp — Leu — Glu — Arg — Asp — His — Arg — Pro — His — Val — Ser — Met — Pro — Gln — Asn — Ala — Asn

CCP-II:

Aap — Met — Ser — Ser — Asp — Leu — Glu — Arg — Asp — His — Arg — Pro — His — Asn* — His* — Cys* — Pro* — Glu* — Glu* — Ser* — Leu*

FIGURE 3 Sequence of human nProCT (*Top*) and the two CCP peptides (*Bottom*).

the study of other pro-peptides can be applied to ProCT. Topographically, highly organized traffic from the endoplasmic reticulum must pass through the Golgi apparatus, the dense-core secretory vesicles, and, eventually, the cell surface. During this process the ProCT is processed to monomeric calcitonin and flanking peptides. When the CT gene is expressed in cell types that do not possess this processing system (e.g., during sepsis), ProCT is the predominant secretory product (Snider *et al*., 1997).

After the biosynthesis and folding of ProCT, subsequent proteolytic processing occurs, both within the Golgi apparatus and, later, within the secretory granules (Chanat and Huttner, 1991) (Fig. 4). During early post-translational processing, the nProCT segment may act as a signal for sorting its parent ProCT molecule to nascent secretion vesicles of the regulated secretory pathway; such a role has been demonstrated for the N-terminal 26-amino-acid peptide of the prohormone of adrenocorticotropin (ACTH), pro-opiomelanocortin (POMC) (Cool and Loh, 1994). Furthermore, chromogranin B may function as a helper protein to favor *trans*-Golgi sorting to the regulated secretory pathway as it does for ACTH.

Within the newly formed secretion vesicles, proteolytic cleavage releases immature CT. Then, as amidation proceeds, mature CT is produced and is concentrated progressively within these vesicles (Treilhou-Lahille *et al*., 1986). The ensuing tight aggregation of hormones within the vesicle causes its subsequent electron-dense appearance. These secretory vesicles are destined to serve as storage repositories for later secretion. Without the appropriate external stimulus, they have a relatively long half-life. Ultimately, in response to the appropriate signal at the plasma membrane, there is a brief increased concentration of cytosol-free Ca^{2+}, which induces secretion. In this process, the secretory vesicles further migrate via an intracellular microtubular system toward the periphery of the cell, fuse with the apical portion of the plasma membrane, and discharge their hormonal contents in a quantal release by exocytosis. Studies in normal humans suggest that these secretory vesicles contain, in addition to mature CT, intact ProCT, nProCT, CT:CCP-I, CCP-I, and probably some free immature CT (Treilhou-Lahille *et al*., 1986; Snider *et al*., 1997).

Secretion

The primary regulatory loop controlling CT secretion is the inverse of that regulating parathyroid hormone secretion. An increase of the serum calcium concentration activates the calcium-sensing receptor (CaSR), a G protein-coupled receptor highly expressed on thyroid C cells, and stimulates the release of calcitonin (Garrett *et al*., 1995; Freichel *et al*., 1996; Fudge and Kovacs, 2004). This is an interesting physiological adaptation where the same receptor expressed by two different cell types (parathyroid hormone- or calcitonin-producing cells) is linked to opposite secretory effects (Fudge and Kovacs, 2004). Most importantly, these regulatory loops lead to maintenance of the serum calcium concentration within a narrow range. Deficiency of parathyroid hormone leads to hypocalcemia; absence of calcitonin causes an exaggerated extracellular calcium increase in response to parathyroid hormone, but the basal serum calcium concentration is normal (Lanske *et al*., 1998; Hoff *et al*., 2002).

There are other stimulants for CT secretion: magnesium, glucagon, gastrin, cholecystokinin, secretin, vasoactive intestinal peptide, and pentagastrin (Cooper *et al*., 1971; Selawry *et al*., 1975; Roos *et al*., 1976). The increase of serum CT in response to gastrin, and perhaps to cholecystokinin and glucagon, raises the question as to whether these gastrointestinal hormones regulate its secretion (Selawry *et al*., 1975). Although there is abundant evidence that gastrin is a potent stimulant of calcitonin secretion when given in pharmacological concentrations, the evidence that it plays a significant physiological role is

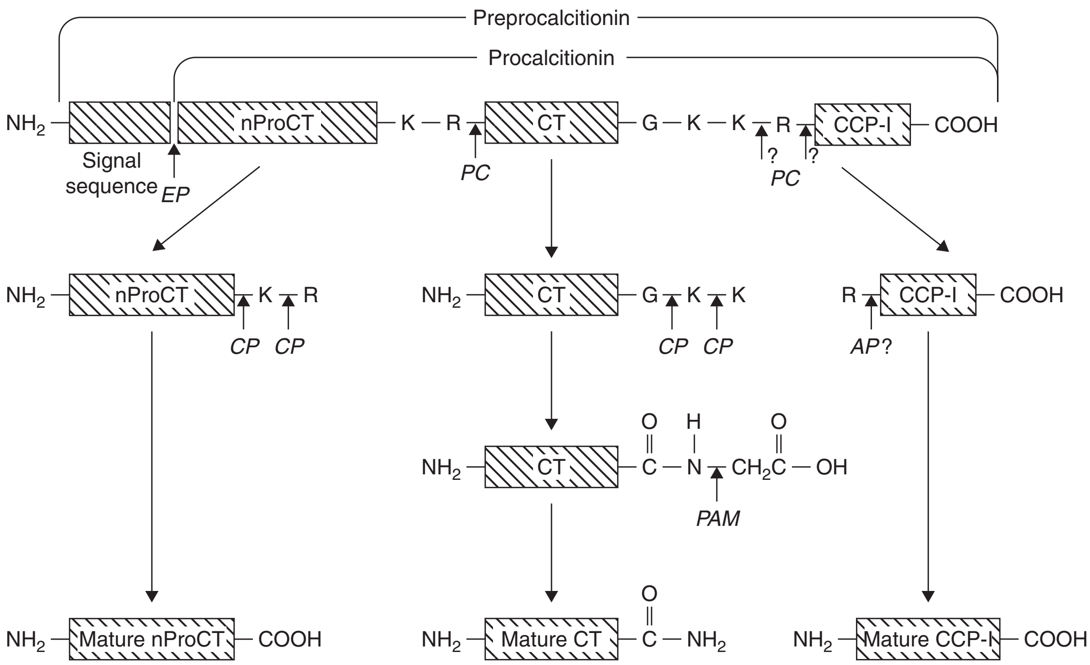

FIGURE 4 Enzymatic processing of pre-ProCT and its constituents. The cleavage of ProCT, and the consequent release of the immature CT, is accomplished by a prohormone convertase (PC) enzyme, which is calcium dependent. The appropriate order of proteolysis is modulated, in part, by autocatalytic self-activation, which in turn may be influenced by neuroendocrine "chaperone" peptides that aid in protein folding. For reasons detailed later, it is likely that the initial or preferential cleavage site of immature CT is at its amino terminus region, yielding a conjoined polypeptide of CT plus the CT carboxyl-terminal peptide (CT:CCP-I). The PC cleaves the pro-peptide at the carboxyl terminus of the dibasic paired Lys-Arg residues between nProCT and immature CT, and either between Lys and Arg or at the end of the Lys, Lys, Arg basic triplet, which is located at the junction between immature CT and CCP-I. If the cleavage is between Lys and Arg, an aminopeptidase enzyme would remove residual Arg. A carboxypeptidase removes residual Lys-Lys (or the Lys-Lys-Arg) from the immature CT prior to the action of PAM and removes Lys-Arg from the carboxyl terminus of nProCT. EP, an endopeptidase; PC, a prohormone convertase; CP, a carboxypeptidase; AP, an aminopeptidase; PAM, peptidylglycine α-amidating monoxygenase and its constituent enzyme peptidyl-α-hydroxyglycine α-amidating lyase (PAL); K, lysine; G, glycine; R, arginine.

limited. One hypothesis is that a large load of calcium in the upper gastrointestinal tract activates calcium-sensing receptors on the gastrin-producing cells, leading to gastrin release which in turn stimulates calcitonin secretion (Ray *et al.*, 1997). Recent studies show that a loading dose of calcium gluconate (1 g) increases both gastrin and calcitonin, lending some credibility to this hypothesis in humans (Bevilacqua *et al.*, 2005). The histamine-2 receptor blockers and proton pump inhibitors used to treat ulcer disease have been demonstrated to stimulate the release of gastrin and calcitonin (Erdogan *et al.*, 2006). On the other hand, in rats who have undergone fundectomy, which leads to hypergastrinemia, CT levels did not rise above normal compared with sham-operated controls (Rumenapf *et al.*, 1998).

Location of Calcitonin in the Body

Systematic studies have been done on immunoreactive CT (iCT) in tissues of humans and monkeys. In humans, the highest concentration of iCT is in the thyroid gland, where it is located within the C cells, mostly found in the central portion of each lobe. Scattered C cells may also be found in adjacent tissues (i.e., parathyroid glands and thymus). However, a survey of approximately 20 tissues of other regions of the human body has yielded iCT values for many tissues that are appreciably higher than in the blood; the highest levels have been found in small intestine, thymus, urinary bladder, lung, and liver (Becker *et al.*, 1979). In mammalian species, calcitonin expression has been demonstrated in thymus, jejunum, lung, urinary bladder, prostate, brain, pituitary gland, mammary gland, endometrium, and testis (Maddineni *et al.*, 2007). When one considers the weights of most of these extrathyroidal tissues, their total iCT content is considerable. In nearly all of the extrathyroidal tissues where appreciable amounts of iCT are found, neuroendocrine cells have been identified, and in some of these tissues, immunohistochemical staining reveals the presence of iCT. These extrathyroidal neuroendocrine cell contents contribute to the serum content of iCT, and these cells can be induced to secrete both locally and distally under the influence of various stimuli.

Mature Calcitonin

Modification of a precursor pro-peptide through a series of enzymatic processes produces mature CT (Fig. 4). Initially,

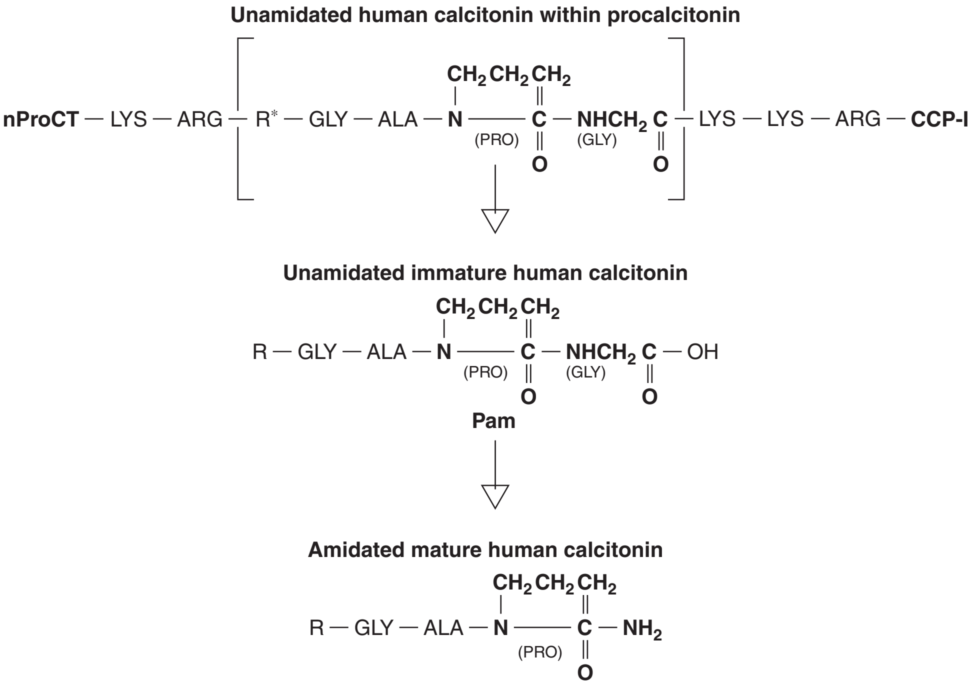
Unamidated human calcitonin within procalcitonin
nProCT — LYS — ARG — R* — GLY — ALA — N — C — NHCH2 C — LYS — LYS — ARG — CCP-I
CH2 CH2 CH2
(PRO)
(GLY)
O
Unamidated immature human calcitonin
R — GLY — ALA — N — C — NHCH2 C — OH
Pam
Amidated mature human calcitonin
R — GLY — ALA — N — C — NH2

FIGURE 5 Sequential steps involved in the amidation of CT (see text). R*, 29 amino acids of human CT not shown.

there is cleavage at an endoproteolytic site (e.g., the Lys-Lys-Arg locus) within ProCT, and there is a prerequisite glycine residue at the carboxyl-terminal side of the amino acid residue that undergoes subsequent amidation. As is the case for a very large number of bioactive peptides, mature CT possesses an α-amide moiety at its carboxyl terminus. Although the exact biological function of the amide is not completely understood, there are indications that it contributes the mature peptide's stability, bioactivity, and interaction with its specific receptor (Walsh and Jefferis, 2006).

The sequential steps leading to amidation are shown in Figure 5. Peptidylglycine α-amidating monooxygenase (PAM) is the key enzyme involved in amidation (Eipper *et al.*, 1992). The preliminary step performed by PAM is oxidation of the α-hydrogen of glycine to form an α-OH. Within the PAM protein, a second enzyme resides, peptidyl-α-hydroxyglycine α-amidating lyase (PAL). This enzyme acts on the intermediate molecule; it catalyzes amidation of the adjacent proline of CT, thus removing glycine in the form of a glyoxylate. The product of this two-step enzymatic action is a mature 32-amino-acid CT with an amidated proline at its carboxyl terminus.

Measuring Mature Serum Calcitonin

Serum immunoreactive calcitonin (iCT) is measured most commonly by a two-site immunoassay. Specificity and sensitivity is provided by use of two monoclonal antibodies that target different epitopes on monomeric CT. One of these antibodies is immobilized on a matrix and binds to a single epitope on monomeric CT; a second antibody targets a second epitope and also contains a detection system (radioactivity, colorimetric, or chemiluminescent molecules). Calcitonin is sandwiched between the two monoclonal antibodies. Assays for ProCT target an epitope in monomeric CT and a second within the precursor portion of the peptide.

The principal clinical use of mature CT measurement is the detection and follow-up of patients with medullary thyroid cancer (MTC). Initial assays using polyclonal antisera identified not only monomeric CT but larger precursor molecules; as monoclonal technology evolved, assays specific for monomeric CT or ProCT were developed (Motte *et al.*, 1988; Seth *et al.*, 1989; Guilloteau *et al.*, 1990; Perdrisot *et al.*, 1990). Normal serum or plasma concentrations using these second- and third-generation assays is <2 pg/mL and may increase to 10–15 pg/mL after calcium or pentagastrin stimulation.

BIOACTIONS OF CALCITONIN

Calcitonin and the Skeletal System

CT plays an important role in skeletal homeostasis by modulating osteoclast action and bone resorption as protection from bone loss during stressful times of potential calcium deficiency (e.g., growth, pregnancy, and lactation) (Friedman and Raisz, 1965; Zaidi *et al.*, 1993; Woodrow *et al.*, 2006). Mammalian osteoclasts contain abundant, high-affinity receptors for CT (Nicholson *et al.*, 1986; Lin *et al.*, 1991; Gorn *et al.*, 1992). The complex process of bone resorption involves osteoclast attachment to the bone surface using podosomes present in an actin-rich ring (the sealing zone) and the formation of the ruffled border, the main resorptive organelle in the osteoclast. CT has been shown to disassemble podosomes, disrupt the actin-rich ring and ruffled border, and eventually lead to osteoclast detachment (Kallio *et al.*, 1972). Recently, it has been shown that this may be mediated by CT modulation of Pyk2 and Src activities (Bruzzaniti and Baron, 2006; Shyu *et al.*, 2007). The hormone inhibits bone resorption by acutely inducing quiescence of osteoclast cell motility (Q effect) within 1 min, followed by a more gradual pseudopod retraction of osteoclasts (R effect), which together decrease cell contact with bone surface (Zaidi *et al.*, 1990; Inzerillo *et al.*, 2002). The Q and R effects are distinct cellular processes mediated by G proteins. The Q effect is associated with an elevation of intracellular cyclic adenosine monophosphate (cAMP) after direct activation of a cholera toxin-sensitive G_s protein by CT (Nicholson *et al.*, 1986). The R effect is thought to be coupled to a pertussis toxin-sensitive G_i protein, which increases intracellular free calcium concentration (Moonga *et al.*, 1992). Retraction of the pseudopods is associated with the formation of intracellular retraction fibers and a cessation of membrane ruffling; the final result is a small, rounded, nonmotile cell (Gravel *et al.*, 1994). CT-induced retraction has also been attributed to protein kinase C activation in osteoclasts (Su *et al.*, 1992). Additionally, *in vivo* studies of patients with Paget's disease of bone found that CT administration led to alteration of the ultrastructure of osteoclasts and a significant decrease in osteoclast number (total per area and within resorptive pits) (Singer *et al.*, 1976; Williams *et al.*, 1978).

CT inhibits other components of the osteoclast, which in turn contributes to its antiresorptive effects. Tartrate-resistant acid phosphatase (TRAP) is specifically expressed in osteoclasts to a high degree; serum concentrations of TRAP correlate with rates of bone resorption (Stepan *et al.*, 1989; Halleen *et al.*, 2002). TRAP release from osteoclasts is potently inhibited by calcitonin, even when incubated with osteoclastic activating factors like receptor activator of nuclear factor kappa B ligand (RANKL) and interleukin-1 (IL-1) (Kirstein *et al.*, 2006). CT decreases carbonic anhydrase II, the enzyme expressed by osteoclasts that leads to the production of protons required for bone resorption, expression in a dose-dependent manner (Zheng *et al.*, 1994). Thus, by various mechanisms, mature CT diminishes osteoclastic activity, a phenomenon that is so marked that its *in vitro* effect on bone resorption can be used as a bioassay to measure picomolar concentrations of the hormone (Zaidi *et al.*, 1994).

However, within 48 hours of continuous or repetitive exposure to CT, there is a loss of inhibitory effects by CT on osteoclast-mediated bone resorption (Wener *et al.*, 1972; Heersche, 1992). The "escape phenomenon" from CT inhibition of bone resorption appears to be associated with reduction of CT-receptor (CTR) expression by osteoclasts, development of rapid resistance by the osteoclast, and desensitization to CT (Samura *et al.*, 2000). Additionally, administration of a monoclonal antibody to Kat1-antigen, a unique cell surface antigen expressed on rat osteoclasts distinct from RANK, led to stimulation of osteoclast formation only when in the presence of calcitonin (Kukita *et al.*, 2001). This indicates a potential role of Kat1-antigen in the "escape phenomenon" from CT inhibition of bone resorption. However, owing to its inhibitory effects on the osteoclast, CT is used successfully in the therapy of disorders of bone loss or of rapid bone turnover. Additionally, its hypocalcemic activity allows it to be used clinically in hypercalcemic disorders. This is further described in the next section.

Calcitonin appears to interact with osteoprotegerin (OPG), RANKL, and RANK (Mancini *et al.*, 2000). Some data suggest that CT may also exert a stimulatory effect on the osteoblast. The hormone increases the concentrations of IGF-I and IGF-II in cultures of human osteoblast cell lines in a dose- and time-dependent manner (Farley *et al.*, 2000).

One of the vexing issues related to the physiology of CT has been the apparent lack of effect of removal of the thyroid gland, the major source of CT, on bone physiology. Numerous studies have failed to document significant effects of thyroidectomy on long-term bone mass (Tiegs *et al.*, 1985; Hurley *et al.*, 1987). A potential explanation lies in the aforementioned expression of the CT gene by neuroendocrine cells in many tissue types. Several rodent models have been developed in recent years to more directly address the question of long-term deficiency of calcitonin.

The first is a model that targeted the CT/CGRP-alpha gene (Hoff *et al.*, 2002; Dacquin *et al.*, 2004). The initial report described animals studied at the F_3 generation that exhibited greater calcemic responses to PTH and a significant increase in bone formation and bone volume. In a subsequent report using the same F_3 generation CT/CGRP-alpha animals, it was shown that the bone mass differences were maintained and the CT/CGRP-alpha-deficient animals developed a robust pattern of trabecular remodeling as they aged (Hoff *et al.*, 2002). Taken together these findings suggested a role for CT in the regulation of bone formation and remodeling. However, subsequent studies utilizing mice backcrossed for 10 generations (F_{10}) to a pure C57B6 background, showed no significant differences in trabecular bone volume, bone formation, trabecular number, or any other measured parameter until 12 months of age. Examination of 12- and 18-month-old female animals demonstrated profound cortical and trabecular thinning, increased cortical tunneling, and biochemical evidence of increased bone resorption (Gagel *et al.*, 2007).

In addition, there was a profound remodeling of cortical bone into trabecular bone near the cortical surface. These results document a profound effect of CT deficiency in older, gonadal-insufficient animals.

Examination of skeletal metabolism in these same animals, bred for 10 generations against a black Swiss mouse background, demonstrated profound skeletal changes during lactation. CT/CGRP-alpha-deficient mice lose approximately twice as much bone mass during lactation and the time for these mice to normalize their bone mass is significantly greater than for the wild-type mice.

There are two other models that address this question. The first is a targeted deletion of the CTR (Dacquin *et al.*, 2004). Homozygous animals have an embryonic lethal phenotype-characterized profound edema of the developing embryo. This is analogous to the model of mouse embryos with targeted deletion of the CRLR, also an embryonic lethal situation caused by a failure of normal lymph system development (Dackor *et al.*, 2006). Studies in this model system performed in younger CTR +/− animals show no evidence of bone loss (Dacquin *et al.*, 2004).

Collectively, these results suggest an important role for calcitonin in the regulation of skeletal resorption and remodeling, particularly during periods of stress such as lactation, exposure to bone resorption stimulators, or gonadal steroid deficiency associated with aging. Experience in this model system identifies an important role of calcitonin in the prevention of cortical bone loss in older individuals. Extension of these observations to humans would suggest that the declining CT secretion seen in older patients may indeed have the physiological consequence of causing increased cortical bone loss. The apparent discrepancy between the CT/CGRP-alpha-deficient and the CTR heterozygous mice needs to be resolved by development of an osteoclast-specific deletion of the CTR.

Calcitonin may also have a protective role in osteoarthritis, a form of degenerative joint disease involving changes in subchondral bone, as well as cartilage. Calcitonin receptors have been identified on chondrocytes. Calcitonin has been demonstrated to increase cAMP levels in chondrocytes which, in turn, mediate the attenuation of the activity of matrix metalloproteinase (Sondergaard *et al.*, 2006; Karsdal *et al.*, 2007). With the findings that calcitonin administration decreases cartilage destruction in animal models, considerable attention has been directed to the potential therapeutic utility of calcitonin for osteoarthritis in humans (El Hajjaji *et al.*, 2004; Manicourt *et al.*, 2006; Sondergaard *et al.*, 2007). The results of ongoing studies are highly anticipated.

Calcitonin and Calcium and Phosphate Metabolism

Since Copp's initial report of the hypocalcemic effect of CT in animals, CT has been considered to have a physiological protective role against hypercalcemia by inhibiting osteoclast-mediated bone resorption and promoting renal calcium excretion (Azria *et al.*, 1995). Secretion of CT is directly related to elevation of extracellular calcium concentration as mentioned earlier (Deftos *et al.*, 1973). Acutely, CT administration decreases the serum calcium of laboratory animals. Although salmon CT administration to sham rats can lead to a significant, acute decrease in calcium levels, the hypocalcemic effect is exaggerated in ovariectomized rats, where the higher bone resorption state associated with estrogen deficiency leads to greater decreases in calcium concentrations (Davey and Morris, 2005).

In human studies, the effects of CT on serum calcium vary with the species from which the hormone was obtained, dosage, method of administration, and concurrent bone turnover rate of the human subject. The hypophosphatemic effect of CT is dose dependent and parallels the hypocalcemic effect. In studies of healthy persons, CT transiently lowers serum calcium and phosphate concentrations and raises urinary excretion of calcium; however, these changes are usually clinically irrelevant (Buclin *et al.*, 1987; Thamsborg *et al.*, 1990; Buclin *et al.*, 2002). In healthy humans and dogs, pharmacological doses of salmon CT result in hypocalcemia that is characterized by marked fluctuations, and paradoxical above-baseline increases of serum calcium; these patterns may be partly owing to parathyroid hormone overcompensation. Interestingly, serum calcium levels are normal in hypercalcitonemic patients with medullary thyroid cancer (MTC) (Deftos and Parthemore, 1974).

In patients with high bone turnover (e.g., Paget's disease of bone, immobilization, multiple myeloma, and hyperparathyroidism), CT exhibits greater hypocalcemic effects (Minkoff *et al.*, 1985). In myeloma patients, greater reduction of calcium levels is predictive for new lytic bone lesions with or without hypercalcemia, indicating the association with greater degrees of hypocalcemia during states of increased osteoclastic resorption (Bataille and Sany, 1982; Bataille *et al.*, 1985). In patients with hypercalcemia of malignancy, human calcitonin given intravenously will normalize calcium concentrations within the first 24 hours of administration, but a sustained effect was not noted after treatment was discontinued (Chevallier *et al.*, 1988). Additionally, hypocalcemia usually does not occur after CT administration in these patients.

There has been documentation of an interrelationship between CT and vitamin D metabolism. Calcitonin significantly increases the production of 1,25-dihydroxyvitamin D_3 in rats, likely through stimulation of 1α-hydroxylase activity in the proximal tubule (Kawashima *et al.*, 1981; Armbrecht *et al.*, 1987; Wongsurawat and Armbrecht, 1991). There is also evidence demonstrating an increase in the expression of the renal 1α-hydroxylase gene in normocalcemic rats by calcitonin (Shinki *et al.*, 1999). Furthermore, intestinal 24-hydroxylase enzymatic expression, which is required for the process of inactivating vitamin D_3 metabolites, is diminished by CT administration and contributes to increased 1,25-dihydroxyvitman D_3 activity (Beckman *et al.*, 1994).

Calcitonin and the Central Nervous System

Calcitonin has specific receptor-binding sites within the central nervous system and pituitary gland. Many studies have identified changes in the hypothalamic or pituitary system in response to CT administration. The physiological significance of these findings is still unclear; however, these studies, as well as others, do support other functions of CT besides regulation of bone metabolism and calcium balance. The intracerebral injection of CT suppresses food and water intake in rats (Chait *et al.*, 1995). The hormone also increases body temperature, acting on specific regions of the thalamus and hypothalamus (Sellami and de Beaurepaire, 1993). In the rat, intracerebral administration of salmon CT and rat CGRP decreases the frequency and amplitude of spontaneous growth hormone secretory pulses, which may have a role in regulating appetite (Tannenbaum and Goltzman, 1985). In humans, large doses of salmon CT reduce the serum concentrations of testosterone, LH, and FSH, probably acting at the hypothalamic level (Mulder, 1993). It has been shown that salmon CT and calcitonin-like pituitary peptide (pit-CT) are inhibitors of prolactin synthesis, secretion, and pituitary lactotroph proliferation (Shah *et al.*, 1999). A transgenic mouse model with overexpression of pit-CT demontrates hypoprolactinemia and, consequently, decreased fertility rates (Yuan *et al.*, 2005)

Calcitonin has been characterized to have analgesic effect on chronic bone pain secondary to osteoporosis, Paget's disease of bone, or malignant processes (Ziegler, 1984; Singer, 1991; Ofluoglu *et al.*, 2007). Other than through the effects on bone resorption, the antinociceptive activity of CT may be mediated, in part, via central cholinergic or serotonergic influences (Colado *et al.*, 1994; Chen and Lee, 1995). Reduced nociception appears to rely on the presence of spinal serotonin receptors, which are reduced in ovariectomized rats but normal in ovariectomized rats treated with calcitonin (Yoshimura, 2000; Yoshimura *et al.*, 2001; Furue *et al.*, 2005). Additionally, salmon CT potentiates the analgesic effectiveness of opioid agonists, especially delta- and kappa-agonists, and of antidepressants (Goicoechea *et al.*, 1999; Ormazabal *et al.*, 2001).

In a recent study, subcutaneous administration of salmon CT for 2 weeks to women with postmenopausal osteoporosis significantly increased plasma beta-endorphin levels and improved pain and quality-of-life scores (Ofluoglu *et al.*, 2007). This is contrasted to the findings of another study of osteoporotic women treated with intranasal calcitonin, where no reduction of chronic back pain intensity was demonstrated (Papadokostakis *et al.*, 2006). A recent meta-analysis evaluating the effectiveness of CT (via intramuscular, intranasal, or rectal routes) compared with placebo for the treatment of acute pain in patients with recent osteoporotic vertebral compression fractures concluded that there was clear benefit imparted by CT with respect to pain relief and earlier mobilization (Knopp *et al.*, 2005). Epidural CT was found to provide greater postoperative analgesia in patients undergoing total hip arthroplasty in comparison with epidural opioid (Gabopoulou *et al.*, 2002). Salmon CT has been reported to relieve postherpetic neuralgia when given intravenously to an elderly man who did not respond to other pharmacotherapies (Visser and Kwei, 2006).

Calcitonin and the Respiratory System

The total amount of immunoreactive CT in normal lungs exceeds that of any tissue of the human body, including the thyroid gland (Becker *et al.*, 1979). The hormone is found within pulmonary neuroendocrine (PNE) cells, which are situated near the basement membrane and often extend to the lumen of the airway extending from the nasal respiratory epithelium, laryngeal mucosa, and throughout the respiratory tract from the trachea to terminal airways (Becker *et al.*, 1980; Weichselbaum *et al.*, 2005). Hamsters exposed to noxious stimuli, such as cigarette smoke or diethylnitrosamine, have demonstrated increased levels of immunoreactive CT in the serum and lung tissue (Linnoila *et al.*, 1984; Tabassian *et al.*, 1989). These findings and the presence of the great number of PNE cells in the fetus and newborn suggest that CT may play a role in pulmonary maturation and pathophysiology. Immunoreactive calcitonin has been found in high concentrations in sputum and lung tissue from cystic fibrosis patients, which may represent either an underlying pathological effect of cystic fibrosis or an inflammatory response to the infectious process present in lung tissue from cystic fibrosis patients (Wolf *et al.*, 1986) Patients with lung cancer of all histological subtypes have demonstrated elevated serum CT concentrations, with extensive small-cell lung carcinoma being associated with the highest levels (Krauss *et al.*, 1981). However, other than in the case of medullary thyroid carcinoma, serum CT is not used routinely as a tumor marker. Recently, more evidence has come to support neuropeptides, such as CGRP, as being important markers and mediators of pulmonary disorders and systemic inflammation, as will be discussed later in this chapter.

Calcitonin and the Gastrointestinal System

Although CGRP-α has become recognized as an important modulator of intestinal function and pathology (discussed later in this chapter), CT was initially noted to have gastrointestinal effects, as well (Lenz *et al.*, 1985). In humans, pharmacological doses of CT increase gastric acid and pepsin secretion, decrease pancreatic amylase and pancreatic polypeptide production, and increase small intestinal motility (Demol *et al*., 1986). Additionally, serum motilin and gastric inhibitory peptide levels are decreased. Somatostatin levels in the serum are increased. The small intestinal secretion of potassium, sodium chloride, and water is augmented. Thus, at high concentrations, CT increases the net secretion of water and electrolytes from the human jejunum and ileum while shortening transit time; it has been postulated that these effects may contribute to the diarrhea seen in some patients with medullary thyroid cancer (Demol *et al*., 1986).

Calcitonin and the Reproductive System

Calcitonin expression occurs in both the mammalian uterus and placenta (Balabanova *et al*., 1987). Calcitonin is present in human, monkey, and rat endometria during the luteal stage of the menstrual cycle (Ding *et al*., 1994; Kumar *et al*., 1998; Diao *et al*., 2002). This period of increased CT expression overlaps with that which is considered the window of uterine receptivity for embryonic implantation, supporting the use of calcitonin as a biomarker of receptivity (Giudice, 1999). In the rat, CT messenger RNA is present in the glandular epithelial cells of the uterus at the time of implantation and may have a regulatory role in early pregnancy after implantation of the embryo (Ding *et al*., 1994). Uterine CT expression is stimulated by progesterone. Furthermore, administration of mifepristone, an antiprogestin drug, decreases CT expression in the uterus and inhibits implantation. Early uterine expression of CT may also be important in preimplantantion embryonic development because calcitonin has been shown to accelerate blastocyst differentiation through elevation of cytosolic ionized calcium concentration (Wang *et al*., 1998; Armant *et al*., 2000). Recently, it was reported that endometrial cell production of tissue transglutaminase type II (tTGase), an enzyme that catalyzes calcium-dependent protein cross-linking, is upregulated upon exposure to progesterone and CT; therefore, tTGase likely represents a downstream target of CT (Li *et al*., 2006).

Calcitonin receptors are present in the human placenta within the syncytiotrophoblast brush border that faces the mother and in the basal plasma membrane that faces the fetus (Lafond *et al*., 1994). The intraplacental presence of CT receptors and immunoreactive CT suggests a role of the CT-CTR system in implantation, regulation of placental function, and/or mineral metabolism during fetal life. A recent study of CT/CGRP-alpha null mice evaluated whether CT and/or CGRP-alpha are required for placental calcium transfer or fetal mineral regulation (McDonald *et al*., 2004). Interestingly, fetal CT/CGRP-alpha null mice demonstrated reduced levels of serum and skeletal magnesium levels. In contrast, there was no disruption of serum ionized calcium concentration, skeletal calcium content, or placental transfer of calcium in these mice. Another finding from this study that highlights the physiological importance of CT is that CT/CGRP-alpha null mice had significantly fewer viable fetuses *in utero* compared with heterozygous CT/CGRP-alpha (+/−) and wild-type mothers; however, viable, developmentally normal pups have been shown to be born to CT/CGRP-alpha null mice (Hoff *et al*., 2002; McDonald *et al*., 2004).

Human breast milk from women who have a history of total thyroidectomies contains large amounts of immunoreactive CT, indicating independent production of CT from the mammary gland (Bucht *et al*., 1986). Calcitonin expression and secretion is identified in mouse mammary glands during pregnancy and is progesterone-dependent; CTR expression is not affected by progesterone (Ismail *et al*., 2004). Calcitonin receptors localized in rat mammary glands are expressed in varying degrees depending on the reproductive status: slightly increased during pregnancy and markedly increased during lactation (Ishii *et al*., 2006). Ishii and colleagues noted that CT inhibited thymidine incorporation into DNA in lactating mammary gland cells. They hypothesized that CTR upregulation during lactation mediates CT's regulation of DNA synthesis to prevent mammary gland cell proliferation. Additionally, the possible role of CT as a modulator of mineral and electrolyte concentrations of milk merits study.

Immunoreactive CT production by breast cancer was first reported by Coombes and colleagues (1975). Although suggested as a potential tumor marker in breast cancer, calcitonin later was found to have very little correlation to stage of disease (Bezwoda *et al*., 1981). In fact, it has been reported recently that there is decreased expression of CTR mRNA in breast cancer cells, particularly with invasive tumors, as compared with matched cancers and unaffected ductal epithelia from the same patients (Wang *et al*., 2004). A follow-up study by the same investigators found that CT administration inhibited breast cancer cell invasiveness (Han *et al*., 2006).

Calcitonin and CTR have been localized to human prostate neuroendocrine cells (Davis *et al*., 1989; Shah *et al*., 1992; Wu *et al*., 1996). Significantly greater levels of CT are found in hyperplastic prostates than in normal specimens (Davis *et al*., 1989). The presence of the CT-CTR system in prostatic tissue indicates a possible role in regulation of prostatic growth. In fact, small-cell carcinoma of the prostate, a rare histological subtype of prostate cancer involving neuroendocrine cells, is associated with increased serum CT concentrations (Sim *et al*., 1996). Calcitonin appears to be involved in the regulation of prostate cancer cell growth as supported by the induction of DNA synthesis and cell proliferation by exogenously administered CT to prostate cell cultures and by the presence of high CT and CTR expression in prostate cancer cells (Chien *et al*., 2001). Further evaluation supports the proliferative effects of CT as the downregulation of CT expression in prostate cancer cell lines leads to diminished oncogenicity, as expressed by cellular growth rates and invasiveness (Thomas *et al.*, 2006).

Calcitonin and the Renal System

The primary action CT has in the human kidney is the enhancement of calcium excretion through inhibition of tubular calcium resorption (Ardaillou *et al.*, 1969; Cochran *et al.*, 1970). In humans, intravenous administration of CT stimulates diuresis and fractional excretion rates of calcium, phosphate, sodium, chloride, magnesium, and potassium (Gnaedinger *et al.*, 1989). However, in some species (e.g., rat), CT stimulates the renal tubular reabsorption of calcium and magnesium, probably within the thick ascending limb of the loop of Henle, and decreases urinary excretion of calcium and magnesium (Di Stefano *et al.*, 1985; Carney, 1992).

The kidney is a principal site of CT degradation (Hysing *et al.*, 1991). Serum CT concentrations are elevated in chronic renal failure; however, postdialysis levels of CT are unchanged or increased when compared with predialysis levels, indicating that some mechanism other than decreased renal clearance contributes to hypercalcitonemia in renal failure patients (Niccoli *et al.*, 1995). In fact, Messa and colleagues demonstrated in patients with varying degrees of renal failure compared with normal controls that CT concentrations varied in response to fluctuations of serum calcium levels, indicating that variable CT secretion by thyroid C cells occurs rather than variable renal excretion (Messa *et al.*, 1995).

Renal effects after binding of CT to CTR are mediated through the activation of protein kinase or cAMP pathways, with selection of a specific pathway occurring in a cell cycle-dependent manner (Chakraborty *et al.*, 1991). Besides regulating renal absorption of calcium, CT is a natriuretic; it decreases sodium reabsorption via effects on the Na/K ATPase and Na/H exchanger in the renal proximal tubule in a cell cycle-dependent manner (Chakraborty *et al.*, 1994).

CLINICAL CONDITIONS WITH INCREASED PRODUCTION OF CALCITONIN PRECURSORS

Humans normally produce measurable, albeit low, levels of procalcitonin (ProCT), amino-terminal peptide (nProCT), conjoined calcitonin-calcitonin carboxyl-terminal peptide (CT:CCP-I), free CCP-I, and mature CT (Snider *et al.*, 1997) (Fig. 6). Collectively, we refer to the component peptides as calcitonin precursors (CTpr). Moreover, in several pathological states (e.g., hyperplasia or malignancy, and states of inflammation or infection), CTpr biosynthesis and secretion are augmented whereas mature CT levels are normal or minimally elevated owing to incomplete posttranslational processing.

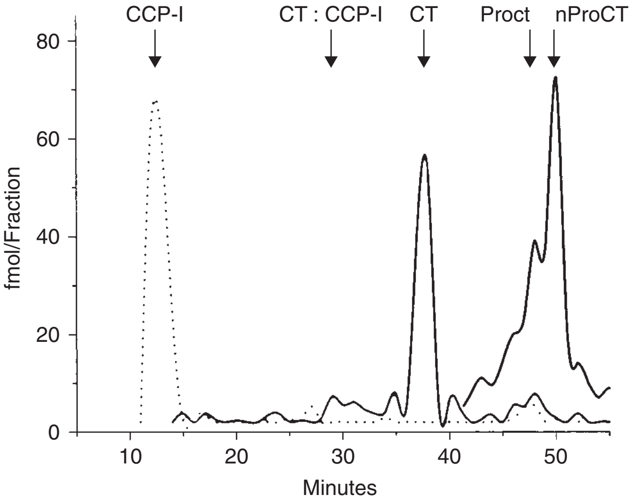

FIGURE 6 Procalcitonin and its component peptides in normal human serum. CT radioimmunoassay (thick line), CCP-I radioimmunoassay (dashed black line), and nProCT radioimmunoassay (thin line). Modified from Snider *et al.* (1997).

Extrathyroidal Neuroendocrine Cell Hyperplasia or Tumors

In addition to C-cell hyperplasia of the thyroid, extrathyroidal neuroendocrine cell hyperplasia is associated with increased serum iCT levels. The study of some chronic nonneoplastic pulmonary conditions with region-specific antisera has demonstrated slight to moderate increases of serum iCT associated with an apparent increase of CT precursors (most prominently, procalcitonin); whereas, levels of free mature CT usually remain within the normal range. These conditions include patients with chronic bronchitis, chronic obstructive pulmonary disease, and chronic pulmonary tuberculosis (Becker *et al.*, 1981; Nylen *et al.*, 1996). Immunoreactive CT-containing PNE cells demonstrate hyperplasia in some chronic lung diseases (Becker *et al.*, 1980; Linnoila *et al.*, 1984). Additionally, increased levels of pro-CT may be related to underlying infections associated with these pulmonary processes. This will be described further in the next section. Insufficient studies have been performed to fully characterize the nature and distribution of the precursor forms secreted in extrathyroidal neuroendocrine cell hyperplasia.

Serum mature CT has proven to be a very important clinical marker to detect and follow the course of medullary thyroid carcinoma (MTC), the neoplasm of the thyroid C cells. In addition, the use of region-specific antisera and separatory techniques has demonstrated that MTC secretes large amounts of high-molecular-weight forms of the hormone (i.e., pro-CT and CCP-II) (Becker *et al.*, 1978; Minvielle *et al.*, 1991) (Fig. 7).

Small-cell lung cancer and bronchial carcinoids, tumors involving pulmonary neuroendocrine cells, can produce large concentrations of iCT, consisting primarily

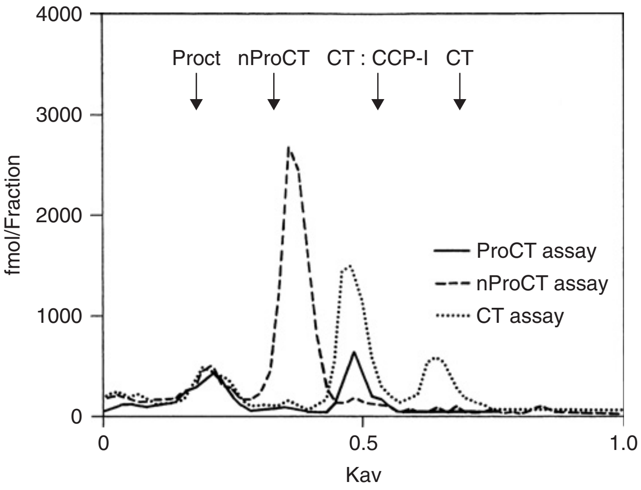

FIGURE 7 Gel filtration of serum of MTC patient. Note that the peak corresponding to the elution position of the CT:CCP-I peptide does not cross-react equally in both CT and ProCT assays. This may be due to the presence of some CT dimer (Tobler *et al.,* 1983), as it has a similar molecular mass.

of CT precursors (Cate *et al.*, 1986). The ratio of levels of high-molecular-weight precursors to mature CT is usually much greater in bronchogenic tumors than MTC (Becker *et al.*, 1978; Bertagna *et al.*, 1978). Other neuroendocrine tumors have been reported to produce iCT *in vitro* and *in vivo* (e.g., pheochromocytoma, neuroendocrine carcinoma of prostate cancer, neuroendoocrine carcinoma of the larynx, carcinoid tumors [Mascolo *et al.*, 2005], and pancreatic neuroendocrine tumor) (Deftos, 1998; Chung *et al.*, 2004; Van den Eynden *et al.*, 2007). Although excessive amounts of immunoreactive CT have been detected in the sera of patients with non-neuroendocrine tumors (e.g., breast cancer), there are few data specifically regarding levels of serum or tissue CTpr in the literature (Coombes *et al.*, 1975).

Severe Inflammation, Infection, and Sepsis

In the United States, sepsis afflicts more than 750,000 persons annually and the in-hospital mortality rate ranges from 29% to 50% (Weycker *et al.*, 2003). Marked insults, such as burns, trauma, surgical procedures, pancreatitis, or bacterial infections, commonly induce a hypersecretion of various proinflammatory cytokines, arachidonic acid metabolites, and other humoral substances that cause a syndrome of systemic inflammation: vasodilation, chemoattraction of hematopoietic cells, activation of macrophages, and capillary endothelial leakage. The appellation of "sepsis" is given to this clinical condition when it is attributable to a microbial source (Bone, 1995). In severe cases, this may lead to "multiple-organ dysfunction" characterized by myocardial insufficiency, circulatory hypoperfusion, hypoxemia, cerebral obtundation, renal failure, coagulopathy, and shock. Essentially, the patient becomes severely ill primarily because of a hyperresponsivity of the humoral reaction to the initial injury.

Over the past two decades, studies have indicated that serum concentrations of CTpr, specifically ProCT, are increased markedly in severe inflammation and are often associated with microbial infections (Assicot *et al.*, 1993; Tang *et al.*, 2007). In sepsis, circulating levels may be increased hundreds- to thousands-fold. Importantly, because of incomplete processing, there is very little or no elevation of serum mature CT, as seen in cases of tumors associated with increased CT production described earlier. In fact, it was recommended that ProCT be included into the international definition of sepsis because it may differentiate sepsis from noninfectious causes of systemic inflammatory response syndrome (SIRS) (Levy *et al.*, 2003). Procalcitonin has been identified as a useful marker of sepsis in various populations or injuries: pediatrics (Assicot *et al.*, 1993), emergency department (Hausfater *et al.*, 2002), ventilator-associated pneumonia (Duflo, 2002), acute respiratory distress syndrome (Brunkhorst, *et al.*, 1999), burn injury (Carsin *et al.*, 1997), post-cardiac surgery (Aouifi *et al.*, 2000), bacterial meningitis (Viallon *et al.*, 1999), secondary peritonitis (Rau *et al.*, 2007), and acute pancreatitis (Rau *et al.*, 2007). However, a recently published meta-analysis evaluating the value of ProCT in critical care settings found that ProCT cannot differentiate sepsis from noninfectious causes of SIRS, limiting its generalized use (Tang *et al.*, 2007). The conclusion of this review should not be applied to the utility of ProCT measurements in identifying sepsis in noncritical care settings or in specific disease processes.

The most proximal stimulus to increased CTpr in sepsis may be endotoxin, a product of gram-negative bacteria. Endotoxin administration to healthy human individuals has been shown to increase tumor necrosis factor-alpha (TNF-alpha) and other cytokines within hours of exposure, correlating with a rise of procalcitonin concentration plateauing at 24 hours and remaining high for more than 7 days after exposure (Dandona *et al.*, 1994; Preas *et al.*, 2001). Gram-negative bacteremia or translocation of *Escherichia coli* (or its endotoxin), normal intestinal flora, from the gut across the bowel wall into the bloodstream may be initial sources of endotoxin to initiate the inflammatory pathway. In the specific case of burn injury, which is highly associated with sepsis and mortality, high levels of endotoxin and cytokines [e.g., TNF-alpha and interleukin-6 (IL-6)] have been found (Guo *et al.*, 1990; Yamada *et al.*, 1996). This may be attributable to increased gut permeability demonstrated within 48 hours of injury causing translocation of intestinal bacteria (Ryan *et al.*, 1992). However, a study of 40 patients with thermal burn injuries found that ProCT and IL-6 levels increased acutely, correlating significantly with severity of skin burn, without any evidence of infection nor increase in endotoxin or TNF-alpha concentrations (Carsin *et al.*, 1997). Thus, increased levels of ProCT may not be related directly to an infectious source or endotoxin exposure in burn injury. Sepsis induced by gram-positive bacteria also has been reported to increase circulating ProCT levels (Feezor *et al.*, 2003). Thus in the presence of contradictory reports, the stimulus for increased ProCT production in sepsis or inflammatory disorders is still unknown.

The source of ProCT in sepsis or systemic inflammation has been studied in animal models and humans. Studies in septic hamsters, pigs, and humans have revealed detectable CT mRNA not only in the thyroid gland, but also in all extrathyroidal tissues studied (brain, gut, kidney, liver, lung, testes, muscle, etc.) (Muller *et al.*, 2001). In contrast, in control animals, CT mRNA is detectable only in thyroid (i.e., C cells) and lung (i.e., PNE cells). In septic humans and animals, *in situ* hybridization studies have demonstrated that multiple cell types within these organs and tissues are involved. Thus, in sepsis, the entire organism is transformed into an endocrine gland (Muller *et al.*, 2001). A similar phenomenon has been found for the expression of CGRP and AM, two other peptides of the CALC gene family (Domenech *et al.*, 2001). It appears that increased gene transcription is induced by microbial infection-specific sepsis response elements in the gene promotor. Thus, these findings indicate that calcitonin gene products can follow either a classical hormonal expression or, alternatively, a cytokine-like expression pathway. In this regard, these gene products are a prototype of "hormokine" mediators (Domenech *et al.*, 2001; Muller *et al.*, 2001). Recent studies have indicated that liver and adipose tissue may be significant sources of ProCT and other calcitonin gene products (Meisner *et al.*, 2003; Linscheid *et al.*, 2005).

The correlation between serum CTpr levels and the severity of systemic inflammation/infection/sepsis suggests that one of these peptides, in particular, ProCT, might be contributing to the morbidity and mortality of the host. Indeed, injection of ProCT into septic hamsters was found to increase mortality greatly, and immunoneutralization of ProCT with antisera raised to different regions of the molecule improved the clinical course of experimentally induced sepsis markedly (i.e., severe peritonitis) in hamsters and pigs (Nylen *et al.*, 1998; Wagner *et al.*, 2002). In contrast, Hoffman and colleagues postulated that the increased production of ProCT seen in sepsis or shock, rather than being directly toxic, may reflect a counterregulatory mechanism to limit the release of nitric oxide, a mediator of vasodilation and cell death typical in septic shock (Hoffmann *et al.*, 2001). The authors demonstrated in an *in vitro* cell culture line that ProCT inhibited (in a dose-dependent manner) TNF-alpha and interferon-gamma stimulation of inducible nitric oxide synthase gene expression, which is stimulated in sepsis and septic shock (Hoffmann *et al.*, 2001). At this time, the function of ProCT in sepsis is unclear and further studies are required in this area.

Calcitonin Gene-Related Peptides Alpha And Beta

The CALC-I gene encodes either CT or CGRP-alpha by tissue-specific alternative processing of the primary RNA transcript (see Fig. 1) (Amara *et al.*, 1982). CGRP-alpha (also termed CGRP-I; molecular mass, 3789 Da) is a 37-aminoacid peptide. In humans, CGRP-beta (or CGRP-II; molecular mass, 3794), the product of the CALC-II gene, differs from CGRP-alpha in three of the 37 amino acids (Steenbergh *et al.*, 1985). There is no alternative processing of the RNA transcript to produce CGRP-beta. As is the case for the structure of CT, both types of CGRP have an amidated carboxy1 terminus and a disulfide bridge at the amino terminus. The first N-terminal residues (1–7) are necessary for receptor activation, and loss of the disulfide bridge linking amino acids 2 and 7 will eliminate the biological activity of CGRP (Tippins *et al.*, 1986; Maggi *et al.*, 1990). In fact, CGRP(8–37), where the first seven N-terminal amino acids are removed, is a CGRP-receptor antagonist with high affinity (Chiba *et al.*, 1989). Additionally, the amidated carboxyl terminus is important for CGRP binding to its receptor (Banerjee *et al.*, 2006).

CGRPs are neuropeptides; they are found mostly in the central and peripheral nervous systems (e.g., brain, ganglia, spinal cord, and peripheral nerves) and the cardiovascular system. They also are located in C cells of the thyroid and in neuroendocrine cells of the respiratory tract, where often they coexist with CT. Serum levels of CGRPs are detectable in normal persons and may arise in the setting of hypercalcemia, but this response does not seem to be physiologically relevant. Both CGRPs also have been identified in cerebrospinal and joint fluid (Wimalawansa, 1996). CGRPs have been located in a variety of tumors: medullary thyroid carcinoma, bronchogenic lung cancer, carcinoid tumor, pheochromocytoma, insulinoma, prostate cancer, parathyroid adenoma, and promyelocytic leukemia (Wimalawansa, 1996).

The two CGRPs exhibit nearly identical biological activities; thus, they will be collectively referred to as CGRP for the remainder of this discussion. CGRP is an extremely potent endogenous vasodilator and can reduce blood pressure. This last function is supported by the finding that the CT/CGRP gene knockout mouse model has elevated baseline blood pressure (Gangula *et al.*, 2000). Involved in the regulation of gastric vascularity, gastric acid secretion, and intestinal motility, CGRP may maintain gastric mucosal integrity (Holzer, 1998; Gyires, 2004). In fact, mice lacking CGRP expression developed adult-onset colitis (Thompson *et al.*, 2007). The hormone may play a role in various pulmonary pathophysiological settings: bronchial asthma or chronic obstructive pulmonary disorder (Groneberg *et al.*, 2004; Springer *et al.*, 2004), and fetal and neonatal lung disease (Johnson *et al.*, 1988). Serum CGRP is increased slightly in sepsis, which may be a counter-regulatory mechanism to inhibit the process of local acute inflammation (Gomes *et al*., 2005). Much attention has focused recently on the association of CGRP with migraine attacks, leading to the study of CGRP antagonists in the treatment of migraines (Edvinsson, 2001; Lassen *et al*., 2002; Olesen *et al*., 2004).

CGRP may cause a slight diminution of bone resorption, the physiological relevance of which is unknown (Owan and Ibaraki, 1994). The hormone inhibits osteoclast bone resorption by inducing quiescence (Zaidi *et al*., 1990). This inhibition is mediated in part via cAMP (Akopian *et al*., 2000). Additionally, CGRP has been shown to have direct proliferative effects on osteoblast cells (Villa *et al*., 2006). CGRP increases the number of bone colonies in cultured rat bone marrow (Bernard and Shih, 1990). Bone tissue contains CGRP-immunoreactive nerves, which increase in concentration at times of bone remodeling and fracture healing (Hukkanen *et al*., 1993; Li *et al*., 2007). Recently, Villa and colleagues demonstrated that CGRP reduces the release of osteoprotegerin (OPG) by osteoblasts, and, consequently, modulates the OPG/RANKL/RANK system to favor osteoclastogenesis and bone resorption (Villa *et al*., 2006). The overall weakly anabolic activity of CGRP may result from a balance of stimulatory actions on both bone formation and resorption. It is clear that CGRP is a neuromodulator with a broad spectrum of biological effects, mediated by receptors that are distributed widely throughout the body.

AMYLIN

Amylin, encoded by the CALC-IV gene located on chromosome 12, is a 37-amino-acid peptide (molecular mass, 3903 Da) that was identified in amyloid deposits in an endocrine pancreatic tumor and in patients with type 2 diabetes mellitus (Cooper *et al*., 1987). Amylin, which is co-secreted with insulin from pancreatic beta cells in response to similar stimuli (e.g., meals, glucose load), essentially delays glucose entry into circulation (i.e., inhibits post-prandial glucagon secretion, delays gastric emptying, and confers early satiety). Serum levels of amylin are elevated in some patients with insulin resistance, obesity, and hypertension. High concentrations of this peptide lead to amylin aggregation and amyloidosis, which has been shown to be cytotoxic to pancreatic beta cells and may be the pathogenic basis in the development of type 2 diabetes (Zhang *et al*., 2003).

In the kidney, the hormone stimulates plasma renin secretion (Cooper *et al*., 1995). Amylin promotes renal sodium excretion by acting on sites within the brain (Mathai *et al*., 2005). Similar to CGRP, amylin has vasodilating and anti-inflammatory properties (Clementi *et al*., 1995). Amylin also has been localized to the gut (primarily in the pyloric antrum), nerves in the dorsal root ganglia (co-localized with CGRP), and osteoblasts (Gilbey *et al*., 1991; Miyazato *et al*., 1991; Mulder *et al*., 1995).

Amylin affects bone and mineral metabolism by inhibiting osteoclastic bone resorption, and hence, leading to hypocalcemia (Datta *et al*., 1989). Amylin causes hypocalcemia in patients with Paget's disease of bone (Wimalawansa *et al*., 1992). Amylin inhibits bone resorption by inhibiting osteoclasts motility (Alam *et al*., 1993). Amylin-knock-out mice models experience bone loss owing to uncontrolled bone resorption (Lerner, 2006). Interestingly, amylin also stimulates rat osteoblast proliferation *in vitro* and increases mineralized bone volume in mice *in vivo* (Cornish *et al*., 1995). Bone loss in the setting of estrogen deficiency was salvaged with amylin administration to ovariectomized rats (Horcajada-Molteni *et al*., 2000).

ADRENOMEDULLIN

Adrenomedullin is a bioactive peptide originally isolated from human pheochromocytoma tissue (Kitamura *et al*., 1993) (see Fig. 2). This 52-amino-acid peptide (molecular mass, 6029 Da) is also amidated at the carboxy1 terminus and possesses an intramolecular disulfide bridge between cysteine residues at positions 16 and 21, thus, forming a six-residue ring structure (Ishimitsu *et al*., 1994). These characteristics, plus its moderate amino acid homology to CGRP and a slight amino acid homology to amylin, suggest that it belongs to the calcitonin gene family of peptides (Wimalawansa, 1997). As is the case for CALC-I, -II, and -III genes, its encoding gene (CALC-V) is found on human chromosome 11. In contrast to CT, CGRP, and amylin, which possess a disulfide ring structure at or near the amino terminus, adrenomedullin contains an additional 15 amino acid residues situated on the amino terminus side of its disulfide ring. This preceding peptide segment is not required for hormonal bioactivity; biological activity depends on the terminal 40 amino acids (Brain and Grant, 2004). On the other hand, within the precursor pro-adrenomedullin molecule (185 amino acids), there is an N-terminal 20-residue peptide segment that may be biologically active through a mechanism distinct from that of adrenomedullin (Shimosawa *et al*., 1995).

Adrenomedullin is found normally in adrenal medulla, heart, lung, kidney, pancreas, intestine, vascular smooth muscle, and plasma (Hinson *et al*., 2000). The hormone activates adenylate cyclase through a G protein-coupled mechanism and mobilizes intracellular Ca^{2+}. Various pharmacological stimulants of its secretion include cortisol, aldosterone, interleukin-1 alpha and beta, TNF-alpha and -beta, and lipopolysaccharide (Sugo *et al*., 1995).

The primary physiological effect adrenomedullin is vasodilation. Adrenomedullin has been shown to increase pulmonary blood flow, reduce mean blood pressure owing to the decrease in peripheral vascular resistance, and increase blood flow in other areas of the body (e.g., heart, retina, and skin)

(Kitamura *et al*., 1993; Lippton *et al*., 1994; Ueda *et al*., 2005; Hasbak *et al*., 2006). It appears that part of the vasodilatory effect is mediated by indirect induction of nitric oxide synthesis (Hattori *et al*., 1999). Other actions include bronchodilation, modulation of hypothalamic-pituitary-adrenal axis, and inhibition of angiotensin-induced aldosterone release (Kanazawa *et al*., 1994; Yamaguchi *et al*., 1995; Shan *et al*., 2003). In the brain, adrenomedullin exerts vasorelaxant and antidipsogenic effects and may function as a neurotransmitter-neuromodulator (Murphy and Samson, 1995). In transgenic mice that overproduce adrenomedullin, adrenomedullin will promote vasodilation, vascular regeneration and neurogenesis after induced-ischemic brain injury, supporting investigation of this hormone in the management of ischemic brain (Miyashita *et al*., 2006).

Increased concentrations of adrenomedullin are seen in cardiovascular diseases (heart failure, hypertension), renal failure, and sepsis. Given the vasodilatory activity of this hormone, it can be surmised that increased adrenomedullin production may be a compensatory process in these conditions. In fact, as was seen with ischemic brain injury, adrenomedullin may have potential therapeutic or modulating properties in other disorders: pulmonary hypertension (Qi *et al*., 2007), diabetic retinopathy (Kaneko *et al*., 2006), heart failure (Nishikimi and Matsuoka, 2005), and ischemic heart disease.

In both *in vivo* and *in vitro* studies, adrenomedullin has been found to be a potent stimulator of osteoblasts (Cornish *et al*., 1997). Adult mice injected with adrenomedullin into the calvariae demonstrated two- to threefold increase in bone formation. Adrenomedullin may be a local regulatory of bone metabolism as osteoblasts express both adrenomedullin peptides and receptors (Naot *et al*., 2001). Indeed, administration of the bioactive portion of adrenomedullin(27–52) to adult male mice led to significant increases in cortical width (21%), trabecular bone volume (45%), and bone strength (Cornish *et al*., 2001). Growth plate thickness also was shown to increase after exposure to adrenomedullin, indicating that chondrocytes may be an additional target. Insulin-like growth factor-1 (IGF-1) receptors on osteoblasts appear necessary to mediate the effects of adrenomedullin, as well as amylin, in the bone (Cornish *et al*., 2004). The study of effect of adrenomedullin on bone development has been difficult to conduct because adrenomedullin knockout mice die *in utero* (Caron and Smithies, 2001). To date, the effect of the hormone on the osteoclast, if any, is unknown.

NOVEL CALCITONIN GENE FAMILY PEPTIDES

A few novel peptides recently have been identified to belong to the calcitonin gene family of peptides: calcitonin receptor-stimulating peptide types-1, -2, and -3 (CRSP-1, CRSP-2, and CRSP-3) and intermedin (IMD, also referred to as adrenomedullin-2) (Katafuchi *et al*., 2003a and 2003b; Roh *et al*., 2004; Takei *et al*., 2004). All subtypes of CRSP were first isolated from porcine brain tissue and found to have approximately 60% amino acid sequence homology to human CGRP-alpha and CGRP-beta. CRSPs are expressed primarily in the thyroid, pituitary, and central nervous system, with only a few sites showing variable subtype expression (Katafuchi *et al*., 2003a). Despite the structural similarity to CGRP, CRSP-1 did not lead to hypotension when administered to rats; however, a transient reduction of plasma calcium concentration occurred (Katafuchi *et al*., 2003b). Besides additional evidence indicating that exogenously administered CRSP-1 may affect renal ion (sodium and calcium) transport and renal cell growth (Hamano *et al*., 2005) and energy homeostasis (Sawada *et al*., 2006), CRSP-1 inhibited the formation of multinucleated osteoclast-like cells in a dose-dependent manner and destroyed the actin ring associated with osteoclast resorptive activity (Notoya *et al*., 2007). CRSP has not been identified in humans or rodents and its biological function remains unclear (Katafuchi and Minamino, 2004).

Intermedin, a 47-amino-acid peptide with a disulfide bridge at the amino terminus, has been localized to submaxillary gland, kidney, stomach, uterus and ovary, lymphoid tissues, and pancreas (Takei *et al*., 2004). Biological activities of intermedin include: vasodilation (of pulmonary, mesenteric, and renal vascular beds) (Burak Kandilci *et al*., 2006; Chauhan *et al*., 2007); suppression of gastric emptying and appetite (Roh *et al*., 2004); and stimulation of plasma renin, aldosterone, and atrial and brain natriuretic peptide levels (Charles *et al*., 2006). Intermedin has been found to inhibit pituitary release of growth hormone mediated by inhibition of growth hormone-releasing hormone (GHRH) effects, an activity not seen with the other members of the CT gene family of peptides (Taylor *et al*., 2006). Effects on bone or mineral metabolism have yet to be reported in the literature.

RECEPTORS OF THE PEPTIDES OF THE CALCITONIN GENE FAMILY

Bioactivity of the peptides of the CT gene family is exerted by binding to their receptors. These are seven-transmembrane-domain G protein-coupled receptors, in which guanidine nucleotide guanosine triphosphate facilitates the actions of the peptide by binding to specific mediator proteins. There are two subgroups of receptors for the CT gene family: CT receptor (CTR) and CT receptor-like receptor (CRLR) (Lin *et al*., 1991; McLatchie *et al*., 1998). Interestingly, CRLRs were not identified until it was recognized that a member of another novel family of single-transmembrane-domain proteins, called receptor-activity-modifying protein (RAMP), must also be expressed to transport the CRLR to the cell membrane and to convey ligand specificity (McLatchie *et al*., 1998). Each of the CT gene family of peptides binds with variable affinities to these receptors. Consequently, some of their bioeffects overlap. Calcitonin is unique in that it alone can activate a specific G protein receptor in the absence of a RAMP. The RAMP together with either the calcitonin receptor (CTR) or calcitonin receptor-like receptor (CRLR) binds the other ligands of this family of peptides (see Table 2).

The complicated and unique overlapping of receptivity of CTRs and CRLRs owes to their multipotentiality when associated with various RAMPs. Three RAMPs (RAMP-1, -2, and -3) have been identified. The presence, concentration, and/or timing of one or more of the three accessory proteins determines the specific phenotype and ligand specificity of the receptor that is ultimately expressed on the cell surface (see Table 1) (McLatchie *et al*., 1998; Buhlmann *et al*., 1999; Muff *et al*., 1999; Leuthauser *et al*., 2000; Hay *et al*., 2004; Roh *et al*., 2004). This process has given rise to seven distinct, molecularly characterized receptors for CT, CGRP, amylin, and adrenomedullin (Lerner, 2006). Additionally, RAMPs modify gene expression of the CTR, transport CRLR to the plasma membrane, and assist with cellular recycling of receptor complexes (Bomberger *et al*., 2005). The profile of RAMP expression and activity is altered by the local milieu and is subject to humoral influences (Frayon *et al*., 2000). Modification of the CRLR also can modify its binding affinity to different peptides; the deletion of a sequence of the CRLR led to continued affinity for CGRP (in the presence of RAMP-1) but eliminated its binding to adrenomedullin (in the presence of RAMP-2) (Koller *et al*., 2004). This extraordinarily elegant and complex system allows for a diversification of receptor function, thus modulating the action of the CT gene products according to ambient needs.

CONCLUSION

In the early 1960s, CT was discovered as a hormone that had hypocalcemic effects but had an undetermined role in human physiology. Since then, it has been found to be only one peptide among a vast array of related circulating peptides comprising the CT gene family of peptides. A valid hypothesis for the function of CT, in part, made more than two decades ago, is that CT maintains bone mineral in emergency situations (i.e., to combat hypercalcemia) and may play a role in the conservation of body calcium stores in certain physiological but stressful states (i.e., growth, pregnancy, and lactation). However, it is clear from the growing body of literature that the CT gene family of peptides, as a whole, has many important physiological and pathological functions. Further investigations into this broad family of peptides should provide great insight into the physiology of the human in healthy and disease states.

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Amylin and Calcitonin Gene-Related Peptide

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INTRODUCTION

Calcitonin gene-related peptide (CGRP) and amylin are homologous 37 amino acid peptides. Their biological activities have been reviewed recently (Wimalawansa, 1997; Young, 2005a). The genes for amylin and CGRP have a common ancestral origin, and both peptides have a 6 amino acid ring structure at the N terminus created by a disulfide bond between cysteine residues at positions 2 and 7 (see Fig. 1). In addition, C termini are amidated. αCGRP is generated by alternative processing of mRNA from the calcitonin gene, located on the short arm of chromosome 11. This gene has six exons, the first four of which produce the mRNA for the precursor of calcitonin, preprocalcitonin. This is subsequently converted to calcitonin itself and N- and C-terminal flanking peptides. An alternative mRNA for the αCGRP precursor, preproCGRP, is formed from exons 1, 2, 3, 5, and 6. This peptide is cleaved to produce an N-terminal flanking peptide similar to that from procalcitonin and αCGRP itself, which is coded by exon 5. The alternative splicing of calcitonin mRNA is tissue specific – calcitonin mRNA is produced mainly in parafollicular cells of the thyroid, whereas in the nervous system, αCGRP mRNA is the predominant form. A second form of CGRP, βCGRP, differs from αCGRP by only one amino acid in the rat and three amino acids in the human (see Fig. 1). It is produced by a separate gene also on the short arm of chromosome 11, thought to have arisen as a result of exon duplication (Zaidi *et al.*, 1987a). Both CGRPs have approximately 20% homology with calcitonin, which is also amidated and contains a disulfide bridge.

Amylin has 43% sequence identity with αCGRP, 49% with βCGRP, and 13% with calcitonin in the human. It was originally isolated from amyloid deposits in the pancreases from patients with insulinoma or diabetes mellitus (Cooper *et al.*, 1987; Westermark *et al.*, 1987). Human amylin appears to be produced from a single gene on the short arm of chromosome 12, consisting of three exons. Like calcitonin and CGRP, amylin is synthesized as a prepropeptide, an 89 amino acid precursor that is subsequently processed to proamylin (67 amino acids) and thence to amylin itself. Amylin is the only known hormonal product of this gene.

Amylin is produced principally in β cells of the islets where its tissue content is less than 1% that of insulin. It is co-secreted with insulin, and evidence shows that insulin and amylin genes share transcriptional regulators (German *et al.*, 1992). Thus, hyperglycemia stimulates amylin secretion (O'Brien *et al.*, 1991), whereas hypoglycemia reduces it (Alam *et al.*, 1992). Destruction of the β cell with streptozotocin reduces amylin secretion (Ogawa *et al.*, 1990). The excursion in circulating insulin levels following a glucose challenge appears to be greater than that seen with amylin (Mitsukawa *et al.*, 1990). Amylin secretion, however, may be more sustained following glucose administration (Mitsukawa *et al.*, 1990). In some experimental models, dissociation of the secretion of the two peptides has been achieved but this does not commonly occur *in vivo*.

Amylin has also been detected in tissues of the gastrointestinal tract, with tissue concentrations about 1% of those in the pancreas being found in the pyloric antrum (Nakazato *et al.*, 1989). Amylin or its mRNA has also been found in lung, dorsal root ganglion, hypothalamus, neuroendocrine tumors, and in an osteoblast cell line (Gilbey *et al.*, 1991).

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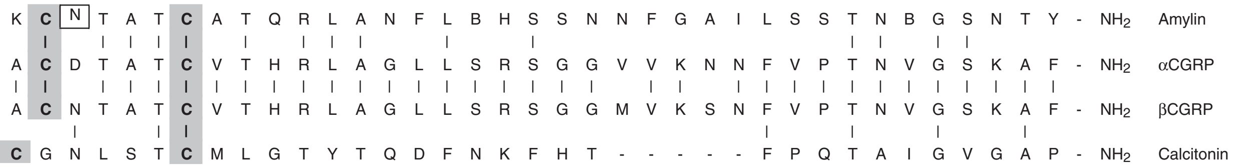
K C N T A T C A T Q R L A N F L B H S S N N F G A I L S S T N B G S N T Y - NH2 Amylin
A C D T A T C V T H R L A G L L S R S G G V V K N N F V P T N V G S K A F - NH2 αCGRP
A C N T A T C V T H R L A G L L S R S G G M V K S N F V P T N V G S K A F - NH2 βCGRP
C G N L S T C M L G T Y T Q D F N K F H T - - - - - F P Q T A I G V G A P - NH2 Calcitonin

FIGURE 1 Amino acid sequences of amylin, αCGRP, βCGRP, and calcitonin in humans. The cysteine residues connected by a disulfide bond in each peptide are shaded. Each peptide is amidated at its C terminus. Identical amino acids are indicated by vertical lines and squares.

EXTRASKELETAL ACTIONS OF AMYLIN AND CGRP

Fuel Metabolism

The effects of amylin and CGRP on fuel metabolism appear to be comparable. In skeletal muscle the primary effects appear to be inhibition of glycogen synthesis and stimulation of glycogenolysis. Thus, muscle glycogen stores are decreased by amylin or CGRP, and this is accompanied by diminished glucose uptake and increased glycolysis, leading to increased lactate production. In the liver, glucose output is increased, probably as a result of increased gluconeogenesis. This may be contributed to by the increased flow of lactate from skeletal muscle, but has also been observed in some studies of isolated hepatocytes, implying an additional direct effect of amylin on liver cell function. Thus, in both skeletal muscle and liver, amylin and CGRP act as noncompetitive antagonists of insulin and produce insulin resistance (Young *et al.*, 1992). Amylin does not influence adipocyte metabolism, however, so the insulin resistance it produces is selective and could lead to a redirection of fuel from carbohydrate storage in liver and muscle to triglyceride storage in adipose tissue.

As well as antagonizing the actions of insulin (Hettiarachchi *et al.*, 1997; Gebre-Medhin *et al.*, 1998), amylin also reduces its secretion (Furnsinn *et al.*, 1994), possibly by altering glucose sensing at the β cell (Wagoner *et al.*, 1993). Overexpression of the amylin gene results in the development of diabetes in transgenic mice (Verchere *et al.*, 1996), but paradoxically, an amylin analog (pramlintide) reduces postprandial blood glucose excursion, probably by retarding gastric emptying (Thompson *et al.*, 1997). In some species, amylin can, in certain conditions, polymerize to form the β-pleated sheets characteristic of amyloid. Amyloid is toxic to pancreatic β cells (Lorenzo *et al.*, 1994), and its presence in type 2 diabetes may contribute further to reduced insulin secretion in that condition. Amino acids 25–29 in human amylin appear to be critical for amyloid formation. There is no homology between amylin and CGRP in this region, which may account for the failure of CGRP to form amyloid deposits.

Cardiovascular System

The important roles of CGRP in the cardiovascular system and particularly in the regulation of blood pressure have been reviewed recently (Deng and Li, 2005). CGRP is one of the most potent vasodilators known and its receptors are distributed widely throughout the vascular system. In some vessels, it appears to act directly on smooth muscle, whereas in others, CGRP receptors are expressed in endothelial cells, which release a diffusible mediator, probably nitric oxide. CGRP-containing nerves are also present in the heart, and CGRP exerts both positive inotropic and positive chronotropic effects. The wide distribution of CGRP-containing nerves throughout the vascular system makes it likely that CGRP has an important role in the regulation of regional blood flow, including renal and cerebral blood flow. CGRP administration is associated with a fall in systemic blood pressure. In a number of αCGRP-deficient mice models the cardiovascular system was unaffected; the mice had normal mean arterial pressure and heart rate (Lu *et al.*, 1999; Deng and Li, 2005). Other strains of αCGRP-deficient mice showed elevation of heart rate, systolic blood pressure, mean arterial pressure and peripheral vascular resistance (Gangula *et al.*, 2000; Oh-hashi *et al.*, 2001). Amylin shares the vasodilatory properties of CGRP but it is significantly less potent (Young *et al.*, 1993). Both peptides cause hypertrophy of cardiomyocytes (Bell *et al.*, 1995).

Nervous System

CGRP is distributed throughout the nervous system, being found in sensory, motor, and autonomic nerves, as well as in the hypothalamus, thalamus, and hippocampus. It is one of the most abundant peptides in the nervous system and it is frequently co-localized with other neuropeptides, such as substance P. This suggests that it may have a neuromodulatory role, acting in concert with other neurotransmitters. CGRP modulates pain perception and also appears to act centrally as an anorexiant (Lutz *et al.*, 1994). αCGRP-deficient mice showed elevated activity of the sympathetic nervous system (Oh-hashi *et al.*, 2001) and were different from wild-type mice in some specific aspects of pain perception, including reduced response to morphine-induced analgesia (Salmon *et al.*, 1999) and reduced pain associated with neurogenic inflammation (Salmon *et al.*, 2001).

Amylin also appears to have a role in the nervous system. It is found in some sensory neurons (Mulder *et al.*, 1995) and modulates appetite, memory, and motor function (Morley *et al.*, 1995; Clementi *et al.*, 1996). Through the formation of oligomers, it may produce neuronal apoptosis (Tucker *et al.*, 1998). Although amylin does not have a central anti-nociceptive effect, when administered subcutaneously or intraperitoneally it was potently analgesic in a mouse model of visceral pain (Young, 2005b).

Other Tissues

These peptides increase renin secretion (Wookey *et al.*, 1996) and may also interact with other hormonal axes, as circulating amylin concentrations are higher in patients with primary hyperparathyroidism (Valdermarsson *et al.*, 1996), and CGRP is a growth hormone secretagogue (Nakamura *et al.*, 1998). They reduce gastric acid secretion (Rossowski *et al.*, 1997) and inhibit gastric emptying (Kolterman *et al.*, 1995; Young *et al.*, 1995). There is also evidence that both peptides can modulate inflammatory responses (Clementi *et al.*, 1995).

PEPTIDE ACCESS TO THE BONE MICROENVIRONMENT

The principal route by which amylin reaches bone is the circulation, which, in turn, derives its amylin from pancreatic secretion. Circulating amylin levels are of the order of 5 pmol/liter, rising to 10–20 pmol/liter following a meal. Amylin secretion is pulsatile, with peaks occurring at about 5-minute intervals (Juhl *et al.*, 2000). Levels are probably higher in obese subjects and those with type 2 diabetes (Butler *et al.*, 1990; Mitsukawa *et al.*, 1990; Hartter *et al.*, 1991; Sanke *et al.*, 1991; Reid *et al.*, 1993), but appear to be decreased by leptin (Karlsson *et al.*, 1998). There is one report of amylin mRNA expression and peptide secretion from a human osteoblast-like cell line (Gilbey *et al.*, 1991), raising the possibility of amylin production locally within the bone microenvironment, but we have been unable to detect amylin mRNA in primary rat osteoblasts and in primary human osteoblast samples from 12 different donors (Naot *et al.*, unpublished data).

Early reports suggested that CGRP circulated in concentrations of 30–40 pmol/liter (Zaidi *et al.*, 1986; Schifter, 1991), but it has been suggested more recently that the concentration is closer to 1 pmol/liter (Born *et al.*, 1991). Circulating concentrations are increased by sex hormone replacement therapy in postmenopausal women (Spinetti *et al.*, 1997). Some circulating CGRP may be secreted by the parafollicular cells of the thyroid and the balance is released by nerve endings. It is likely that bone may be exposed to significantly higher concentrations of CGRP as a result of local release of CGRP from nerve terminals. Sensory nerve fibers containing CGRP are widely distributed in bone, including bone marrow (Bjurholm, 1991; Hukkanen *et al.*, 1992; Ahmed *et al.*, 1994; Irie *et al.*, 2002). Innervation is richest at the epiphysis and periosteum (Hill and Elde, 1991). Immunostaining of CGRP-containing nerve fibers in bone tissue demonstrated changes in distribution during bone development and regeneration (Irie, 2002). When bone defects are created surgically, the development of CGRP-containing nerves is noted several days later, often in association with new blood vessels (Aoki *et al.*, 1994), suggesting a role in callus formation and bone healing. Similar responses are seen following fractures (Hukkanen *et al.*, 1993). It is interesting to note that CGRP-containing nerves are also seen in the growing deer antler (Gray *et al.*, 1992) and surrounding developing teeth (Fristad *et al.*, 1994). It is possible that CGRP aids bone growth in all these circumstances through its direct effects on osteoblast function. The intimate association of these nerves with blood vessels suggests that they may also have a role in regulating blood flow to sites of bone healing or growth.

There has been a report of CGRP mRNA in osteosarcoma cell lines and in human osteoblasts, raising the possibility that this peptide is produced locally in bone (Drissi *et al.*, 1997).

EFFECTS ON OSTEOCLASTS

CGRP

Following the discovery of CGRP, its common origin and sequence homology with calcitonin led to an investigation of its effects on bone resorption. This was first approached by injection of the peptide into intact animals. Tippins *et al.* (1984) found that CGRP had a calcitonin-like effect in the rat and rabbit, lowering circulating calcium concentrations. In the rabbit, it was approximately equipotent with calcitonin, although in the rat, concentrations 100- to 1,000-fold higher than those of calcitonin were required to produce hypocalcemia. However, in the rabbit, higher concentrations of CGRP produce hypercalcemia. In the chicken, the peptide causes only hypercalcemia.

These results led to more detailed assessments of CGRP effects on bone resorption. Yamamoto *et al.* (1986) studied the effect of human CGRP on ^{45}Ca release from prelabeled neonatal mouse calvariae (Fig. 2). CGRP produced a comparable degree of inhibition of both basal and parathyroid hormone-stimulated resorption, but the half-maximal concentration of CGRP was 500-fold higher than that of human calcitonin. Others have produced similar results in fetal rat bone organ cultures (D'Souza *et al.*, 1986; Roos *et al.*, 1986; Tamura *et al.*, 1992; Zhang *et al.*,

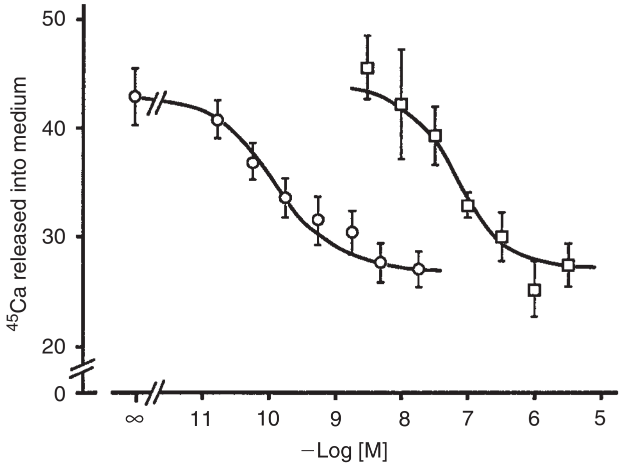

FIGURE 2 Effect of human calcitonin and human CGRP on PTH-stimulated bone resorption in neonatal mouse calvariae. Data are mean ± SE, $n = 6$. From Yamamoto *et al.* (1986), with permission.

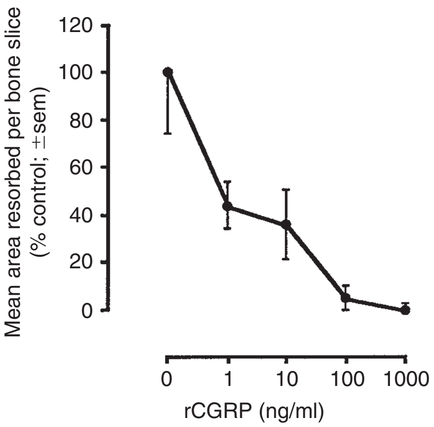

FIGURE 3 Effect of rat CGRP on bone resorption by isolated rat osteoclasts; 1 ng/ml CGRP = 0.26 nmol/liter. From Zaidi *et al.* (1987), with permission.

1994). The antiresorptive effect of CGRP blocks the stimulation of bone resorption produced by a variety of osteolytic factors.

Studies of disaggregated neonatal rat osteoclasts have confirmed that both αCGRP and βCGRP directly inhibit bone resorption in these cells (Fig. 3). These two peptides are equipotent, but the effect requires nanomolar concentrations, in contrast to calcitonins, which require only picomolar concentrations to produce comparable inhibition (Zaidi *et al.*, 1987b; Zaidi *et al.*, 1987c). More recent studies in isolated osteoclasts have further characterized the effects of CGRP on these cells (Alam *et al.*, 1991; Alam *et al.*, 1993a; Alam *et al.*, 1993b). It inhibits osteoclastic bone resorption by inhibiting cell motility, probably via cAMP production, as this effect is reproduced by forskolin. Inhibition of cell motility is blocked by the CGRP fragment, CGRP-(8–37). Osteoclast retraction, which appears to be mediated by changes in intracellular calcium, is not seen with CGRP, although it is produced by calcitonin. These data imply that in this experimental system CGRP activates only the cAMP intracellular messenger system, whereas calcitonin also acts on osteoclasts via changes in intracellular calcium. This may contribute to the greater potency of calcitonin in inhibiting bone resorption.

CGRP has also been shown to be active in other osteoclast models. Tamura *et al.* (1992) demonstrated cAMP production in osteoclast-like multinucleated cells formed in cocultures of mouse osteoblasts and bone marrow cells in the presence of calcitriol. Concentrations 60-fold higher than those of human calcitonin were required, and CGRP in high concentrations displaced calcitonin from its specific binding site.

Evidence also suggests that CGRP may act on osteoclast precursors. Specific binding of CGRP to mouse bone marrow cells (Mullins *et al.*, 1993) and macrophages has been demonstrated, and CGRP inhibits the development of osteoclasts in macrophage-osteoblast cocultures (Owan and Ibaraki, 1994). We have addressed this question in cultures of mouse bone marrow, where CGRP inhibits the formation of mononuclear cells staining with tartrate-resistant acid phosphatase, as well as inhibiting the subsequent fusion of these cells to form multinucleated osteoclasts. Similar effects were seen with calcitonin at 1,000-fold lower concentrations (Cornish *et al.*, 2001). Akopian *et al.* (2000) have reported reduced formation of human osteoclasts following treatment with CGRP. These data suggest that CGRP binds specifically to osteoclast precursors and regulates osteoclast development.

Despite this consistent evidence of an effect of CGRP on osteoclastic resorption *in vitro*, a study of *in vivo* injection of CGRP over the calvariae of adult mice detected no significant inhibition of resorption (Cornish *et al.*, 1995; Cornish and Reid, 1999) (Fig. 4). However, Valentijn *et al.* (1997) have demonstrated incomplete suppression of post-ovariectomy increases in bone resorption in rats with CGRP treatment. This required the use of CGRP in a dose 500 times higher than that of calcitonin, which produced a greater therapeutic effect. Thus CGRP is antiresorptive *in vivo*, but its low potency suggests that this is unlikely to contribute to normal skeletal physiology except, possibly, in the region immediately adjacent to CGRP-producing cells.

Amylin

Within a short time of the description of amylin, its effect on indices of bone resorption had been assessed in studies paralleling those just described for CGRP. Thus its hypocalcemic actions in rats (Datta *et al.*, 1989; MacIntyre, 1989; Zaidi *et al.*, 1990), rabbits (Datta *et al.*, 1989), and humans (Gilbey *et al.*, 1991; Wimalawansa *et al.*, 1992)

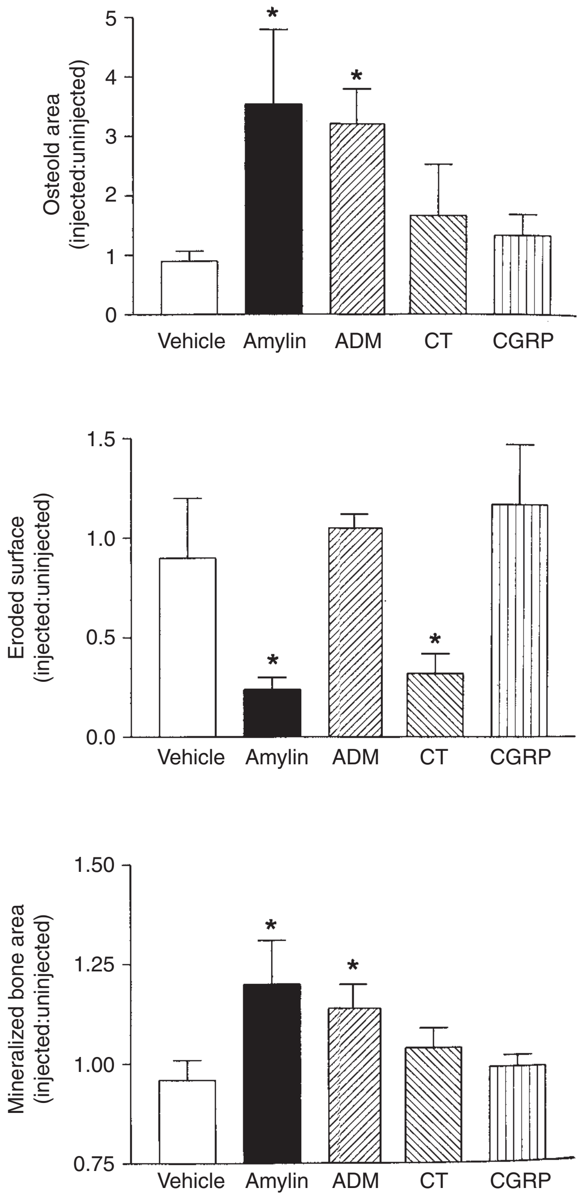

FIGURE 4 Comparison of the *in vivo* effects of rat amylin, human adrenomedullin, rat calcitonin (CT), and rat CGRP on bone histology in adult mice. Animals were injected daily with 4.1×10^{-9} mol of each peptide over the periosteum of one calvaria for 5 days and then sacrificed 1 week later. Data are expressed as the ratio of each index measured in the injected hemicalvaria to that measured in the contralateral, uninjected hemicalvaria. $n = 5$ in each group. Data are mean ± SE. Significant differences ($p < 0.05$) between injected and uninjected hemicalvariae are indicated by asterisks. From Cornish *et al.* (1999), with permission.

were reported (Fig. 5), and similar effects have been described in the goat (Min *et al.*, 1999). In the rat, 30-fold higher doses of amylin were required to produce comparable hypocalcemia to that of calcitonin, and human studies showed a similar trend. The effects of amylin on serum calcium in the rat, however, were significantly greater than those of CGRP (Zaidi *et al.*, 1990).

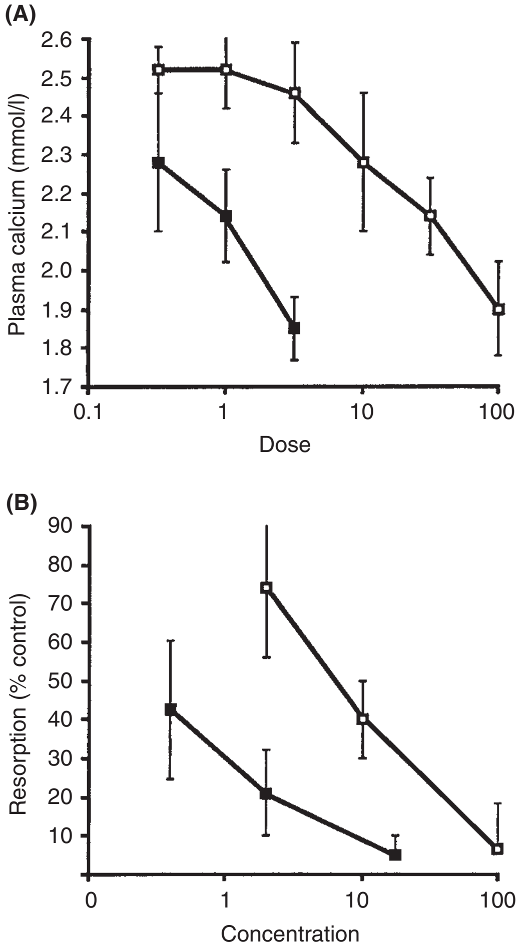

FIGURE 5 Effect of human amylin and human calcitonin on **(A)** plasma calcium in the rat and **(B)** bone resorption by isolated osteoclasts *in vitro*. Doses are given in pmol/rat **(A)** and in pmol/liter **(B)**. Data are mean ± SE. From MacIntyre (1989), with permission.

More detailed studies of the action of amylin on isolated osteoclasts have shown that its actions are qualitatively similar to those of CGRP. Thus amylin inhibits the motility of mature osteoclasts by way of increasing intracellular cAMP concentration. It is tenfold more potent than CGRP but an order of magnitude less potent than human calcitonin (Alam *et al.*, 1993). Amylin does not produce the osteoclast retraction seen with calcitonin. CGRP and amylin were equipotent in stimulating cAMP production in osteoclasts produced by coculture of osteoblasts and bone marrow cells (Tamura *et al.*, 1992), although 60-fold less potent than human calcitonin. In macrophages, amylin stimulated cAMP production but was 100-fold less potent than CGRP (Owan and Ibaraki, 1994). This effect was blocked by the CGRP antagonist, CGRP-(8–37). We have also studied the effect of amylin on osteoclast development in mouse bone marrow cultures (Cornish *et al.*, 2001).

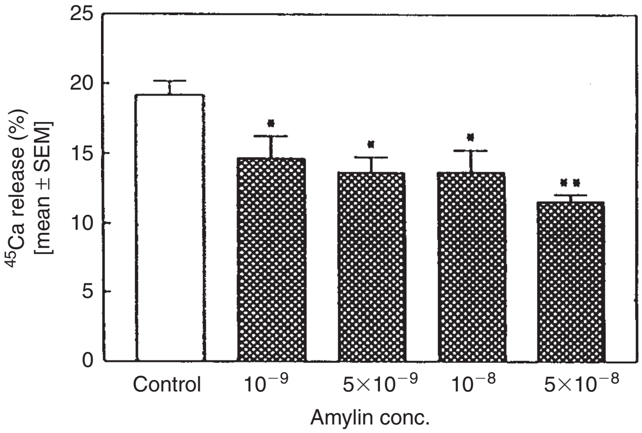

FIGURE 6 Effect of amylin on basal bone resorption in neonatal mouse calvariae. Based on data in Cornish *et al.* (1998b).

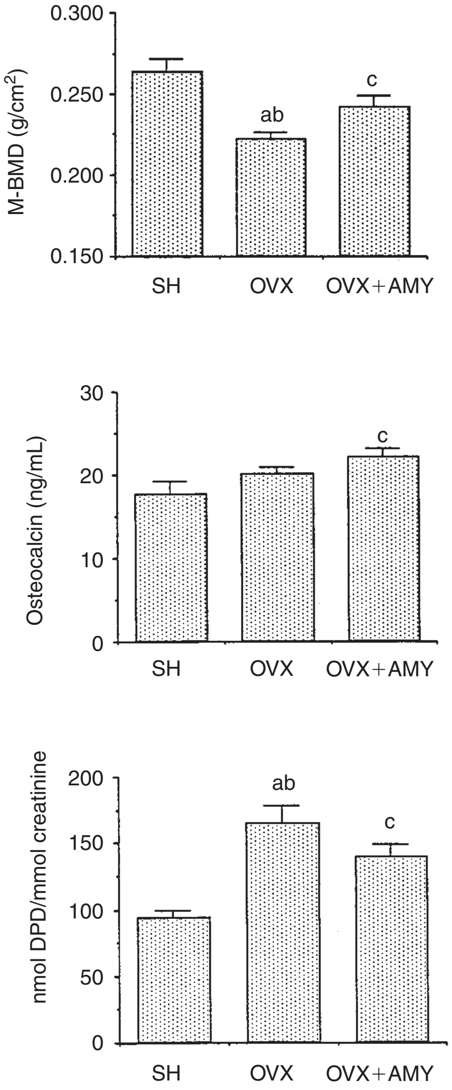

FIGURE 7 Effects of daily systemic administration of amylin (3 μg/100 g) to ovariectomized rats for 90 days. Indices assessed were distal metaphyseal bone mineral density of the femur (M-BMD), serum osteocalcin concentration, and urinary excretion of deoxypyridinoline (DPD). SH, sham operated; OVX, ovariectomized; AMY, amylin. a, significantly different from sham animals, $p < 0.01$; b, significantly different from amylin-treated group, $p < 0.05$; c, significantly different from sham, $p < 0.05$. Data from Horcajada-Molteni *et al.* (2000), with permission.

As with CGRP, there is an inhibition of both the formation of mononuclear osteoclast-like cells and the fusion of these cells. Dacquin *et al.* (2004) showed that amylin inhibition of osteoclastogenesis requires the activation of the extracellular signal-regulated protein kinase 1/2 (ERK1/2). In a bone marrow culture system, amylin inhibition of osteoclast differentiation was associated with a transient phosphorylation of ERK1/2 and moreover, when a negative dominant form of ERK1/2 was expressed in the cells, amylin was unable to inhibit osteoclast formation. The study also demonstrated that amylin had no effect on bone marrow cell proliferation, but in the presence of amylin osteoclasts were smaller and contained fewer nuclei.

Amylin has also been shown to reduce bone resorption in organ culture. It is approximately equipotent with CGRP in inhibiting calcitriol-stimulated resorption in fetal rat long bones (Tamura *et al.*, 1992). Amylin reduces both basal and parathyroid hormone-stimulated bone resorption in neonatal mouse calvariae, and cAMP production is also stimulated in this model (Pietschmann *et al.*, 1993; Cornish *et al.*, 1998b). In the studies of Cornish and colleagues (Fig. 6), inhibition of resorption was seen at concentrations as low as $10^{-9}M$.

These results should be interpreted in the light of the marked propensity for amylin to adhere to the surfaces of laboratory plasticware (Young *et al.*, 1992), suggesting that the actual concentrations of amylin in all *in vitro* experiments may be one to two orders of magnitude less than the amount added to the media. Thus, both osteoclast and calvarial studies imply that amylin may regulate bone resorption at physiological concentrations. This activity is dependent on the presence of the carboxyl-terminal amide group. In its absence, the potency of amylin in reducing osteoclastic resorption is comparable to that of CGRP (Datta *et al.*, 1989; Alam *et al.*, 1993). Amylin's inhibition of bone resorption in neonatal mouse calvariae only occurs with the intact molecule, in contrast to the situation with amylin action on osteoblasts. Amylin fragments, which act as antagonists in the osteoblast, do not modify the effect of amylin on osteoclasts (Cornish *et al.*, 1998b).

The effect of amylin on resorption *in vivo* has been studied histomorphometrically in several different models. Cornish *et al.* (1995) demonstrated 60–70% reductions in indices of bone resorption following daily local administration of amylin over the calvariae of adult mice. Very similar changes in resorption indices were seen following systemic administration of amylin to adult male mice for one month (Cornish *et al.*, 1998a) although the aminoterminal octapeptide of amylin is without effect on resorption in the same model (Cornish *et al.*, 2000). In ovariectomized rats, intact amylin reduces urinary excretion of deoxypyridinoline and reduces bone loss (Horcajada-Molteni *et al.*, 2000) (Fig. 7). In contrast, the earlier experiment of Romero *et al.* (1995) in which amylin was administered systemically to rats, showed only a nonsignificant trend toward reduced resorption, and Borm *et al.* (1999) found no change in resorption markers in 23 diabetic patients receiving the amylin analog pramlintide for 1 year. The latter study needs to be interpreted with caution, as it used an amylin analog of unknown activity on bone and it was uncontrolled.

EFFECTS ON OSTEOBLASTS

CGRP

At the time that the effects of CGRP as a calcitonin analog in bone were being investigated, data began to emerge suggesting that it may also have an effect on osteoblasts. Michelangeli *et al.* (1986) demonstrated that CGRP increased cAMP formation in an osteogenic sarcoma cell line (UMR 106–01) that had an osteoblastic phenotype and was not calcitonin responsive. The same group subsequently studied mixed bone cell cultures obtained by sequential digestion of neonatal chicken, rat, or mouse calvariae and again demonstrated the presence of a cAMP response to CGRP when none to calcitonin was detectable (Michelangeli *et al.*, 1989). Other studies have demonstrated CGRP binding (Seitz *et al.*, 1986) and cAMP production (Thiebaud *et al.*, 1991; Bjurholm *et al.*, 1992; Tamura *et al.*, 1992) in normal or transformed osteoblast-like cells. Amino-terminal peptides and the disulfide bridge were both necessary to maintain activity of CGRP on osteoblasts (Thiebaud *et al.*, 1991).

There is some evidence that CGRP stimulates osteoblast proliferation. Cornish *et al.* (1995) showed small increases in proliferation in primary rat osteoblast-like cells in response to CGRP, but compared to amylin, a 1,000-fold higher concentration of CGRP was required to produce a similar effect (Fig. 8). In primary cultures of human osteoblast-like cells, CGRP stimulated proliferation with a maximal effect at 10nM. The proliferating activity of CGRP was not inhibited by the two antagonists, CGRP-(8–37) or amylin-(8–37) (Villa *et al.*, 2000). *In vivo*, Cornish *et al.* (1995) found no significant effect of local injection of CGRP on osteoblast indices in adult mice (Fig. 4) and Valentijn *et al.* (1997) found no change in bone formation rates in ovariectomized rats treated with CGRP. However, transgenic mice overexpressing CGRP in osteoblasts under the regulation of an osteocalcin promoter, showed increases in bone formation indices, bone volume, and bone density (Ballica *et al.*, 1999).

Several intracellular signaling pathways are activated by CGRP in osteoblast-like cells. Gupta *et al.* (1994) demonstrated a stimulation of Na^+/H^+ exchange in UMR 106 cells.

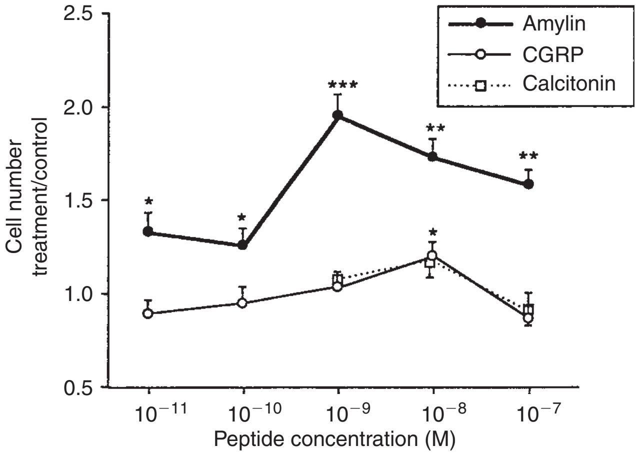

FIGURE 8 Dose dependence of the effects of rat amylin (●) rat CGRP-1 (○), and rat calcitonin (□) on numbers of primary rat osteoblast-like cells in culture over 24 hours. $n = 6$ in each group. Data are mean ± SEM. Statistical significance of differences from control: $*p \leq 0.05$; $**p < 0.005$; $***p < 0.001$. Reprinted with permission from Cornish *et al.* (1995).

Kawase *et al.* (1995), using the same cell line, showed that CGRP induces a transient twofold increase in intracellular calcium concentrations by mobilization of calcium from intracellular stores. Similar results were obtained in the human preosteoblastic cell line MG-63, where CGRP initially stimulated calcium discharge from intracellular stores in a cAMP-independent mechanism, and subsequently stimulated calcium influx in a cAMP-dependent manner (Burns *et al.*, 2004). In the osteosarcoma cell line OHS-4, CGRP increased intracellular calcium concentrations but had no detectable effect on cAMP (Drissi *et al.*, 1999). CGRP was also shown to stimulate potassium efflux, inducing membrane hyperpolarization that results in rapid changes in cell morphology (Kawase and Burns, 1998). Villa *et al.* (2003) demonstrated that calcitonin, CGRP, and amylin induced proliferation in a primary culture of human osteoblasts, and all three peptides significantly activated protein kinase C. Using the specific inhibitor, staurosporine, they showed that the activation of protein kinase C is necessary for the proliferative effect of CGRP in these cells. Of all three peptides, calcitonin, CGRP, and amylin, only CGRP stimulated cAMP production in the primary human osteoblasts.

There is also evidence that CGRP may act on preosteoblasts (Thiebaud *et al.*, 1991; Tamura *et al.*, 1992), influencing their development from precursor cells (Mullins *et al.*, 1993). Bernard and Shih (1990) have demonstrated that the number and size of bone colonies developing in bone marrow cultures are increased by CGRP and that systemic treatment with CGRP increases the number of bone colonies developing in marrow cultures.

The effects of CGRP on cytokine/growth factor production have been assessed. Sakagami *et al.* (1993) found that CGRP increased cAMP and interleukin-6 production in a preadipocyte-like stromal cell line. CGRP also inhibited the proliferation of these cells. In primary osteoblasts, however, CGRP increased IGF-1, IGF-2, and osteocalcin mRNA, and this effect on IGF-1 has been confirmed by others (Vignery and McCarthy, 1996). In primary rat osteoblast cultures, CGRP has also been shown to substantially reduce tumor necrosis factor-α production and to weakly stimulate interleukin-6 production by these cells (Millet and Vignery, 1997). Thus CGRP may regulate the function of both precursor cells and mature osteoblasts by modulating the production of cytokines and growth factors. In a recent publication, Villa *et al.* (2006) reported that CGRP dose-dependently inhibited the expression and secretion of osteoprotegerin (OPG) from primary cultures of human osteoblast-like cells. This effect could be blocked by CGRP(8–37) and by an inhibitor of protein kinase A. The inhibition of OPG by CGRP is unexpected, as previous studies showed that CGRP inhibited bone resorption whereas decreased OPG suggests activation of osteoclasts.

We have raised the possibility that these relatively weak effects of CGRP on osteoblast function may be mediated by receptors that have a higher affinity for amylin than for CGRP (Cornish *et al.*, 1999b). In cultures of fetal rat osteoblasts, amylin increased cell number, thymidine, and phenylalanine incorporation at 100-fold lower concentrations than those of CGRP and its maximal effects were about twice as great as those of CGRP. There was no additivity between maximal doses of the peptides on these indices. The CGRP receptor blocker, CGRP-(8–37), completely blocked the effect of CGRP at blocker concentrations $\leq 10^{-9}$ *M*. In contrast, the amylin receptor blocker, amylin-(8–37), completely blocked the effects of CGRP when the blocker was present in concentrations as low as 10^{-11} *M*. In converse experiments studying the blockade of maximal doses of amylin, amylin-(8–37) 10^{-10} *M* was effective, whereas a 100-fold greater concentration of CGRP-(8–37) was necessary to achieve the same effect. It was concluded that amylin and CGRP probably act through a common receptor to stimulate osteoblast growth and that this receptor has a higher affinity for amylin than for CGRP.

Amylin

Shortly after its discovery, amylin was shown to stimulate cAMP production in a preosteoblastic cell line, possibly through binding of a putative CGRP receptor (Tamura *et al.*, 1992). Cornish *et al.* (1995) demonstrated stimulation of proliferation of fetal rat osteoblasts by amylin in concentrations as low as 10^{-11} *M*, as shown in Fig. 8. Similar effects have been shown in human osteoblasts by Villa *et al.* (1997) and by ourselves (unpublished data). Amylin-(1–8) also stimulates osteoblast proliferation, although its half-maximally effective concentration is tenfold higher than that of the intact peptide (Cornish *et al.*, 1998b). This octa-peptide also stimulates thymidine incorporation in neonatal mouse calvariae (Cornish *et al.*, 1999). In rat primary osteoblasts, amylin activated the ERK1/2 signaling pathway, most likely through activation of Gi proteins (Cornish *et al.*, 2004). Phosphorylation of ERK1/2 was required for the mitogenic effect of amylin in these cells, as PD-98059, a specific inhibitor that prevents the phosphorylation of ERK1/2, also inhibited amylin-induced proliferation. Interestingly, the mitogenic effect of amylin in osteoblasts requires the presence of an IGF-1 receptor, although there is no direct binding of amylin to this receptor, nor a paracrine effect of osteoblast-derived IGF-1 (Cornish *et al.*, 2004). *In vivo*, daily injections of amylin induced a transient elevation of serum osteocalcin in rats (Romero *et al.*, 1995). Cornish *et al.* (1995) (Fig. 4) have shown two- to fourfold increases in histomorphometric indices of osteoblast activity in adult mice to whom amylin was administered locally over the calvariae daily for five days. Systemic administration of amylin over one month produced an increase of 30–100% in these indices (Cornish *et al.*, 1998a). Horcajada-Molteni *et al.* (2000) demonstrated increases in serum osteocalcin concentrations in ovariectomized rats treated systemically with amylin (Fig. 7), although the small human study with pramlintide did not detect any changes in osteoblast function (Borm *et al.*, 1999).

When amylin-(1–8) was administered by local injection over the calvariae of female mice, this peptide increased the double-labeled surface threefold. The effect was dose dependent from 0.4 to 40 n*M* and greater than that of an equimolar dose of hPTH-(1–34). The mineral apposition rate was increased by 40 n*M* amylin-(1–8) but not by hPTH-(1–34). Daily systemic administration of this peptide to sexually mature male mice for four weeks almost doubled histomorphometric indices of osteoblast activity (Cornish *et al.*, 2000). Thus, a number of studies have found evidence of an anabolic action of amylin and its amino terminus in osteoblasts.

EFFECTS ON BONE MASS

The effects of amylin and CGRP on the total amount of mineralized tissue in bone have been assessed in several experimental systems, including local injections, systemic administration, and more recently, in genetically modified animals.

CGRP

In ovariectomized rats that were given daily subcutaneous injections of CGRP for 28 days, Valentijn *et al.* (1997)

reported decreased bone resorption and a modest reduction in post-ovariectomy bone loss from 60% to 46%. In a study of transgenic mice overexpressing the CGRP gene in osteoblasts, Ballica *et al.* (1999) demonstrated a 5% increase in distal femoral bone density at the age of 12 weeks. A quite different approach to determining the effect of CGRP on bone mass was used by Hill *et al.* (1991). Reasoning that most of the CGRP that gains access to bone does so via sensory nerves, they studied the effect of sensory denervation using capsaicin treatment in rats. This intervention produced no change in tibial histomorphometry although the osteoclast surface in the mandible was decreased. In a more recent study, Offley *et al.* (2005) showed that capsaicin treatment destroyed the unmyelinated sensory axons containing CGRP and the reduced CGRP signaling was associated with a decrease in bone mass.

The first knockout mouse model that was used to study the physiological effect of CGRP deficiency was missing the *Calca* gene that encodes for both calcitonin and αCGRP (Hoff *et al.*, 2002). Previously, many *in vitro* and *in vivo* studies had shown that the hypocalcemic hormone calcitonin is an inhibitor of bone resorption (Zaidi *et al.*, 1987c; Cornish *et al.*, 2001). Binding of calcitonin to the calcitonin receptor on osteoclasts results in inhibition of osteoclast activity, and therefore it was expected that *Calca*-deficient mice would have an osteoporotic phenotype due to accelerated bone resorption. The bone phenotype of the animals was unexpected. There was no change in osteoclast number or bone resorption markers, and moreover, the animals had higher bone volume and trabecular number with a decrease in trabecular spacing. Double calcein-labeling studies in the knockout animals showed that the high bone volume was a result of a significant increase in bone formation, rather than a decrease in bone resorption. The unexpected finding of increased bone formation in the *Calca*-deficient mice suggests a yet unrecognized effect of calcitonin or αCGRP to inhibit bone formation.

To differentiate between the contributions of the calcitonin deficiency to that of αCGRP, the bone phenotype of the *Calca*-deficient mice was compared to that of specific αCGRP-deficient mice (Schinke *et al.*, 2004). αCGRP-deficient mice had osteopenia caused by reduced bone formation rate. Therefore, studies in these two mice strains imply that *in vivo*, calcitonin inhibits osteoblast activity and bone formation, whereas αCGRP is a physiological activator of bone formation. A subsequent long-term study of the bone phenotype of these animals showed that the osteopenia displayed by the αCGRP-deficient mice was also evident at 6, 12, and 18 months of age (Huebner *et al.*, 2006). In the *Calca*-deficient mice the bone phenotype developed with age, and at 12 and 18 months, along with the increased bone formation there was evidence for increased bone resorption. The high bone turnover resulted in hyperostotic lesions in 20% of the *Calca*-deficient mice.

Amylin

As discussed previously, local injection of intact amylin over the calvariae of adult mice produced a substantial decrease in bone resorption, an increase in bone formation, and a 20% increase in mineralized bone area after only five daily injections (Fig. 4) (Cornish *et al.*, 1995). Romero and colleagues (1995) administered amylin systemically to normal male rats for 18 days, producing no changes in histomorphometric indices of formation or resorption but increasing cancellous bone volume of the proximal tibia by 25%. In a similar study in normal male mice, Cornish *et al.* (1998a) showed an increase of 70% in total bone volume in the proximal tibia (Fig. 9), as well as increased cortical width, tibial growth plate width, tibial length, body weight, and fat mass. A similar experiment using amylin-(1–8) increased bone volume by 36%, tibial cortical thickness by 8%, and resulted in increased bone strength as assessed by three-point bending (Cornish *et al.*, 2000). In the study of

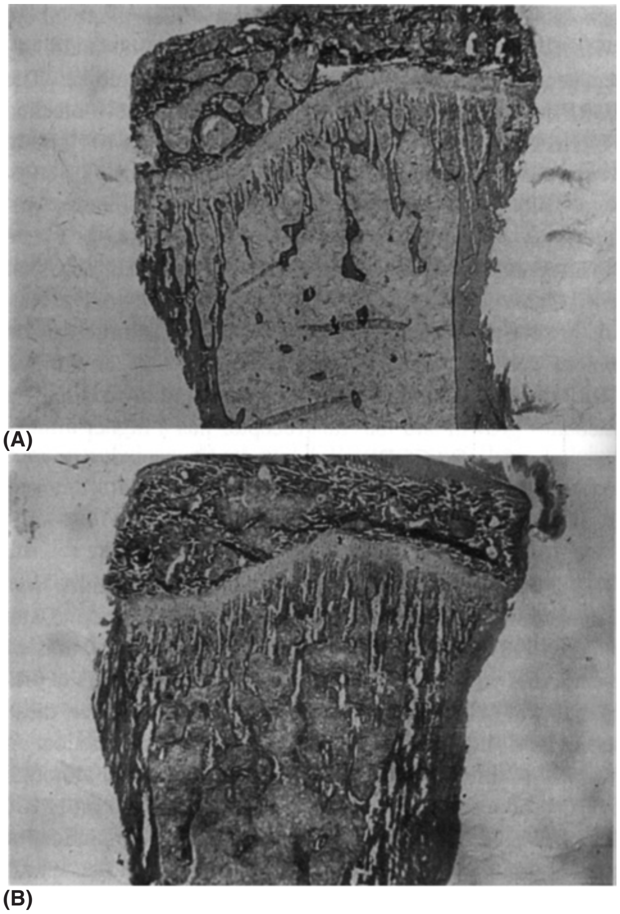

FIGURE 9 Photomicrographs of proximal tibiae of mice treated systemically with **(A)** vehicle or **(B)** amylin 10μg/day for 4 weeks. Trabecular bone volume is increased 70% in amylin-treated animals. Reprinted from Cornish *et al.* (1998a), with permission.

systemic administration of intact amylin to ovariectomized rats, reported by Horcajada-Molteni *et al.* (2000), both distal metaphyseal and total femoral bone densities were higher in animals receiving peptide (Fig. 7).

The phenotype of *amylin*-deficient mice was described by Dacquin *et al.* (2004). Analysis of the animals showed that in this model system amylin was dispensable for regulation of food intake, body weight, and glucose metabolism. At the age of 24 weeks, the *amylin*-deficient mice had a typical osteoporotic phenotype; showing decreased bone density of long bones, low bone mass, and a 50% decrease in trabecular bone volume. The number of osteoblasts, as well as the bone formation rate, assessed by double calcein injection, were similar between the *amylin*-deficient mice and the wild-type controls, suggesting that the osteoporotic phenotype was not a result of a defect in bone formation. In contrast, the *amylin*-deficient mice had an increased number of osteoclasts and an increase in degradation products of collagen in the urine, suggestive of accelerated bone resorption. Further studies of *amylin*-deficient mice demonstrated that the bone effects were sex-dependent. *Amylin*-deficient males showed increased trabecular thickness at 4 and 6 weeks of age and increased femoral length at 26 weeks, while female mice were no different from the wild type (Davey *et al.,* 2006).

In summary, the bone phenotypes of the calcitonin, αCGRP, and amylin knockout mice were quite unexpected, and changed our understanding of the possible physiological role of these peptides. Thus, the main effect of calcitonin on bone appears to be inhibition of bone formation. αCGRP has the opposite effect, acting as a stimulator of bone formation, whereas amylin turns out to be the most robust inhibitor of bone resorption within this group of peptides. Further studies will be required to fully understand the physiological role of the peptides and their mechanisms of action.

EFFECTS ON CALCIUM METABOLISM *IN VIVO*

Serum calcium levels were within the normal range in *amylin*-deficient mice (Dacquin *et al.*, 2004), αCGRP-deficient mice (Schinke *et al.*, 2004), and *Calca*-deficient mice (Hoff *et al.*, 2002). Therefore, amylin, αCGRP, and calcitonin do not appear to have an important role in the regulation of baseline serum calcium levels in mice. However, when injected with either PTH or 1,25(OH)$_2$-vitamin D$_3$, *Calca*-deficient mice showed significantly higher levels of serum calcium in comparison to wild-type animals, a response that could be reversed by calcitonin but not by CGRP. Therefore, the hypocalcemic effect of calcitonin seems to be important under calcium stress.

In earlier experiments that tested changes in systemic calcium metabolism in response to injection of amylin or CGRP, the most apparent effect of amylin or CGRP was the induction of hypocalcemia, which has been demonstrated in a number of species, as discussed earlier. For amylin, this is probably substantially attributable to inhibition of osteoclastic bone resorption. However, amylin, like calcitonin, may have a direct calciuretic effect on the kidney. Rat amylin binds to the renal porcine calcitonin receptor with an affinity comparable to that of porcine calcitonin itself. Both peptides comparably stimulate cAMP production via this receptor (Sexton *et al.*, 1994). CGRP does not compete for binding to this receptor. However, in rat renal tubular membranes, CGRP and amylin stimulated cAMP production with comparable half-maximal concentrations, and the effects of both peptides were blocked by CGRP(8–37) (Osajima *et al.*, 1995). Consistent with these results is the finding of Miles *et al.* (1994) that amylin infusion doubles urinary calcium excretion in dogs. However, the increased urinary loss of calcium accounted for less than 10% of the fall in serum calcium, suggesting that reduced osteoclastic resorption was the principal contributor to the hypocalcemic effect. These changes were accompanied by a doubling of PTH but no change in circulating calcitonin concentrations.

In the rat, CGRP, like amylin, is hypocalcemic with a potency slightly greater than that of amylin (Young *et al.*, 1993). However, in the chicken it has the opposite effect, elevating serum calcium within 20 minutes of administration (Ancill *et al.*, 1990; Bevis *et al.*, 1990). The mechanism of this effect has not been elucidated.

RECEPTORS

Specific amylin and CGRP receptors are formed by heterodimerization of the seven transmembrane domain calcitonin receptor or calcitonin receptor-like receptor (CRLR) with a receptor activity-modifying protein (RAMP). Calcitonin receptor belongs to the type II seven transmembrane G protein-coupled receptors, and has been cloned initially from porcine (Lin *et al.*, 1991) and subsequently from rat (Sexton *et al.*, 1993), human (Kuestner *et al.*, 1994; Gorn *et al.*, 1995), and other species. In most species, calcitonin receptor RNA is alternatively spliced, resulting in the expression of several isoforms (Sexton *et al.*, 1993; Kuestner *et al.*, 1994; Albrandt *et al.*, 1995; Gorn *et al.*, 1995). The various isoforms differ from each other not only structurally, but also in their tissue distribution, affinity for ligands and their signaling pathways (Egerton *et al.*, 1995; Moore *et al.*, 1995). CRLR shares about 55% amino acid sequence homology with calcitonin receptor, and was initially described as an orphan receptor (Fluhmann *et al.*, 1995). McLatchie *et al.* (1998) cloned a CGRP receptor from a human neuroblastoma cell line, and discovered that specific CGRP binding requires the coexpression of two proteins: CRLR and RAMP1. RAMP1 belongs to a family of proteins with a single transmembrane domain, which includes three members: RAMP1, RAMP2, and RAMP3. Further studies established that different combinations of calcitonin receptor and CRLR with RAMPs produce receptors with different affinities for peptides of the calcitonin family. Thus, heterodimerization of the calcitonin receptor with each of the three RAMPs produces receptors with high affinity for amylin, the combination of CRLR with RAMP1 creates a CGRP receptor, and CRLR with either RAMP2 or RAMP3 acts as an adrenomedullin receptor (Lerner, 2006).

The way in which RAMPs control the specificity of calcitonin receptors and CRLR for their ligands is still not fully understood. Cross-linking experiments with radioactive ligands demonstrated that RAMP and its seven transmembrane-domain partner are in very close association on the cell surface, forming complexes that are maintained following ligand binding (McLatchie *et al.*, 1998; Christopoulos *et al.*, 1999). RAMPs were also shown to regulate the glycosylation state of the receptors, which affects their transportation to the cell membrane. For example, when CRLR was coexpressed with RAMP2, it had only core glycosylation, while together with RAMP1, it was terminally glycosylated (McLatchie *et al.*, 1998). In contrast, other studies showed that the glycosylation state does not affect the specificity for the ligand (Aldecoa *et al.*, 2000; Hilairet *et al.*, 2001).

The pattern of expression of RAMPs, calcitonin receptor, and CRLR in bone cells has been investigated by several groups. RAMP1–3 and CRLR are expressed ubiquitously, and mRNA for all four have been identified in primary calvarial osteoblasts from rat and mouse as well as in the osteoblastic cell lines UMR106–06 and MC3T3–E1 (Naot *et al.*, 2001; Uzan *et al.*, 2004). Expression of CRLR and RAMP2 was also demonstrated in osteoclast-like cells by laser capture microdissection and RT-PCR (Nakamura *et al.*, 2005). Calcitonin receptor is highly expressed in osteoclasts. Its expression is induced by the activation of receptor activator of nuclear factor kappa B (RANK), and mRNA for the calcitonin receptor can be first identified on progenitor cells just prior to multinucleation (Lee *et al.*, 1995). Most osteoblasts and osteoblast-like cell lines do not express the calcitonin receptor (Sexton *et al.*, 1993; Izumi *et al.*, 2001; Naot *et al.*, 2001). Using the sensitive method of real-time PCR and RNA from cells of eight different donors we were unable to detect calcitonin receptor expression in these cells (unpublished results). In contrast, Villa *et al.* (2003) demonstrated the expression of calcitonin receptor in human osteoblast-like cells using RT-PCR.

Using specific amylin agonists and antagonists it was possible to dissociate the effect of amylin on bone formation from its effect on bone resorption (Cornish *et al.*, 1998b). Proliferation of primary osteoblasts was induced by intact amylin and by the peptide amylin-(1–8), whereas amylin-(8–37), terminally deamidated amylin and reduced amylin acted as antagonists. In contrast, inhibition of bone resorption in neonatal mouse calvariae only occurred with the intact amylin molecule and was not antagonized by any of these peptides, suggesting that amylin acts through two separate receptors on osteoclasts and osteoblasts (Cornish *et al.*, 1998b).

A number of studies showed that in various experimental systems the effects of peptides of the calcitonin family are transient, and prolonged exposure results in desensitization of the treated cells. In bone, this effect has been investigated mainly in osteoclasts treated with calcitonin. Early studies showed that stimulation of bone resorption in organ cultures by PTH could be inhibited by the addition of calcitonin, but over time the cells managed to 'escape' this inhibitory effect (Wener *et al.*, 1972). This 'escape phenomenon', was lated found to be due to ligand-induced internalization of the receptor, as well as inhibition of de novo synthesis of the calcitonin receptor (Takahashi *et al.*, 1995; Wada *et al.*, 1995). Similar escape from the inhibitory effect of amylin on PTH-stimulated bone resorption has also been described (Pietschmann *et al.*, 1993). Interestingly, cells treated with amylin for 48 hours were desensitized to further inhibition by amylin, but addition of calcitonin at this time point produced an inhibitory effect. Therefore, it appears that the mechanisms involved in desensitization to amylin are not identical to those involved in calcitonin desensitization (Pietschmann *et al.*, 1993). The escape phenomenon has not been studied directly in osteoclasts treated with CGRP, but there is some evidence from studies in microvascular endothelial cells and in vascular smooth muscle cells that exposure to CGRP also results in desensitization to further CGRP treatment (Drake *et al.*, 2000; Nikitenko *et al.*, 2006).

Recently, knockout animals have been used to study the physiological role of the calcitonin receptor and CRLR and their contribution to the activity of peptides of the calcitonin family in bone. *Calcr* (calcitonin receptor) homozygous deficiency and *Calcrl* (CRLR) homozygous deficiency are both embryonic lethal. *Calcrl*-deficient embryos have very similar phenotype to embryos that carry a targeted deletion of adrenomedullin, indicating that CRLR is the primary receptor through which adrenomedullin acts during the embryonic development (Dackor *et al.*, 2006). Heterozygous mice, with deletion of one copy of the calcitonin receptor gene, are viable and have a reduced expression of calcitonin receptor in their osteoclasts. These mice had high bone mass due to an increase in bone formation, similar to the calcitonin knockout mice. In contrast, *amylin*-deficient mice display a low bone mass phenotype and increased bone resorption. The difference between the *amylin*-deficient phenotype and that of the calcitonin-receptor heterozygous knockout animals indicates that the calcitonin receptor is not the main receptor through which amylin affects bone remodeling *in vivo* Dacquin *et al.*, 2004.

In summary, although the recently identified heterodimeric receptors for peptides of the calcitonin family have been investigated and characterized in detail, the bone effects of amylin and CGRP cannot be fully explained by their interactions with these receptors, and it seems that other receptors have yet to be identified.

SIGNIFICANCE OF AMYLIN AND CGRP TO BONE PHYSIOLOGY

What role amylin and CGRP play in normal bone metabolism and bone pathology in humans remains to be determined. It has been hypothesized that amylin secretion following a meal directs the absorbed calcium and protein from the meal into new bone synthesis by increasing bone growth at a time when the substrates are available (MacIntyre, 1989; Zaidi *et al.*, 1993). Amylin may also contribute to the relationship between body mass and bone density. Body mass, or more particularly fat mass is the principal determinant of bone density in women (Reid *et al.*, 1992a; Reid *et al.*, 1992b). While this might be mediated to some extent by the effect of weight on skeletal load bearing and by adipocyte production of estrogen, neither of these explanations is adequate to explain the published results (Reid *et al.*, 1992a; Reid *et al.*, 1992b; Reid *et al.*, 1993; Reid *et al.*, 1994; Reid *et al.*, 1995; Reid, 2002). Because both insulin and amylin are hypersecreted in obesity and because both may potentially act directly or indirectly to increase bone mass, they may contribute significantly to this relationship. Indeed, circulating insulin levels are directly related to bone density in normal postmenopausal women (Reid *et al.*, 1993), and because amylin is co-secreted with insulin, it would seem likely that a similar relationship for this peptide exists.

Because most of the CGRP gaining access to bone does so via sensory nerves, it is likely that this peptide is involved in the response of bone to injury and to other stimuli, such as exercise. The association of CGRP-containing nerves with healing and growing bone supports this contention.

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Chapter 40

Estrogens and Progestins

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INTRODUCTION

Sex steroids influence sexual dimorphism and reproductive functions of the skeleton. In the process they modify bone growth and remodeling. Estrogens regulate osteoblast-mediated bone formation and osteoclast-mediated bone resorption at multiple levels, including progenitor cell recruitment, proliferation, differentiation, and programmed cell death. In contrast to estrogen, the role of progesterone in bone physiology is less well understood. However, recent studies in progesterone receptor null mice suggest that progestins have novel actions on bone growth and turnover. This chapter reviews the actions of estrogen and progesterone on bone, emphasizing progress since the publication of the second edition of this book in 2002.

The effects of sex steroids on the skeleton are complex. Multiple receptor isoforms for the estrogen receptor (ER) and progesterone receptor (PR) have been identified in bone cells. Added to these variables is the existence of steroid receptor coregulators, including nuclear transcription factors that modulate steroid receptor interactions with the transcriptional machinery, tissue-specific distribution of enzymes that metabolize sex steroids, and membrane receptor- and nonreceptor-mediated actions of these hormones on target cells. Estrogen and progesterone also have indirect effects (e.g., through the hypothalamic-pituitary-gonadal axis) on bone and mineral homeostasis at multiple levels. With the complexity of molecular and cellular events that mediate the actions of estrogen and progesterone, it is easy to lose sight that these hormones' primary function is in reproduction.

The topics covered in this chapter include: the skeleton as a reproductive organ, ER and PR structure and function, roles of the receptor isoforms and receptor coregulators, receptor isoform expressions in skeletal tissues, alternative pathways of estrogen action, effects of treatment with estrogen and progesterone on bone cells *in vitro* and on the skeleton *in vivo,* sex steroid metabolism, and consequences of ER and PR gene deletions on skeletal structure and bone metabolism. Additionally, owing to the increasing clinical and scientific interest in these substances, the actions of estrogen-related compounds (selective estrogen receptor modulators or SERMs, estrogen metabolites, and phytoestrogens) on the skeleton are discussed.

THE SKELETON AS A REPRODUCTIVE ORGAN

The skeleton has primary as well as secondary roles in reproduction. The essential function of the skeleton in reproduction is most clearly evident in birds. Female birds produce medullary bone prior to the start of their egg-laying cycle. This bone has no biomechanical function. Instead, it provides an essential source of minerals for eggshell formation. In turn, the eggshell is partially resorbed during embryonic development, thereby providing the majority of the minerals for skeletal development. Following the reproductive period any remaining medullary bone is resorbed. Sex steroids play a pivital role in regulating medullary bone formation and utilization. The critical role of estrogen for reproductive success in birds is illustrated by the devastating effect of the pesticide DDT on

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exposed populations. DDT acted as an endocrine disrupter because of its ability to bind to and compete with estrogen for ER, impairing mobilization of calcium from bone for eggshell formation (Kupfer and Bulger, 1976; Turner and Eliel, 1978). The reader is referred to a prior review for a more complete discussion of the important and unique effects of sex steroids on the skeleton during reproduction in oviparous vertebrates (Turner and Riggs, 1994).

The skeleton is also important in mammalian reproduction. Bone provides a significant source of mineral for the fetal skeleton and is a major source of mineral during lactation. Estrogen (and perhaps progesterone) plays a vital role in the accumulation of minerals in the skeleton beyond what is required to fulfill bones' biomechanical function. Changes in estrogen levels likewise play a significant role in the appropriate mobilization of stored minerals from maternal bone during pregnancy and lactation.

The skeleton exhibits species-specific secondary sex characteristics that are in part determined by estrogen (and perhaps progesterone). The sex difference in bone mass is evident in our own species. Although the evolutionary explanation for the more robust skeleton in men is subject to debate, the consequences are clear. The net suppressive effect of estrogen on the accumulation of cortical bone prior to skeletal maturity is one of the factors that predisposes women to a higher risk for fractures later in life. This predisposition is exaggerated by the effects of menopause on the skeleton, which may recapitulate the temporary physiological effects of reduced estrogen signaling during pregnancy and lactation.

STEROID HORMONE MECHANISMS OF ACTION

Estrogen and progesterone are produced primarily in the gonads and released into circulation. Additional estrogens are produced locally in tissues, including bone, owing to the aromatization of circulating androgens to estrogens (Miki *et al.*, 2007). Circulating estrogen is preferentially bound to sex steroid-binding globulin and progesterone is preferentially bound to corticosteroid-binding globulin. The concentrations of these steroid-binding globulins in serum influence the metabolism and bioavailability of the steroid hormones. Steroids diffuse passively into cells and are retained intracellularly by complexing specific receptor proteins, which are steroid and target tissue-specific.

Steroid hormone receptor (ER and PR) concentration in bone is low relative to reproductive tissues. However, the absence of enzymes that inactivate estrogen in skeletal tissues may accentuate the responsiveness of bone cells to low physiological levels of the hormone compared to some reproductive tissues.

The diffusion of steroids into cells and the formation of the ligand-receptor complex are rapid, occurring within minutes of steroid injection into an animal. Following steroid-receptor complex formation (1–4 minutes post-treatment) the complex binds to nuclear binding sites at target gene promotors (2–5 minutes). This nuclear binding regulates the transcription of early (~60 minutes) or late (~8 hours and beyond) genes. Changes in protein profiles owing to regulation of late gene expression begin to occur 12 to 24 hours post-treatment. The major physiological effects of steroids in reproductive and skeletal tissues begin to occur by 24 to 48 hours after steroid treatment. Cell and tissue responses that occur at 36 to 72 hours after treatment probably represent downstream effects of steroid-regulated paracrine/autocrine factors. The 2- to 3-hour period elapsing between steroid receptor binding to the nuclear acceptor sites and the delayed, but major, transcriptional response of the late structural genes has been termed the lag phase. This is a period during which early response genes, coding for transcription factors and other proteins, are activated to subsequently regulate late gene expression. The latter process has been termed the cascade model of steroid hormone action (Landers and Spelsberg, 1992).

Steroid hormone receptors are a subclass of a larger (~100 member) family of zinc finger-containing, nuclear hormone receptors that function as transcriptional regulators (Tsai and O'Malley, 1994). The ligands bind with high affinity (K_d 10^{-10} to 10^{-8} M), but reversibly, to their respective receptors. Ligand binding triggers receptor activation, which induces a conformational change, dissociation of accessory proteins, receptor dimerization, and oligomerization and, in some cases, post-translational modifications such as phosphorylation. Activated receptors either bind directly to regulatory DNA elements (hormone response elements) in the promoter region of target genes or bind indirectly with other DNA-binding transcription factors (e.g., AP-1 factors) via protein-protein interactions, which then bind to the promoter. Once bound, either directly or indirectly, to the DNA, the activated receptor complex recruits transcription factors, termed coregulator proteins. These, in turn, bind to the core proteins of the transcriptional activation complex to regulate gene transcription (Bevan and Parker, 1999; McKenna *et al.*, 1999; Robyr *et al.*, 2000).

ESTROGEN

Estrogen Receptor Isoforms

Two ERs, ERα and ERβ, have been identified. The discovery of ERβ, a product of a different gene than the first described estrogen receptor (ERα) has revealed an additional level of complexity to estrogen action in the skeleton and other tissues (Kuiper *et al.*, 1996; Mosselman *et al.*, 1996). As depicted in Fig. 1, ERα and ERβ possess a high degree of homology in their DNA-binding domains and a moderate degree of conservation in their C-terminal ligand-binding domains (Kuiper and Gustafsson, 1997). The former explains why the two receptor isoforms interact with the same DNA response elements and the latter explains

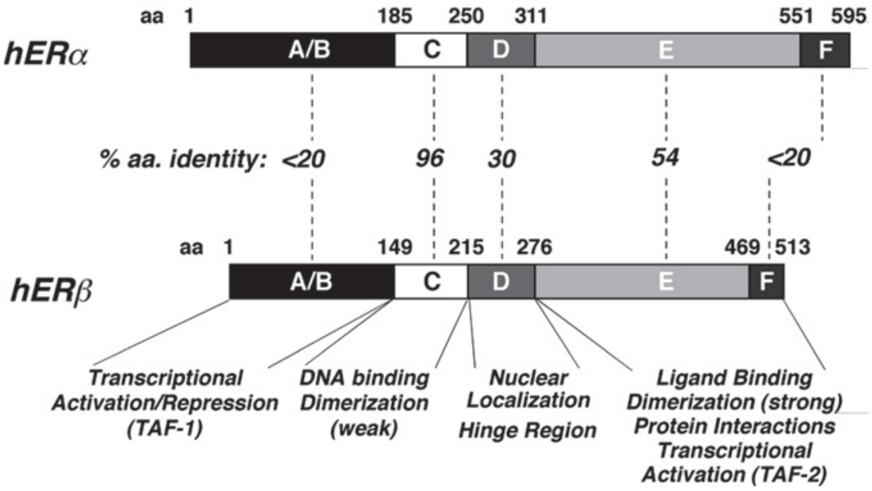
aa 1
185
250
311
551
595
hERα
A/B
C
D
E
F
% aa. identity: <20
96
30
54
<20
aa 1
149
215
276
469
513
hERβ
A/B
C
D
E
F
Transcriptional Activation/Repression (TAF-1)
DNA binding Dimerization (weak)
Nuclear Localization Hinge Region
Ligand Binding Dimerization (strong) Protein Interactions Transcriptional Activation (TAF-2)

FIGURE 1 Model of human estrogen receptor isoforms, their domains, functions, and homologies.

why both isoforms bind many natural estrogens, SERMs, and antiestrogens, but sometimes with different affinities (Kuiper and Gustafsson, 1997). ERα and ERβ are most divergent in their N-terminal A/B domains, which harbor the ligand-independent AF-1 transactivation function and that interact with proteins of the core transcriptional machinery to regulate gene expression. The tissue distributions of ERα and ERβ proteins also differ. Concentrations of the ERβ isoform are high in prostate, ovary, vasculature, brain, and bladder, but low or absent in uterus, breast, kidney, pituitary, and epididymis (Kuiper *et al.*, 1996; Kuiper and Gustafsson, 1997), tissues that contain high levels of ERα. In certain cell types, transfection-reporter gene analysis using an estrogen-response element reporter gene construct indicates that ERβ is a weaker regulator of gene transcription than ERα (Kuiper *et al.*, 1996; Kuiper and Gustafsson, 1997; Paech, *et al.*, 1997; Hall and McDonnell, 1999). Also, in response to estrogen treatment, ERα interacts with AP-1 factors and binds to AP-1 regulatory elements, a site where ERβ is inactive when complexed with estrogen (Paech *et al.*, 1997). Interestingly, ERβ activates AP-1 regulatory elements in response to treatment with SERMs, including tamoxifen and raloxifene (Paech *et al.*, 1997). Similarly, transactivation through Sp1 elements has been noted for both ER*a* and ERβ (Saville *et al.*, 2000). In some systems, however, the partial agonist activity of tamoxifen manifested through ERα is completely abolished upon coexpression of ERβ (Hall and McDonnell, 1999). Additionally, ERβ has 5 known carboxyl-terminal isoforms (ERβ1-5) derived from alternative splicing. ERβ1 is a full-length receptor, whereas the ERβ 2, 4, and 5 variants do not have full-length helix 11 and do not form homodimers. The truncated variants of ERβ heterodimerize with ERβ1 and alter its transactivation in a ligand-dependent manner (Leung *et al.*, 2006). ERβ2 lacks the activation function (AF)-2 core required for ligand-dependent transcriptional activation but behaves as a dominant-negative receptor affecting the function of ERα (Chakravarty *et al.*, 2007).

Distinct patterns of estradiol-dependent gene expression are induced by ERα and ERβ in U2OS osteosarcoma cells (Monroe, *et al.*, 2003; Monroe *et al.*, 2005; Monroe *et al.*, 2006). These findings suggest that during osteoblast differentiation a unique pattern of gene responses will occur when either receptor type predominates. Similar results have been shown for breast cancer cells (Secreto *et al.*, 2007). A pattern of gene expression occurs when both ERα and ERβ are present as heterodimers in U2OS cells (Monroe *et al.*, 2005) which differs from cells containing exclusively ERα or ERβ homodimers.

Steroid Receptor Coregulators

The activity of steroid nuclear receptors is modulated by a family of steroid receptor coregulators composed of coactivators and corepressors (Bevan and Parker, 1999; McKenna *et al.*, 1999; Robyr *et al.*, 2000). Each receptor isoform (ERα and ERβ, or A and B isoforms of PR) adopts a unique conformation that is dependent on the particular isoform and the agonist/antagonist/mixed agonist nature of the ligand (Brzozowski *et al.*, 1997; Paige *et al.*, 1999). The unique conformation adopted by each receptor isoform following ligand binding determines the relative interaction of the receptor with either coactivators or corepressors, and thereby the differential transactivation of hormone-responsive promoters. Coactivators, when bound to active receptor conformations (usually formed with agonists*):* mediate favorable interactions with the basal transcriptional machinery, stabilize the preinitiation complex and stimulate gene transcription. Conversely, corepressors bind preferentially to inactive receptor conformations (usually formed with antagonists or unliganded receptors) and prevent the interaction of the receptor with coactivators, resulting in nonproductive transcription factor complexes, which suppress gene transcription.

There is little information on the differential regulation of transcription by ERα andERβ homo- or heterodimers on osteoblasts. ERα andERβ coexpression was found to decrease transcriptional capacity compared to each ER isoform alone on an estrogen response element-dependent reporter gene. Overexpression of the steroid hormone coactivator 1 (SRC1) resulted in preferential transcriptional enhancement by ERβ as well as coexpressed ERα and ERβ· SRC2 overexpression preferentially enhanced ERα transactivation. SRC3, on the other hand, failed to increase estrogen-dependent transactivation of any ER combination in osteoblasts. These findings suggest the transactivation capacity of various ER isoforms is dependent on SRC (Monroe, Johnsen *et al.,* 2003).

Expression and Distribution of ERα and ERβ in Bone Cells and Skeletal Tissue

The important effects of estrogen on bone growth and turnover were recognized well before estrogen receptors were first detected in bone cells. After the initial discovery of ERα in cultured osteoblastic cells (Eriksen *et al.,* 1988; Komm *et al.,* 1988*):* the mRNAs for ERα and ERβ were identified in several osteosarcoma and immortalized osteoblast cell lines (Etienne *et al.,* 1990; Masuyama *et al.,* 1992; Lau *et al.,* 2006) and *in vivo* in osteoblast lineage cells (Zaman *et al.,* 2006).

Estrogen receptor abundance is low in bone cells compared to reproductive tissues and liver. Although many studies have monitored ERα and ERβ mRNA levels, little is known regarding the number and turnover of functional estrogen receptors in bone cells. ERα mRNA has been detected in a murine bone marrow stromal cell line, and the levels were upregulated by 1,25-dihydroxyvitamin D_3 (Bellido *et al.,* 1993). The expression of ERα mRNA was increased following glucocorticoid-induced differentiation of human bone marrow stromal cells (Oreffo *et al.,* 1999), consistent with findings from rat calvarial osteoblasts. Although estrogen modulated the expression of certain osteoblast marker genes in an osteogenic murine stromal cell line (Mathieu and Merregaert, 1994): proliferation and osteogenic differentiation of primary rat bone marrow stromal cells were unaffected by estrogen (Rickard *et al.,* 1995). However, developmental expression of ERα mRNA bone marrow stem cells was reported to correlate with osteogenic differentiation of these cells (Wang *et al.,* 2006). It is clear that ERs are present within differentiated osteoblasts, but it remains uncertain whether osteoblast progenitor cells express ERα and/or ERβ and thus represent additional targets for estrogen action.

The expressions of ERα and ERβ typically increase during osteoblast differentiation *in vitro.* In fetal rat calvarial-derived osteoblast cultures, the level of ERα mRNA was initially low in proliferating cells but increased at the onset of alkaline phosphatase expression and progressively increased thereafter to reach a maximum level in fully differentiated osteoblasts (Bodine *et al.,* 1998). In a human fetal osteoblast line, expression of ERβ mRNA also increased steadily as cells differentiated and then deposited a mineralized matrix (Arts *et al.,* 1997). In contrast, the level of ERβ transcripts in primary rat calvarial cultures was maintained at a high level throughout differentiation (Onoe *et al.,* 1997).

As discussed in the next few paragraphs, the skeletal distribution of ER is not limited to osteoblasts. The expression of ERα mRNA and protein has been reported in isolated avian and human osteoclasts and giant cell tumors of bone (Oursler *et al.,* 1991; Oursler *et al.,* 1994). The human leukemic cell line FLG 29.1, which can be induced by phorbol esters to form cells exhibiting many features typical of osteoclasts, possesses ERα (Oursler *et al.,* 1991; Oursler *et al.,* 1994; Fiorelli *et al.,* 1995; Fiorelli *et al.,* 1997). In addition, osteoclast precursors present in murine hematopoietic blast cell cultures have been shown to express ERα mRNA (Kanatani *et al.,* 1998). The presence of ERβ in osteoclasts and osteoclast precursors has also been reported (Sorensen *et al.,* 2006) but corroborating studies are needed. Estrogen attenuates human osteoclastogenesis through an ERα-mediated pathway, but has no effect on resorption by mature osteoclasts (Sorensen *et al.,* 2006). In contrast, Cre-lox-ERα studies strongly support a role for ERα in osteoclastogenesis and regulation of bone resorption *in vivo* in mice (Nakamura *et al.,* 2007).

Although the distribution of ERα or ERβ in the skeleton is incompletely known, the expression pattern of the two isoforms appears to overlap considerably. Both ERα and ERβ have been localized to chondrocytes in growth plate cartilage, but the exact distribution of ER isoforms within the growth plate may depend upon species and growth stage (Ben-Hur *et al.,* 1993; Kusec *et al.,* 1998; Nilsson *et al.,* 1999). ERα and ERβ are expressed in the human growth plate throughout puberty. In children, both ERα- and ERβ-positive cells were detected at a greater frequency in resting and proliferative zones than in the hypertrophic zone (Nilsson *et al.,* 2003).

In rats, the expression of ERα protein and mRNA was examined in the temporomandibular joint of adult males by immunocytochemistry and *in situ* hybridization. Intense ERα immunoreactivity was localized in the synovial lining cells, stromal cells in the articular disc, and chondrocytes in the temporomandibular joint (Yamada *et al.,* 2003). In contrast, ERβ but not ERα was expressed in high amounts in normal human synovia (Dietrich *et al.,* 2006).

The general consensus for ER distribution in bone is that both receptor isoforms are localized predominantly to osteoblasts and lining cells of cancellous bone, with lower levels in osteocytes and osteoclasts. However, other patterns of distribution of ERα have been obtained. For example, immunofluorescence detection of ERα in human female and pig bone revealed strongest reactivity in about 50% of the osteocytes, with indistinct staining of osteoblasts and lining cells (Braidman *et al.*, 1995). Using a more sensitive *in situ* reverse transcriptase (RT) polymerase chain reaction (PCR) technique to identify ERα mRNA in actively remodeling human fracture callus, Hoyland *et al.* (1997) reported that osteoblasts expressed the highest levels of ERα, whereas osteocytes and osteoclasts expressed lower levels. Similar results were reported in another study in which the ERα mRNA and protein were predominantly localized to osteoblasts and lining cells of cancellous bone from human and rabbit, with no clear expression in osteocytes or osteoclasts (Kusec *et al.*, 1998).

The *in situ* RT-PCR technique has detected a loss of ERα mRNA expression from osteoblasts and osteocytes in males with idiopathic osteoporosis, suggesting estrogen resistance contributed to the bone loss (Braidman *et al.*, 2000). In an immunolocalization study of mouse and human trabecular bone and fracture callus, ERβ was identified in osteoblasts, osteocytes, and osteoclasts, with the latter cells exhibiting cytoplasmic rather than nuclear immunoreactivity (Vidal *et al.*, 1999). Similar results were obtained for ERβ mRNA in neonatal rat bone where the transcripts were expressed predominantly by osteoblasts covering cancellous bone surfaces with lower signals in osteocytes and bone marrow (Windahl *et al.*, 2000).

In rats, the relative levels of ERα and ERβ mRNA are lower in cortical than cancellous bone, with ERα being dominant at both locations (Onoe *et al.*, 1997; Lim *et al.*, 1999). The weak expression of ERβ in cortical bone is an intriguing observation given that the most striking skeletal phenotype in ERβ knockout mice is increased cortical bone mineral content in postpubertal females only (Windahl *et al.*, 1999). In these mice, more than 90% of tibial osteocytes express ERα, and the level per osteocyte is higher in cortical than cancellous bone. Ovariectomy is associated with decreased ERα protein expression/osteocyte, suggesting that estrogen regulates ER expression levels.

In summary, these *in vitro* and expression studies indicate mRNA levels for ERα are generally higher than ERβ in osteoblasts at all stages of differentiation. The levels for ER isoforms in osteoclasts, cartilage, and connective tissues associated with bone are more variable. It remains unknown what concentration of each receptor isoform is required for responsiveness in target cells or whether one isoform modulates the activity of the other when both are expressed.

Effects of Estrogens on Bone *In Vivo*

The important role of gonadal hormones in bone metabolism is well established. Sex steroids influence most if not all aspects of bone development, growth, and remodeling. The physiological actions of sex steroids contribute to: (a) sexual dimorphism of the skeleton, (b) timing of epiphysealclosure, (c) peak bone mass, (d) maintenance of mineral homeostasis during reproduction, and (e) maintenance of bone mass, architecture, and mineral homeostasis in adults. Despite recognition that gonadal insufficiency is the most important risk factor for osteoporosis, the mechanisms of action of sex steroids on bone *in vivo* are poorly understood and have received surprisingly moderate attention by investigators. Because human studies have provided only a limited understanding of the underlying cellular and molecular mechanisms that mediate hormonal action, studies in laboratory animals have proven extremely useful in identifying an impressive range of physiological actions of gonadal hormones on bone and mineral homeostasis. The strengths and weaknesses of the most well-established animal models for osteoporosis have been reviewed (Iwaniec and Turner, 2008).

Sexual Dimorphism of the Skeleton

Bone growth, modeling, and remodeling represent the major processes that determine bone mass and architecture. Bone growth and modeling are most important prior to achieving peak bone mass. In contrast, remodeling and modeling are important in adults. Elongation of the skeleton by longitudinal bone growth ceases shortly after puberty, whereas increases in bone cross-sectional volume owing to radial bone growth continues at a slow rate throughout the remainder of life.

The sexual dimorphism of the skeleton is species-specific (see Table I) and is acquired prior to and during puberty. In humans, males have a taller stature and more robust skeleton than females, a larger tooth size, and greater muscularity (Frayer and Wolpoff, 1985). The origin of human sexual dimorphism is hotly debated but, unfortunately, beyond the scope of this review. However, it is clear that sex differences in the accretion of bone prior to achieving peak bone mass play an underappreciated role in the etiology of osteoporosis.

Premenopausal women have a higher cortical bone mineral density compared to their male counterparts (Kontulainen *et al.*, 2006). However, the excess bone in pubertal girls is largely endocortical, a location inconsistent with it having an important mechanical function but consistant with it being a storage site for bone that can be mobilized as required during gestation and lactation. In contrast, during puberty there is a disproportionate increase in bone size and strength in males compared to females (Macdonald *et al.*, 2006). At age 67–69, men had 25–32% larger cross-sectional bone size than women, supporting the hypothesis that sex differences in bone size and strength continue into old age (Sigurdsson *et al.*, 2006).

Sex steroids play a role in mediating gender differences in peak bone mass and bone architecture that define skeletal sexual dimorphism. Based on the principle that increased estrogen levels during puberty accelerate epiphyseal closure, the hormone was used to reduce stature

TABLE I Species, Age, and Compartment-Specific Effects of Estrogen on Bone Metabolism

Effect of Deficient Estrogen Signaling on the Growing Skeleton*

Species/site	Bone length	Cortical Bone	Cancellous Bone
Human	Increases	Increases#	Decreases$
Rat	Increases	Increases#	Decreases$
Mouse	Decreases	Decreases#	Decreases$

Effect of Deficient Estrogen Signaling on the Adult Skeleton*

Species/site	Bone length	Cortical Bone	Cancellous Bone
Human	No Change	Decrease##	Decreases$
Rat	No Change	Decrease##	Decreases$
Mouse	No Change	Decrease##	Decreases$

Effect of Estrogen Treatment on the Growing Skeleton**

Species/site	Bone length	Cortical Bone	Cancellous Bone
Human	Decreases	Decreases#	Increases$$
Rat	Decreases	Decreases#	Increases$$
Mouse	Increases	Increases###	Increases$$

Effect of Estrogen Treatment on the Adult Skeleton**

Species/site	Bone length	Cortical Bone	Cancellous Bone
Human	No Change	No Change	No Change or Slight
Rat	No Change	No Change	No Change
Mouse	No Change	Increases###	Increases$$$

Estrogen deficiency induced by menopause, ovariectomy, antiestrogens (e.g., ICI 182,780), or aromataase inhibitors; **Estrogen treatment is 17β-estradiol, premarin, diethylstilbestrol, etc.*

#Change largely owing to effect on periosteal expansion; ##Change largely owing to increased endocortical resorption; ###Change largely owing to increased endocortical bone formation and decreased endocortical bone resorption.$Change owing to increased resorption of primary spongiosa;

$$Change owing to decreased resorption of secondary spongiosa;

$$$Change owing to a tissue level increase in bone formation.

in tall girls (Venn *et al.,* 2004). In contrast, administration of androgens to boys with delayed puberty increased height (Ahmed *et al.,* 2004). A prospective, randomized, placebo-controlled study evaluated whether suppression of estrogen synthesis in pubertal boys delays bone maturation and ultimately results in increased adult height. During the 18-month follow-up, an increase of 5.1 cm in predicted adult height was observed in boys who received testosterone and the aromatase inhibitor letrozole, with no change seen either in boys who received testosterone alone or in untreated boys. These findings suggest pubertal increases in estrogen levels antagonize bone growth in males as well as females (Dunkel and Wickman, 2002). Finally, in 19-year-old males, free estradiol in serum was a negative predictor while free testosterone was a positive predictor of cortical bone size (Lorentzon *et al.,* 2005).

In summary, increased estrogen levels at puberty advance epiphyseal closure in males and females. Additionally, androgens increase bone length and cross-sectional area. Thus, the smaller bone size in females compared to males is because, at least in part, of the inhibitory actions of estrogen on bone growth. The generally earlier menarche in girls would enhance the hormonal-mediated sex differences in the skeleton. Physiologically, estrogen-mediated decreases in bone mass, size, and strength during puberty are also important because they may predispose women to osteoporosis later in life.

Effect of Ovariectomy on Bone Metabolism

Similar to humans, there are profound sex differences in bone size in rats (Fig. 2a). In rat tibiae, the volume of the

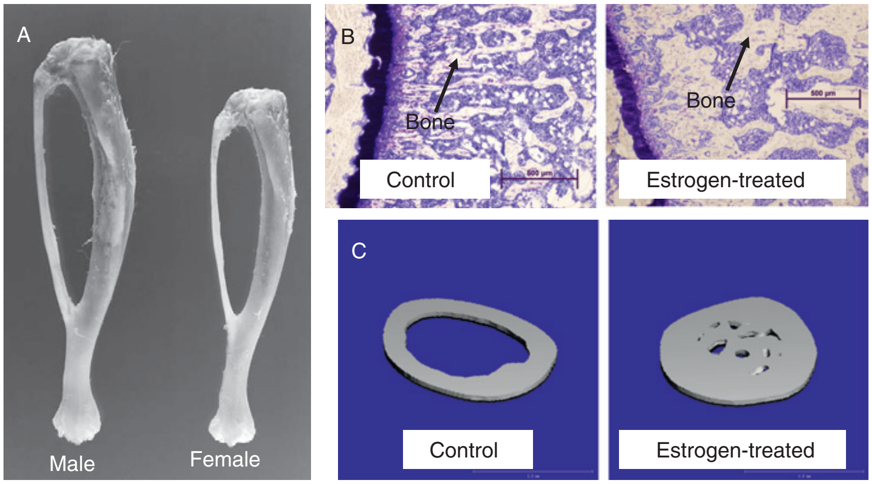

FIGURE 2 Effects of estrogen on bone architecture and histology. A) Sexual dimorphism of the rat tibia showing the size difference between an adult male and an adult female. The bone from the male is longer and more robust. The sex difference is greatly reduced by prepubertal gonadectomy. Bones from gonadectomized rats are intermediate between intact males and females in size. B) Estrogen increases cancellous bone volume in growing animals by preventing the resorption of primary spongiosa. C) Estrogen treatment results in osteosclerosis in mice. The observed endocortical bone formation is not observed in humans. (See plate section)

medullary canal is enlarged following ovariectomy owing to a net increase in bone resorption (Turner, Wakley *et al.*, 1987; Kalu, *et al.*, 1989). Osteoclast number is increased on the endocortical bone surface (Turner, Wakley *et al.*, 1987) and bone formation remains unchanged or increased (Turner, Wakley *et al.*, 1987; Turner, Colvard *et al.*, 1990). In contrast, there is increased bone formation at the periosteal surface (Turner, Wakley *et al.*, 1987). As a result of the opposing changes in radial growth and endocortical modeling, cortical bone volume generally does not decrease in ovariectomized rats (Turner, Wakley *et al.*, 1987; Turner, Colvard *et al.*, 1990). Cortical (and total) bone volume may, in fact, increase in rapidly growing rats following ovariectomy because the addition of extra bone to the periosteal surface of bone often exceeds the increase in endocortical resorption (Turner, Colvard *et al.*, 1990). The cellular mechanism for the differential response of the periosteal and endocortical bone surfaces of the midshaft of rat long bones appears related to the different populations of cells that comprise the two bone envelopes.

Diaphyseal osteoclasts are uncommon on the periosteum and are not notably increased following ovariectomy. This low level of resorption contrasts with the endocortical surface that undergoes bone modeling during growth, to increase the volume of the marrow cavity, and remodeling during adulthood, to repair microdamage and adapt to changing physiological needs (e.g., reproduction or changes in mechanical loading). In turn, endocortical bone turnover is generally much lower than cancellous turnover. Estrogen suppresses radial bone growth by inhibiting periosteal bone formation (Turner, Riggs *et al.*, 1994) while ovariectomy accelerates periosteal bone formation in growing and adult rats. Periosteal expansion occurs in aging humans and is the principal adaptive factor compensating for age-related endocortical and intracortical bone loss. However, this process does not appear to accelerate following menopause (Szulc *et al.*, 2006). The effects of menopause on intracortical (Haversian) bone remodeling have not been extensively investigated. The few studies reported to date suggest aging women have increased turnover of cortical bone, resulting in a negative cortical bone balance. However, the contribution of these changes to bone fragility is unknown.

Theoretically, it is possible to explore the significance of sex steroid action on cortical bone remodeling using animal models. Unfortunately, the skeletons of small rodents do not normally undergo extensive cortical bone remodeling. Cortical bone remodeling occurs in dogs and other large animals. Ovariectomy increases endocortical bone resorption surface and cortical porosity in dogs (Karambolova *et al.*, 1987): however, bone balance appears to be preserved because no net loss of cortical bone was reported (Martin *et al.*, 1987). Additional studies in large animals are necessary to adequately characterize the possible effects of sex steroids on Haversian bone remodeling. In this regard, a recent study suggests that ovariectomy increases periosteal bone turnover in aging monkeys (Bliziotes *et al.*, 2006).

As discussed earlier, estrogen deficiency owing to ovariectomy leads to a bone deficit in adult rodents and primates (Lindsay *et al.*, 1978; Jayo *et al.*, 1990; Kalu *et al.*, 1991). These changes are because of destructive endocortical bone modeling, and destructive cancellous bone modeling and remodeling (Turner *et al.*, 1987; Wronski, *et al.*, 1988). Destructive modeling and remodeling occur when bone resorption exceeds bone formation. Similar changes occur in rats (Goulding and Fisher, 1991) and monkeys

(Mann *et al.*, 1990) following administration of GnRH agonists, a chemical intervention that decreases serum estrogen levels comparable to ovariectomy. The important role of estrogen is further implicated by the observation that the skeletal changes following treatment of rats with ICI 182,780, a potent estrogen receptor antagonist, are similar to ovariectomy (Sibonga *et al.*, 1998). The conclusion that estrogen is critical is further supported by the consistent finding that the skeletal changes that follow ovariectomy are antagonized by pharmacological replacement with the hormone.

Ovariectomy results in (1) severe cancellous osteopenia in long bones and vertebrae of rats (Wronski *et al.*, 1989)*:* (2) strain-specific but generally mild to moderate osteopenia at these sites in mice (Iwaniec *et al.*, 2006)*:* and (3) variable bone loss in vertebrae of monkeys (Jerome *et al.*, 1986; Longcope *et al.*, 1989). The response to ovariectomy in dogs has been inconsistent, with no change (Snow and Anderson, 1985) and bone loss (Martin *et al.*, 1987) reported. The rate of bone loss from rat vertebrae occurs more slowly than from long bones (Wronski *et al.*, 1988; Wronski, *et al.*, 1989). Furthermore, there appears to be regional differences within bones. Cancellous osteopenia in rat tibia is much more prominent in the proximal metaphysis than the distal metaphysis or proximal epiphysis. However, it is important to note that the rate of bone loss may be related to differences in the cellular distribution within bone marrow and prevailing levels of mechanical loading (Westerlind *et al.*, 1997). Additional evidence suggests there is overlap between mechanical signaling and estrogen action on bone (Damien *et al.*, 1998; Tomkinson *et al.*, 1998). In addition, estrogen influences the differentiation and function of neurons, adipocytes, hematopoietic cells and immune cells, each of which, in turn, can affect bone cells. Thus, site-specific actions of estrogen on bone metabolism involve the integrated tissue-level actions of the hormone on a complex assortment of cells.

Ovariectomy increases osteoblast-lined perimeter, osteoclast-lined perimeter, and osteoclast size in long bones of rats (Wronski *et al.*, 1986; Turner *et al.*, 1988). There are simultaneous increases in the mineral apposition and bone formation rates, suggesting that ovariectomy results in high bone turnover. Cancellous bone turnover remains elevated in rats at some skeletal sites a year or more after ovariectomy (Wronski *et al.*, 1989; Sibonga *et al.*, 2000). Bone formation is increased in ovariectomized monkeys (Jerome *et al.*, 1986; Longcope *et al.*, 1989)*,* suggesting bone loss in nonhuman primates is also associated with increased bone turnover. Dempster *et al.* (1995) reported temporal changes in cancellous bone architecture of the distal rat femur after ovariectomy. These investigators demonstrated that the primary mechanism of ovariectomy-induced bone loss is osteoclastic perforation and removal of the trabecular plates. Generalized, gradual trabecular thinning is not observed. Interestingly, a similar process is responsible for removal of the most distal trabeculae during normal bone elongation (Turner, 1994).

Effects of Estrogen Replacement on Bone Metabolism in Ovariectomized Laboratory Animals

Estrogen replacement prevents the ovariectomy-induced increase in the size of the medullary cavity of a rat long bone by reducing osteoclast number (Turner, Wakley *et al.*, 1987). Similarly, estrogen replacement prevents increases in periosteal bone formation (Turner, Vandersteenhaven *et al.*, 1987), and, as a result, decreases bone cross-sectional area and cortical bone area. Because there are very few osteoclasts on the periosteal surface at most skeletal sites, it is unlikely this inhibitory effect of estrogen on bone formation is coupled to decreased bone resorption. Estrogen reduces the number of preosteoblasts in the S phase of the cell cycle, suggesting that the hormone inhibits the proliferation of osteoblasts. At the same time, estrogen decreases steady-state mRNA levels for bone matrix proteins and IGF-I, reduces bone matrix synthesis and osteoblast number, and increases the population of bone-lining cells (Turner *et al.*, 1990; Turner, *et al.*, 1992). Thus, estrogen influences bone growth by decreasing bone formation, as well as bone resorption.

In growing ovariectomized rats, estrogen replacement increases cancellous bone mass by inhibiting the resorption of primary spongiosa (Fig. 2b). In contrast, in adult ovariectomized rats estrogen replacement stabilizes cancellous bone volume by reducing bone turnover, reestablishing a neutral or positive balance between bone resorption and bone formation, and preventing the destruction of trabecular plates (Turner *et al.*, 1988; Wronski *et al.*, 1988; Kalu *et al.*, 1991; Wronski *et al.*, 1993). Estrogen accomplishes these actions, in part, by antagonizing the initiation of new sites of bone remodeling. Osteoclast number is reduced in estrogen-treated rats by a combination of decreased fusion of the osteoclast precursors (Turner, Evans *et al.*, 1994) and reduced osteoclast life span (Kameda *et al.*, 1997). The former may be important in preventing initiation of remodeling, whereas the latter may improve remodeling balance.

The effects of estrogen on cancellous bone formation involve direct as well as indirect actions of the hormone. There is general agreement that the inhibitory effects of estrogen on initiation of bone remodeling leads to an overall coupled decrease in bone formation as well as improved bone remodeling balance. The latter change may be owing to an increased osteoblast life span as well as the previously mentioned decreased osteoclast life span (Gohel *et al.*, 1999). Time course studies have shown that estrogen results in rapid decreases in mRNA levels for bone matrix proteins and collagen synthesis, suggesting that the hormone also has a direct inhibitory effect on bone formation (Turner, 1999). As a consequence of direct and indirect inhibition of bone formation, estrogen does not increase bone volume in adult rats with established bone loss (Wronski *et al.,* 1993).

The Mouse as a Model for Postmenopausal Osteoporosis

The ovariectomized rat is a well-characterized and validated preclinical model for postmenopausal osteoporosis (Iwaniec and Turner, 2008). Because of the proven value of the mouse as a genetic model, there has been great interest in the mouse as an alternative rodent model for postmenopausal bone loss. To date, the results have been discouraging. In general, ovariectomy results in accelerated cancellous bone turnover in mice (Beamer *et al.,* 1996; Gong *et al.,* 2001; Springer *et al.,* 2003; Forlino *et al.,* 2005). However, the magnitude of the change is strain-dependent and much less consistent than in rats (Kuivaniemi *et al.,* 1991; Clark and Rowe, 1996; Lazner *et al.,* 1999). The minute amount of cancellous bone found in mice, especially in long bones, is also a major disadvantage. This limitation is exasperated by age-related cancellous bone loss, which begins in some commonly studied mouse strains shortly after peak bone mass is achieved at around four months of age (Lazner *et al.,* 1999). The net result is too little starting cancellous bone at many important skeletal sites to accurately measure changes in indices of bone mass and turnover following ovariectomy. Unfortunately, one of the most challenging mice to evaluate is C57BL/6, a strain commonly used in genetic manipulations. This strain exhibits especially low cancellous bone volume. For example, the mean cancellous bone volume at the distal femur of seven-month-old ovary intact C57BL/6 mice is often as low as 3% (Iwaniec *et al.,* 2006). Measurement of dynamic histomorphometry and osteoblast perimeter and osteoclast perimeter is problematic because of the paucity of bone. To reliably detect bone loss following ovariectomy in skeletally mature C57BL/6 mice would require reducing the bone volume to near 0%, making it even more unlikely that measurement of fluorochrome labeling and bone cells will accurately reflect changes in bone turnover. Other than agents that induce *de novo* bone formation, it is unlikely that a model system in which there is so little cancellous bone can be reliably used to evaluate the efficacy of pharmacological interventions to restore bone mass in an osteopenic skeleton.

The limitations discussed here can be at least partially addressed by investigating skeletal sites, such as lumbar vertebrae, which have a higher cancellous bone volume. Even so, the vertebral bone loss 3 months following ovariectomy is modest, averaging about 27% (change in bone volume from ~12 to ~9%) (Iwaniec *et al.,* 2006).

There are major differences between human and mouse physiology regarding the actions of estrogens and estrogen analogs such as tamoxifen (Modder *et al.,* 2004). Administration of estrogen to mice induces endocortical bone formation often resulting in near total obliteration of the marrow cavity (Sims *et al.,* 2003) (Fig. 2c). This pathological condition does not occur in humans that overproduce estrogen or are treated with high levels of the hormone. Tamoxifen shows far less tissue discrimination in mice than in humans. These species differences often contraindicate the use of the mouse in studies in which it is important to accurately model the human skeletal response to estrogen (Table I).

Skeletal Phenotypes of Mice with Targeted Disruption of ER Genes

The development of mice exhibiting targeted gene deletions (knockout) in ERα, ERβ, or both genes simultaneously has proven to be a valuable tool for investigating ER signaling. As a note of caution, the mutant phenotype of knockout animals may result from multiple influences that occur as a consequence of the targeted mutation, and therefore the mechanisms responsible for the phenotype may not be restricted solely to the specific mutation. Additionally, disturbing ER signaling in all tissues from conception may destabilize the feedback loops regulating the synthesis of other sex steroids in addition to estrogen, thereby leading to changes in the circulating levels of these hormones, some of which also possess major activity on bone. For example, the ERα knockout female mouse has markedly elevated levels of estrogen, whereas the ERα knockout male has increased levels of testosterone (Couse and Korach, 1999). As a further complexity, phenotypes in single ER gene mutants may arise as a result of the β isoform being able or unable to compensate for the loss of the α isoform or, alternatively, suppress ERα activity. In the latter case, loss of ERβ could conceivably enhance rather than suppress estrogen responsiveness in a cell type that normally expresses both ER isoforms.

Severe loss of function mutations in ER isoforms resulting in tissue resistance to estrogens is very rare in humans and, therefore, is not a significant cause of osteoporosis. In contrast to ER knockout mice, women with postmenopausal osteoporosis have greatly reduced estrogen levels. Because of the substantial species-specific actions of sex steroids on skeletal development, growth and turnover (Table I), extrapolation of ER knockout data generated in mice to human physiology should generally be avoided.

Homozygous deletion of the ERα gene results in reduced cortical bone density and cortical bone formation in both male and female mice, suggesting that estrogen stimulates cortical bone formation via ERα (Korach *et al.,* 1997). There is delayed closure of the epiphyseal growth plate in ERα null mice. However, linear growth in long bones is generally reduced and the decrease is more pronounced in females (Korach *et al.,* 1997; Schmidt *et al.,* 2000). The effect of ERα ablation on cancellous bone is less certain. Using independently generated animals, cancellous bone has been either modestly decreased in both sexes (Korach *et al.,* 1997) or unaffected in females but showing an age-related increase in males (Sims *et al.,* 2003). In contrast to ER knockout mice, both a man with aromatase P450 deficiency (causing complete estrogen deficiency) and a man with a mutation in the ERα gene (causing partial estrogen resistance) exhibited continued longitudinal bone growth. The increased stature of these individuals was accompanied by delayed epiphyseal growth plate closure. In addition, these men had marked cancellous osteopenia and elevated biochemical markers for bone remodeling (Smith *et al.,* 1994; Carani *et al.,* 1997). Consequently, the lack of marked cancellous bone loss suggests that in mice ERβ compensates for loss of ERα.

Female ERα knockout mice lose bone following ovariectomy to the same extent as the wild-type mice. However, estrogen responsiveness in the ERα knockout female appears to be reduced, because higher estrogen concentrations were required to prevent bone loss compared to ovariectomized wild-type mice (Ederveen and Kloosterboer, 1999).

Similar to ERα deletion, deletion of the ERβ gene results in delayed growth plate closure. However, in adult female ERβ knockout mice, the longitudinal bone growth for some long bones is increased (Windahl *et al.,* 1999). Studies suggest the cortical and cancellous bone parameters of male ERβ knockout mice do not differ from wild-type mice (Vidal *et al.,* 2000). In contrast, female ERβ knockout mice exhibit increased whole body bone mineral content owing to increased cortical bone, although this is only observed in the postpubertal stage (Windahl *et al.,* 1999). The increased cortical bone results from elevated periosteal (radial) bone formation, suggesting that ERβ is required for pubertal feminization of the mouse skeleton.

Older (~4 months to 1 year old) female ERβ knockout mice are partially protected against age-related cancellous bone loss, as these animals exhibit higher cancellous bone volume than either male ERβ knockout or wild-type mice (Ederveen and Kloosterboer, 1999; Windahl *et al.,* 2000). The increase in cancellous bone is thought to be owing to enhanced bone formation rather than to changes in growth plate kinetics or altered bone resorption because osteoblast markers and ERα transcript levels are elevated (Windahl *et al.,* 2000). Increased cancellous bone in female ERβ knockout mice may be caused by removal of the dominant-negative function of ERβ on ERα activity (Sims *et al.,* 2002). Female ERβ knockout animals lose cortical and cancellous bone after ovariectomy similarly to wild-type mice (Windahl *et al.,* 1999). Moreover, estrogen treatment prevents ovariectomy-induced bone loss in ERβ knockout female mice to the same extent as in wild-type mice (Ederveen and Kloosterboer, 1999; Ke *et al.,* 2002). Thus, ERβ seems to be nonfunctional in the skeleton of male mice, whereas in females, ERβ mediates the estrogen-induced suppression of periosteal bone growth. In female mice, ERβ is not required for the maintenance of cancellous bone in the presence of ERα, but is required in the absence of ERα. Supporting the hypothesis that ERβ does not mediate estrogen responses in cancellous bone in males, testosterone, but not estrogen, completely prevented the decrease in bone at cancellous sites in orchidectomized ERα knockout males (Vandenput *et al.,* 2001). However, if ERβ is truly nonfunctional in the male mouse skeleton, it is not clear why the ERα knockout male does not exhibit estrogen resistance, characterized by elevated bone turnover rates and osteopenia, rather than the unaltered or slightly increased cancellous bone characteristic of the knockout phenotype (Sims *et al.,* 2003).

Deletion of both ER isoforms in double (ERα/β) knockout mice generates a skeletal phenotype in the male very similar to that caused by ERα single deletion; namely reduced postpubertal longitudinal and periosteal (cortical) bone growth and normal cancellous bone (Vidal *et al.,* 2000). Interestingly, the reduced bone growth in the male knockout has been correlated with decreased serum IGF-1 levels (Vidal *et al.,* 2000). Similarity of the skeletal defects resulting from ERα or ERα plus ERβ gene inactivation in the male knockout is further evidence for the lack of involvement of the β isoform in growth and remodeling of the male skeleton.

In contrast to knockout males, the bone defects in the ERα/β knockout female mice are distinct from those produced by deletion of either ER isoform alone, thus supporting a role for both isoforms in the female skeleton. In postpubertal ERα/β knockout females, cortical thickness and cancellous bone are reduced because of decreased bone formation (Sims *et al.,* 2002). Both ER isoforms participate in normal cancellous bone remodeling. Because female ERα knockout animals fail to exhibit cancellous bone loss, ERβ in females can compensate for the loss of ERα.

Overall, the data suggest ERα and ERβ likely perform different functions in cortical and cancellous bone and that the relative importance of the two isoforms differs between the sexes. However, it is unclear whether the ER isoforms perform identical functions during skeletal growth and turnover in humans. Perhaps the most surprising result arising from the ER knockout analysis is that double ERα/β knockout mice are viable with grossly normal skeletons, demonstrating that both ER isoforms are dispensable for the normal development of cartilage and bone in mice.

Effects of Estrogen on Bone Cells *in Vitro*

Estrogen Regulation of Early and Late Response Genes

As outlined earlier, sex steroids regulate the expression of early response genes within 30 minutes to 2 hours following hormone exposure. The best examples of this are the nuclear proto-oncogenes, including c-myc, c-jun, jun-B, c-fos, N-myc, and the TGFβ-inducible early gene, TIEG (Spelsberg *et al.,* 1992; Tau *et al.,* 1998). These early response genes are expressed and regulated by sex steroids in many different reproductive (Murphy *et al.,* 1987; Fink *et al.,* 1988) and skeletal tissues (Eriksen *et al.,* 1988; Oursler *et al.,* 1991; Oursler *et al.,* 1994; Harris *et al.,* 1995; Tau *et al.,* 1998; Monroe, Getz *et al.,* 2003; Monroe, Johnsen *et al.,* 2003; Monroe *et al.,* 2006; Hawse *et al.,* 2007). Thus, these genes are excellent markers for steroid actions and interactions. Because early response genes are regulated by steroids in all tissues containing steroid hormone receptors, and most of these early gene products in turn regulate the gene expression of late genes, a role for nuclear protooncogenes as early response (regulatory) genes in a cascade model of steroid action in bone and reproductive tissues has been proposed (Landers and Spelsberg, 1992; Spelsberg *et al.,* 1992).

In contrast to these rapidly induced genes, which are universal markers of steroid actions, many late responsive (24–48 hour) structural genes are specific to different cell types and some, like the growth factor/cytokine genes, mediate the effects of estrogen on various target cells and tissues. Control of late gene expression by estrogen has been investigated in numerous osteoblast cell culture systems. Unfortunately, despite extensive efforts, only a few putative target genes have shown consistent regulation. The most reliable responses are increases in type I collagen, transforming growth factor (TGF)β, and insulin-like growth factor (IGF)-I production (for a review, see Spelsberg *et al.,* 1999). In fact, estrogen has been shown to induce TGFβ production by both osteoblasts and osteoclasts (Oursler, 1998). These findings suggest estrogen has a direct bone anabolic effect on cultured osteoblasts. TGFβ plays an important role in chondrogenesis and TGFβ and IGF-I promote the proliferation and differentiation of osteoblast precursors. Under some conditions, TGFβ inhibits the formation of osteoclasts. In addition to regulating IGF-I synthesis, estrogen may modulate IGF bioactivity by regulating the production of IGF-binding proteins, including IGFBP-2, -3, and -4, which in turn enhance or suppress the activities of IGF-I and IGF-II. TGFβ and IGF-I are both sequestered in bone matrix at relatively high concentrations. See Fig. 4 for a detailed list of estrogen responses in cultured osteoblasts and osteoclasts.

In vivo studies in humans and most animal models provide overwhelming evidence that estrogen inhibits intramembranous as well as endochondral bone growth, and as a consequence suppresses bone formation and reduces peak bone mass. Although less completely characterized, the effects of estrogen on the expression of early and late response genes in skeletal tissues *in vivo* follows a time course similar to that in cultured osteoblasts (Turner *et al.,* 1999). However, in contrast to its action *in vitro*, in rats estrogen reduces mRNA levels for bone matrix proteins and decreases bone collagen synthesis. The large discrepancy between osteoblast culture and *in vivo* suggests that the direct actions of the hormone on osteoblasts are modified by other cells and regulatory pathways that are not present in the cell culture environment. As a consequence, cell culture data, although essential for characterizing molecular pathways of action, should not be relied upon to accurately predict the *in vivo* actions of estrogen on bone metabolism.

Estrogen Regulation of Cytokine Synthesis

A hallmark of estrogen deficiency is an elevated rate of bone turnover in which both osteoclast-mediated bone resorption and, perhaps secondarily, osteoblast-mediated bone formation occurs at an increased rate. Physiologically, reduced estrogen levels during pregnancy and lactation may facilitate mobilization of calcium stores from bone for deposition into the fetal and neonatal skeleton, respectively. There is evidence to suggest that release of an estrogen-imposed suppression of bone-resorbing cytokine synthesis plays a role in the accelerated bone turnover. The osteoblast and/or monocyte/macrophage lineage produces cytokines capable of increasing osteoclast formation, differentiation, or activity that are inhibited by estrogen. However, the cytokines that are most important for mediating the skeletal actions of estrogen remain elusive. One hypothesis proposed that bone marrow monocyte/macrophage-derived interleukin (IL-1) and tumor necrosis factor (TNF) induce the production of other cytokines, including macrophage colony-stimulating factor (M-CSF): granulocyte-macrophage CSF (GM-CSF): and IL-6 by osteoblasts and hematopoietic cells, which together promote osteoclast resorptive activity. Evidence in support of this pathway is as follows: (1) release of IL-1, TNF, and GM-CSF by peripheral blood monocytes from untreated osteoporotic women is higher than from estrogen-treated osteoporotic and nonosteoporotic women (Pacifici *et al.,* 1989; Pacifici *et al.,* 1991): (2) production of M-CSF by osteoblastic/stromal cells is increased by ovariectomy in mice and is dependent on bone marrow synthesis of IL-1 and TNF (Kimble *et al.,* 1996): (3) neutralization of TNF activity either by the inhibitory TNF-binding protein (TNFbp) or by expression of an inhibitory soluble TNF type 1 receptor in mice prevents ovariectomy-induced bone loss (Ammann *et al.,* 1997*)*: (4) type 1 IL-1 receptor-null mice fail to lose bone following ovariectomy (Lorenzo *et al.,* 1998): and (5) simultaneous blockade of both IL-1 and TNF activity by IL-1 receptor antagonist (IL-1ra) and TNFbp completely prevents ovariectomy-induced bone loss in rats (Kimble *et al.,* 1995; Kimble *et al.,* 1997).

An alternative proposed pathway favors IL-6 as the mediator of bone loss in an estrogen-deficient state. Evidence for this pathway is as follows: (1) production of IL-6 is inhibited by estrogen in cells of the stromal/osteoblastlineage *in vitro* (Girasole *et al.,* 1992; Passeri *et al.,* 1993): although this is not a universal finding (Rickard *et al.,* 1992; Rifas *et al.,* 1995): (2) the production of IL-6, as well as the number of osteoclast precursors and osteoclast formation is increased in bone marrow cultures from ovariectomized mice compared to sham control animals (Miyaura *et al.,* 1995): (3) all of these effects, as well as the increased population of osteoclasts present in cancellous bone of ovariectomized mice, can be prevented by treatment with estrogen or an anti-IL-6 antibody (Jilka *et al.,* 1992): (4) estrogen may reduce the responsiveness of osteoblast lineage cells and possibly also osteoclasts to IL-6 by decreasing expression of the ligand-binding gp80 and signal-transducing gp130 subunits of the IL-6 receptor (Lin *et al.,* 1997): and (5) IL-6-deficient mice are protected from ovariectomy-induced bone loss (Poli *et al.,* 1994).

Other factors such as osteoprotegerin (OPG), a soluble member of the TNF receptor family, may also mediate the inhibitory effect of estrogen on bone resorption. OPG binds to and inhibits the activity of the endogenous ligand of RANK (RANKL), a TNF-related cytokine produced by bone marrow stromal cells, osteoblasts, megakaryocytes, mast cells, and B cells (Bord *et al.,* 2004; Ali *et al.,* 2006; Kawai *et al.,* 2006; Rauner *et al.,* 2007), which is essential for osteoclast formation. Deletion of the OPG gene in mice generates severe osteopenia in both cancellous and cortical bone (Bucay *et al.,* 1998*):* whereas mice overexpressing OPG develop osteopetrosis and do not lose bone as a result of estrogen deficiency (Simonet *et al.,* 1997). Estrogen increases OPG expression in osteoblasts overexpressing the ERα gene (Hofbauer *et al.,* 1999). This effect may be mediated by TGFβ, which has been shown to increase OPG production by bone marrow stromal osteoblast precursor cells (Takai *et al.,* 1998). Although OPG may be a key mediator of the antiresorptive effects of estrogen, compelling *in vivo* evidence to support this hypothesis is lacking. In fact, OPG levels are increased in women following menopause and are decreased in postmenopausal women treated with estrogen (Khosla *et al.,* 2002; Han *et al.,* 2005).

The studies described here focused on respective roles of osteoblasts and monocytes as sources of proinflammatory cytokines such as IL-1, IL-6, and TNFα. More recent work suggests that other cells may play an important and perhaps predominant role in their generation. These include T cells, mast cells, and adipocytes. T cells and mast cells differentiate from bone marrow-derived hematopoietic stem cells, whereas adipocytes differentiate from bone-marrow derived mesenchymal stem cells (Blair *et al.,* 2007; Hallgren and Gurish, 2007). Estrogen deficiency increases the populations of each of these cell types within the marrow cavity. In addition to the bone-regulating cytokines discussed so far, T cells and mast cells produce RANKL (Ali *et al.,* 2006; Kawai *et al.,* 2006) and mast cells produce histamine and heparin. Histamine-deficient mice are resistant to bone loss following ovariectomy and heparin is an endogenous inhibitor of OPG (Fitzpatrick *et al.,* 2003; Irie *et al.,* 2007).

Adipocytes, in addition to being important sources of proinflammatory cytokines, produce numerous additional adipokines, several of which have been shown to influence bone metabolism by acting directly on bone cells or by acting through the sympathetic arm of the central nervous system. The bone active adipokines include adiponectin (Luo *et al.,*2006*):* resistin (Thommesen *et al.,*2006*):* visfatin (Xie *et al.,* 2007*):* and leptin. The most well studied of the adipokines is leptin. Leptin-deficient ob/ob mice are obese and have bone compartment-specific abnormalities in bone mass and architecture (Hamrick *et al.,* 2004). Although infertile, these mice have normal or elevated cancellous bone mass. Central leptin gene therapy reverses the skeletal abnormalities in growing ob/ob mice without increasing circulating levels of the hormone (Iwaniec *et al.,* 2007). Some studies suggest that leptin inhibits bone formation by increasing sympathetic tone (Karsenty, 2001). However, this proposed mechanism is not supported by the observations that leptin increases bone growth (Steppan *et al.,* 2000) and that neonatal sympathectomy does not alter bone growth (Hill *et al.,* 1991). Furthermore, leptin receptors are located on chondrocytes and osteoblasts (Steppan *et al.,* 2000; Iwamoto *et al.,* 2004) and systemic leptin was found to reduce bone loss in rats following ovariectomy (Burguera *et al.,* 2001). Thus, leptin can potentially have direct effects on bone and cartilage cells, as well as indirect effects that are mediated through the central nervous system. Although no consensus exists regarding the precise role of leptin in regulating bone metabolism, most studies are consistent with the hypothesis that leptin is primarily a permissive factor. According to this view, leptin levels reflect energy reserves and low circulating levels of the hormone initiate adaptive mechanisms to cope with starvation. These adaptations include reduced gonadal hormone secretion that prevent pregnancy and decreased bone growth and turnover (Hamrick, 2004).

Although generally not considered a cytokine, the skeletal growth factor IGF-I functions as an autocrine, paracrine, and hormonal factor. IGF-I is arguably the most important physiological regulator of bone growth and turnover in the postnatal skeleton. IGF-I is produced not only by osteoblasts but also by adipocytes (Zizola *et al.,* 2002). Adipocytes reside within the bone marrow compartment and the local concentration of IGF-I in the vicinity of osteoblasts and osteoclasts could exceed the circulating levels of the growth factor. The principle regulator of systemic and local IGF-I is growth hormone (LeRoith and Yakar, 2007). Growth hormone secretion, in turn, is regulated by estrogen (Meinhardt and Ho, 2006). Hypophysectomy, in addition to preventing the synthesis of growth hormone, results in gonadal insufficiency comparable to ovariectomy. In contrast to ovariectomy, hypophysectomy results in greatly decreased bone turnover and cessation of bone growth

(Yeh *et al.,* 1997). Administration of estrogen to hypophysectomized rats has minimal effects on bone metabolism (Kidder *et al.,* 1997; Yeh *et al.,* 1997). Thus, the actions of sex steroids on the skeleton require growth hormone/IGF-I signaling. Based on the dramatic skeletal effects of growth hormone and the knowledge that there are gender differences in its regulation, it is surprising that the role of growth hormone/IGF-I signaling in the etiology of postmenopausal osteoporosis has received so little attention.

In summary, there is no shortage of bone active cytokines, hormones, and skeletal growth factors whose synthesis is influenced by estrogen. The data do not favor the regulation of a single agent or a single cell lineage as responsible for mediating the physiological actions of estrogen on the skeleton. It seems more likely that multiple cytokines, hormones, and growth factors produced by a plethora of cells contribute to the complexity of estrogen action on bone.

Molecular Mechanism of Estrogen Regulation

The molecular mechanisms involved in the estrogen regulation of several cytokine/late gene promoters produced by bone cells have been elucidated. Many of these promoters lack consensus estrogen response elements (EREs) and thus have revealed alternative mechanisms of transcriptional control by the ER. In some instances, these do not require direct binding of the ligand-activated ER to DNA. Instead, ER interacts with other transcription factors and coregulators to modulate the binding affinity of these proteins to their cognate regulatory sites in the DNA. For example, repression of the IL-6 promoter by estrogen occurs because protein-protein interactions between the ligand-activated ERα and NF-*K*B and C/EBPβ prevent binding of the latter factors to their respective sites on DNA (Stein and Yang, 1995; Galien *et al.,* 1996). In contrast, estrogen stimulates transcription by the IGF-I promoter via enhanced binding of fos/jun heterodimers to AP-1 elements by interaction with activated ERα (Umayahara *et al.,* 1994). Numerous genes, including retinoic acid receptor-α1 (RAR-α1): c-fos protooncogene, cathepsin D, IGFBP-4, and the epidermal growth factor receptor gene, among others, are modulated by estrogen through the binding of ER/Sp1 transcription factor complexes with either Sp1 sites alone or Sp1/ERE half-sites (Krishnan *et al.,* 1994; Rishi *et al.,* 1995; Salvatori *et al.,* 2000).

Estrogen Regulation of Bone Cell Metabolism

Similar to the conflicting reports on the estrogen regulation of late response genes in cultured osteoblasts, there is a lack of a general consensus with regard to the effects of estrogen treatment on osteoblast proliferation. Estrogen stimulates cell proliferation in primary and immortalized fetal rat calvarial cells (Ernst *et al.,* 1989): normal adult human osteoblasts (Scheven *et al.,* 1992): mouse MC-3T3E1 cells (Majeska *et al.,* 1994): and synchronized human osteosarcoma cells (Ikegami, Inoue et al., 1994). Other investigators report no effect of the hormone on primary cultures of osteoblasts from normal adults (Keeting *et al.,* 1991; Rickard *et al.,* 1993) and no alteration of cell growth in osteoblasts stably transfected with the ERα gene (Watts *et al.,* 1989; Kassem *et al.,* 1996; Robinson *et al.,* 1997). *In vivo*, estrogen decreases osteoblast proliferation in cortical and cancellous bone compartments of growing female rats (Turner *et al.,* 1992; Westerlind *et al.,* 1993; Turner *et al.,* 1999).

The effect, if any, of estrogen on the proliferation of less differentiated osteoblast progenitor cells is unclear. The proliferation rate of bone marrow-derived osteoblast precursor cells, isolated from ovariectomized rats, increased compared to cells from sham-operated animals (Modrowski *et al.,* 1993). However, the *in vitro* treatment of rat bone marrow stromal cells with estrogen showed no effect on cell growth (Rickard *et al.,* 1995).

In vivo, osteoblast and osteoclast formation is tightly coupled owing, at least in part, to the dependence of osteoclast precursor development on support from cells of the stromal/osteoblast lineage through the provision of cytokines and other stimuli, and of osteoblast precursor development on factors released from bone matrix by osteoclast activity. As discussed earlier, estrogen deficiency causes not only increased osteoclast formation possibly as a result of increased production of bone-resorbing cytokines, but also stimulates osteoblast formation (Turner *et al.,* 1992; Turner *et al.,* 1993; Westerlind *et al.,* 1997). In support of this, ovariectomy in rodents increases the number of bone marrow-derived osteoblast progenitor cells (Scutt *et al.,* 1996; Jilka *et al.,* 1998). In mice, this increase was transient and partially independent of increased bone resorption, suggestive of a possible direct action of estrogen on osteoblast proliferation and/or differentiation (Jilka *et al.,* 1998). Such mechanisms may partially account for the elevated bone turnover immediately after ovariectomy/menopause. However, estrogen has been shown to have inhibitory effects on bone formation in rats that occur much too quickly to be caused by a prior decrease in bone resorption (Turner *et al.,* 1999). Based on biomarkers, the stimulated rates of bone remodeling owing to gonadal steroid deficiency are thought to be only temporary and with time after menopause bone remodeling declines to levels approximating premenopausal rates. However, this hypothesis has never been verified by tissue level (histomorphometric) analysis. In this regard, no age-related decrease in bone formation was detected in iliac crest biopsies of healthy postmenopausal women (Turner, unpublished data). In rats, long-term effects of ovariectomy on bone re-modeling are site-specific; a transient increase in bone turnover was noted at the proximal tibial metaphysis, whereas bone turnover remained greatly elevated in the epiphysis. Biomechanical signaling may explain the divergent responses of the two bone compartments to estrogen deficiency. Metaphyseal bone, at least in rodents, functions primarily in mineral homeostasis. In contrast, the structural function of the epiphysis greatly outweighs its role in mineral homeostasis. Estrogen deficiency results in elevated turnover of the metaphysis and epiphysis but the higher strain energy levels of the latter bone appear to protect against bone loss. Harold Frost suggested that estrogen influenced the set point of the mechanical sensing system in bone (Frost, 1987). In this regard, recent studies suggest that the mechanical transduction system of bone is dependent upon ER signaling (Lee *et al.,* 2004; Zaman *et al.,* 2006; Armstrong *et al.,* 2007; Saxon *et al.,* 2007).

Estrogen has opposite effects on apoptosis in osteoclasts and osteoblasts. In osteoclasts, apoptosis is stimulated by estrogen via a TGFβ-mediated mechanism (Hughes *et al.,* 1996; Kameda *et al.,* 1997). However, apoptosis is prevented by estrogen in osteocytes (Tomkinson *et al.,* 1997; Tomkinson *et al.,* 1998) and other osteoblast lineage cells (Gohel *et al.,* 1999; Manolagas, 1999). Physiologically, osteoclast apoptosis plays an important role in the rapid removal of osteoclasts during the bone remodeling reversal at termination of lactation (Miller and Bowman, 2007). Thus, the enhancement of osteoclast apoptosis could contribute to the antiresorptive effects of estrogen.

The physiological significance of a decrease in apoptosis in osteoblast linage cells is less clear. The great majority of osteoblast lineage cells are osteocytes and bone lining cells, cells that are not producing bone matrix. As a consequence, prevention of apoptosis in osteocytes and bone lining cells should have no direct effect on bone formation. At present, there is no evidence to suggest that apoptosis occurs in osteoblasts actively synthesizing bone matrix. Therefore, an increase in apoptosis of osteoblast lineage cells during estrogen deficiency may be associated with a reduced interval of bone matrix synthesis by osteoblasts. However, the deficiency in bone formation resulting from a reduced osteoblast life span would have to compensate for the observed increased rate of matrix production by osteoblasts following ovariectomy (Turner *et al.,* 1992; Turner *et al.,* 1993).

Increased apoptosis of osteocytes may play an important indirect role in mediating estrogen-induced bone loss by promoting increased bone turnover (Tomkinson *et al.,* 1998). Also, apoptosis may play an especially important role during cell turnover by limiting the number of daughter cells that undergo further differentiation, for example in the proliferative zone of the growth plate (Sibonga *et al.,* 2002). However, the level of estrogen regulation of apoptosis on the differentiation of mesenchymal stem cells to osteoblasts has not been extensively evaluated.

Recent studies by Kato and coworkers (Nakamura *et al.,* 2007) using a Cre-lox osteoclast-specific knockout of ERα, show that ERα located in osteoclasts is required for maintenance of cancellous bone in female, but not male mice. In addition to indirect effects of estrogen on the formation, differentiation, and activity of osteoclasts via regulation of cytokine synthesis by osteoblasts, estrogen has direct effects on osteoclasts and their precursors (see Fig. 3). Early studies demonstrated not only the presence of ERα in osteoclasts, but also the regulation of gene expression by estrogen in these cells (Oursler *et al.,* 1993; Oursler *et al.,* 1994). Subsequent studies have shown that the bone-resorbing activity of avian, rabbit, and human osteoclasts

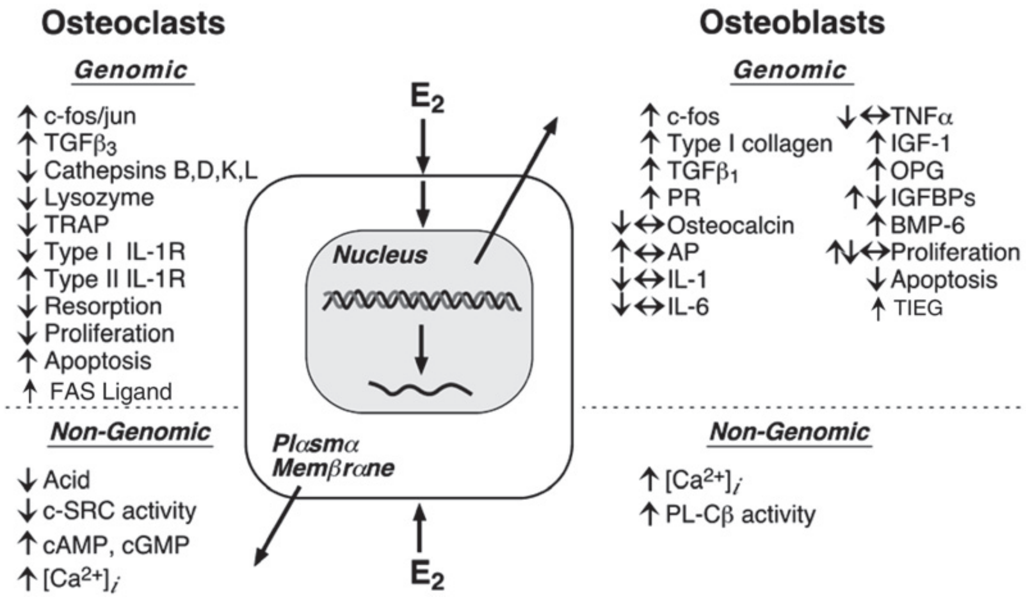

FIGURE 3 Model outlining the reported effects of estrogens on osteoblast and osteoclast functions. Arrows indicate the changes induced by estrogen. Double/triple arrows indicate published differences in reported responses.

is decreased by estrogen treatment, which is owing, in part, to a reduced population of osteoclasts via apoptosis (Hughes *et al.,* 1996; Kameda *et al.,* 1997). Estrogen may further inhibit bone resorption by acting directly on osteoclast progenitors to reduce osteoclast formation, as demonstrated by (1) the ability of estrogen to reduce proliferation and increase apoptosis of FLG 29.1 preosteoclastic cells (Zecchi-Orlandini *et al.,* 1999)*:* (2) the antagonism by estrogen of the differentiation of murine hemopoietic blast cells into multinucleated osteoclasts induced by GM-CSF and PTH/PTH-related peptide (Kanatani *et al.,* 1998)*:* and (3) the finding that estrogen suppresses the production of TRAP-positive, multinucleated osteoclasts from primary murine bone marrow myeloid progenitors and the mouse monocytic cell line RAW 264.7 cultured in the presence of soluble RANKL and M-CSF, but in the absence of stromal/osteoblast support cells (Shevde *et al.,* 2000). This latter effect of estrogen may be owing to downregulation of RANKL-induced JNK activity in osteoclast progenitors (Srivastava *et al.,* 2001).

Estrogen inhibits the expression/secretion of matrix-degrading agents by isolated osteoclasts, including acid and the lysosomal enzymes cathepsin B, D, K, and L, and lysozyme (Kremer *et al.,* 1995; Mano *et al.,* 1996). For a review, see Oursler (1998). Similar to the studies on osteoblasts, the effects of estrogen on isolated avian osteoclasts *in vitro* appear to be determined by the level of ERα (Pederson *et al.,* 1997). Furthermore, the antiresorptive effects of estrogen on osteoclasts may, in part, be mediated by reducing the responsiveness of osteoclasts to resorption-stimulatory cytokines through the modulation of cytokine receptor levels and/or postreceptor signaling. For example, the IL-1 pathway is a stimulator of bone resorption. In isolated human osteoclasts, estrogen directly reduces mRNA expression of the signaling type I IL-1 receptor while simultaneously increasing levels of the decoy type II IL-1 receptor and release of soluble IL-1RII (Sunyer *et al.,* 1999).

Nongenomic Effects of Estrogen on Osteoblasts and Osteoclasts

The cascade model explains many apparent nongenomic actions of steroids that are, in fact, genomic processes. However, there is a growing body of evidence that steroids also influence the cell surface by nongenomic effects, which involve responses to steroids that are too rapid to be explained by genomic processes (Moss *et al.,* 1997; Revelli *et al.,* 1998). For instance, a possible cardioprotective effect of estrogen has been shown to occur through the induction of endothelial nitric oxide synthase in human vascular endothelial cells via a ligand-dependent interaction between ERα and phosphatidylinositol-3-OH kinase (Simoncini *et al.,* 2000). In osteoblasts, treatment with estrogen has been shown to cause a rapid (within seconds) increase in intracellular calcium ion concentration owing to influx across the plasma membrane and release from intracellular stores (Lieberherr *et al.,* 1993). Additionally, estrogen stimulates the hydrolysis of phosphatidylinositol phospholipids with the generation of diacylglycerol and inositol 1,4,5-trisphosphate, which in turn activate other second messenger pathways. In female rat osteoblasts, this effect of estrogen is mediated by the activation of phospholipase C-β2 via a pertussin toxin-sensitive G protein β subunits, further supporting the existence of a nonclassical membrane ER (Le Mellay *et al.,* 1999). Activation of MAP kinases and increases in cAMP and cGMP has been reported in osteosarcoma cells (Endoh *et al.,* 1997).

Several nongenomic effects of estrogen in osteoclasts have been reported. Treatment of isolated chick osteoclasts with estrogen inhibited the basal and PTH-stimulated production of acid within 15 minutes (Gay *et al.,* 1993). This effect appears to be mediated at the plasma membrane because membrane-impermeable conjugates of estrogen produced the same response (Brubaker and Gay, 1994). Estrogen treatment of avian osteoclasts is also associated with rapid and transient changes in cell shape and level of phosphotyrosine proteins at the plasma membrane (Brubaker and Gay, 1999). One of the phosphorylated proteins has been identified as pp60src, a tyrosine kinase required for the bone resorptive function of osteoclasts. Treatment with estrogen affects the degree of phosphorylation, and hence activity, of *src* as well as its subcellular distribution (Pascoe and Oursler, 2001). However, translocation of the *src* protein both to (Brubaker and Gay, 1999) and away (Pascoe and Oursler, 2001) from the cell surface has been reported. Active *src* kinase phosphorylates many substrates, including ras and the MAP kinases, which are stimulated via a nongenomic pathway in estrogen-treated MCF-7 breast cancer cells and rat osteosarcoma cells (Migliaccio *et al.,* 1996; Endoh *et al.,* 1997). Interestingly, constitutive activation of *src* decreases lysosomal enzyme secretion similar to treatment with estrogen, further implicating *src* as an intermediate in the action of estrogen in osteoclasts (Pascoe and Oursler, 2001). Finally, specific cell surface binding sites for estrogen have been reported in chick osteoclasts and FLG 29.1 human preosteoclastic cells in which treatment with estrogen affected intracellular calcium ion concentrations and pH within seconds to minutes (Brubaker and Gay, 1994; Fiorelli *et al.,* 1996).

The nongenomic effects of estrogen have been attributed to classical ER residing in cell membranes as well as to nonclassical receptors (de Wilde *et al.,* 2006; Heberden *et al.,* 2006). In the latter case, estrogen can interact with a variety of proteins. For example, estrogen activates signal transducer and activator of transcription-1 (STAT-1) in human fetal osteoblasts not having classical ER by a mechanism dependent on *src* kinase activity (Kennedy *et al.,* 2005). Also, estrogen can be metabolized to metabolites that act through alternative pathways. In some cases these involve receptors for other classes of sex steroids.

For example, the prodrug tibolone is metabolized in a tissue-specific manner to metabolites that interact with ER, androgen receptor, and PR (Kloosterboer, 2004). Physiologically, testosterone is metabolized in a tissue-specific manner to estrogen and dihydrotestosterone, metabolites that interact with ER and androgen receptor, respectively. Additionally, estrogen is metabolized to 2-methoxyestradiol (2ME) by two sequential enzymatic reactions, hydroxylation of the 2 position on the A ring followed by methylation of the 2-position, which leads to loss of binding of the molecule to ER but confers novel activity. 2ME activates non-ER-mediated cell-specific signaling pathways. Although having no detected effects on primary osteoblasts, 2ME induced interferon gene expression and apoptosis in cultured osteosarcoma cells (Maran *et al.*, 2002). Additionally, double-stranded RNA-dependent protein kinase is involved in 2ME-mediated cell death of osteosarcoma cells (Shogren *et al.*, 2007). Importantly, these effects were not mimicked by estrogen or inhibited by the antiestrogen ICI 182,780. *In vivo*, 2ME slows bone loss in ovariectomized rats (Sibonga *et al.*, 2003). In cell culture, 2ME inhibits differentiation and is cytotoxic to osteoclasts. The 2ME-mediated decrease in osteoclast survival was partially inhibited by anti-lymphotoxinβ antibodies, suggesting that 2-ME-dependent effects involve lymphotoxinβ (Maran *et al.*, 2006). Although the biological significance of endogenously produced 2ME remains to be determined, the anti-tumor, anti-angiogenic, and anti-osteoclastic actions of this estrogen metabolite are under investigation for its therapeutic potential for treatment of primary cancer and tumor metastasis to bone (Cicek *et al.*, 2007).

A nongenomic mechanism involving the *src* tyrosine kinase and MAP kinase signaling pathway has been reported to mediate the anti-apoptotic effects of estrogen on primary and immortalized osteoblastic and osteocytic cells (Kousteni *et al.*, 2001). Intriguingly, the anti-apoptotic effect of both estrogen and the androgen dihydroxytestosterone were mediated equivalently via both ER receptors or by the androgen receptor (AR). Furthermore, only the ligand-binding domain of the receptor protein appeared to be required and the anti-apoptotic function was dissociated from the receptors' transcriptional activity (Kousteni *et al.*, 2001). One possible interpretation of these observations is that ER and/or AR proteins localized at the plasma membrane exhibit unique properties and perform distinct functions from that of the nuclear localized receptor. A compound, 4-estren-3alpha,17beta-diol (estren) was reported to selectively activate the nongenomic pathway and prevent bone loss in mice (Kousteni *et al.*, 2002). However, subsequent studies have shown that estren activates ER-mediated genomic pathways and also acts through androgen receptors (Krishnan *et al.*, 2005; Windahl *et al.*, 2006). Many of the reported tissue selective effects of estren, whereby the compound is more active on bone than in the uterus, are consistent with the well-established actions of weak estrogens (Hewitt *et al.*, 2006). Furthermore, osteoblasts produce the potent androgen 19-nortestosterone from estren by way of a 3α-hydroxysteroid dehydrogenase-like activity (Centrella *et al.*, 2004).

In summary, the response of the target cell to estrogen may include both genomic and nongenomic effects, although the physiological importance of the nongenomic effects is still unclear. The putative membrane receptors for steroid hormones remain unknown and only a few non-ER alternative targets for estrogen have been identified.

Effects of SERMs, Estrogen Metabolites, and Phytoestrogens

SERMs

Despite the success of hormone replacement therapy in the treatment of postmenopausal osteoporosis, prolonged treatment with estrogen, even when supplemented with a progestin, is associated with an increased risk of heart disease, and breast and uterine cancer. Consequently, there has been a tremendous effort to develop synthetic tissue-specific, partial estrogen agonists that possess the desirable estrogen agonist properties in bone without the undesirable growth-promoting effects in reproductive tissue. The benzothiophene derivative raloxifene (LY139481 HC1) largely satisfies these criteria in both laboratory animals and postmenopausal women.

Although raloxifene binds with similar affinity to ERα and ERβ (Kuiper *et al.*, 1998) the compound induces distinctly different conformational changes in ERα (Brzozowski *et al.*, 1997) and ERβ (Paige *et al.*, 1999) when it binds to the ligand-binding domain. The fact that the conformational differences affect the coregulatory factors recruited by each receptor provides a molecular explanation for the divergent effects of estrogen and raloxifene on gene transcription from various estrogen-inducible promoters (e.g., AP-1 sites) depending on which ER isoform is present (Paech *et al.*, 1997; Watanabe *et al.*, 1997). The tissue-selective responses to raloxifene and other partial estrogen agonists may be explained by a combination of the relative level of ERα to ERβ, the repertoire of coregulators present in the cells (described earlier) and the type of DNA response element present in the promoter.

In the ovariectomized rat model of postmenopausal osteoporosis, raloxifene prevented bone loss from sites of both cancellous and cortical bone and reduced serum cholesterol, but did not stimulate uterine hyperplasia (Black *et al.*, 1994; Turner, Sato *et al.*, 1994). Similar to estrogen, raloxifene prevented the increases in longitudinal and radial bone growth as well as cancellous bone resorption induced by ovariectomy (Evans *et al.*, 1994). In adult rats with established osteopenia, raloxifene prevented additional bone loss but, as with estrogen, was unable to replace lost bone (Evans *et al.*, 1996). The prevention of cancellous bone loss by raloxifene occurred by a mechanism mimicking the anti-resorptive action of estrogen in which osteoclast numbers and eroded trabecular perimeters were reduced.

Analogs of raloxifene that are more potent antagonists in the uterus prevented ovariectomy-induced bone loss when administered to rats immediately following ovariectomy, as well as blocked continued bone loss when administered to osteopenic animals (Li *et al.,* 1998). Raloxifene only partially inhibited bone loss during high turnover phase after ovariectomy, but completely prevented bone loss during the subsequent slower phase of post-ovariectomy bone loss. In clinical trials of postmenopausal women, raloxifene, comparable to the effects of conjugated estrogens, reduced markers of bone resorption and formation and increased bone mineral density relative to patients receiving the placebo (Draper *et al.,* 1996; Delmas *et al.,* 1997). Additionally, raloxifene therapy reduced the serum concentrations of total and low-density lipoprotein (LDL) cholesterol but, unlike estrogen, did not stimulate the endometrium.

Although the *in vivo* effects of raloxifene have been studied extensively and in bone appear to closely parallel those of estrogen, comparatively little is known about the cellular and molecular effects of SERMs on bone cells *in vitro* (Bryant *et al.,* 1999). In osteoblast lineage cells, raloxifene stimulates creatine kinase activity (Fournier *et al.,* 1996) total protein, and type I collagen α-chain synthesis (Qu *et al.,* 1999): and the expression of TGFβ3 mRNA (Yang *et al.,* 1996). Similar changes have been reported with estrogen. Lin *et al.* report that raloxifene increased bone nodule formation in cultures of SaOS-2 osteosarcoma cells and blunted the inhibitory effect of parathyroid hormone on nodule formation and alkaline phosphatase activity (Lin *et al.,* 2004). Also, raloxifene produced a decrease in mRNA IL-6 expression in cultured human osteoblasts (Mendez-Davila *et al.,* 2004).

To date, the few known actions of raloxifene on osteoclast lineage cells are very much analogous to those of estrogen. Treatment of ovariectomized mice with raloxifene decreased the number of bone marrow-derived preosteoclasts (GM-CFU) (Liu *et al.,* 2000). This effect of raloxifene may be direct, given that specific high-affinity-binding sites for raloxifene have been demonstrated in a human leukemic preosteoclastic cell line (Fiorelli *et al.,* 1997). Furthermore, raloxifene suppressed osteoclastic differentiation induced by a combination of M-CSF and OPGL in the osteoclastogenic mouse monocytic cell line RAW264.7 (Shevde *et al.,* 2000). Finally, raloxifene inhibited osteoclast differentiation in human bone marrow mononuclear cultures (Ramalho *et al.,* 2002).

Estrogen Metabolites

The importance of steroid hormone metabolism in conferring tissue selectivity is well established but often underappreciated. Steroid hormone activity can be increased or decreased in tissues by enzymatic activity. Circulating testosterone acts largely as a prohormone that is a substrate for aromatase, which converts it to estrogen, and 5α-reductase, which converts it to the potent androgen dihydrotestosterone. Although aromatase is primarily located in the gonads, the enzyme is present in numerous cells and, in some cells there is sufficient aromatase activity to increase intracellular levels of estrogen to values that greatly exceed circulating hormone levels (Brueggemeier *et al.,* 2007; Yamaguchi, 2007). In the bone environment, osteoblasts and adipocytes have significant levels of aromatase activity and are thus capable of converting androgens to estrogens (Belanger *et al.,* 2002; Enjuanes *et al.,* 2005; Pino *et al.,* 2006).

Steroid sulfotransferase and sulfatase can act on sex steroids to produce inactive sulfated derivatives or to activate the sulfated derivatives, respectively. There is very little sulfotransferase activity in bone compared to uterus. This is a likely explanation for the observed tissue discrimination of the weak estrogen 16α-hydroxyestrone. Subcutaneous implantation of a continuous release pellet containing the steroid prevented bone loss in the ovariectomized rat but had minimal uterotrophic activity. In contrast, subcutaneous injection of 16α-hydroxyestrone resulted in a dose-dependent increase in uterine wet weight, presumably because the higher circulating levels owing to the rapid release of the estrogen exceeded the capacity of the uterine sulfotransferase to sulfate it. Whereas the reproductive tissues of mammals are highly sensitive to changes in the circulating levels of sex steroids that occur during the menstrual/estrus cycle, bone is much less responsive. From a physiological point of view, the absence of sulfotransferase in bone may confer increased sensitivity of the skeleton to low circulating levels of estrogen.

As mentioned earlier, tibolone is an example of a novel synthetic steroid that has tissue-selective estrogenic activity owing to differential metabolism (Fig. 4) into compounds with estrogenic, progestogenic, and androgenic activities. Tibolone has estrogenic activity on bone, vagina, and brain but not breast and the endometrium (Gallagher *et al.,* 1991; de Gooyer *et al.,* 2001; Kloosterboer, 2004; Reed *et al.,* 2004). Tibolone is rapidly metabolized to the 3α-hydroxy metabolite and the 3β-hydroxy metabolite, which bind to ER and have a half-life of ~7 hours. These putative estrogenic compounds are further metabolized to sulfated compounds; ~80% of circulating tibolone consists of 3α-and 3β-hydroxy mono- and disulfates. An additional metabolite (Δ^4 isomer) is produced, but is rapidly cleared from the circulation (Timmer and Huisman, 2002). The parent compound and Δ^4 isomer bind to PR and androgen receptors while the 3α-and 3β-hydroxy tibolone exclusively bind to ER with a preference for ERα over ERβ (de Gooyer *et al.,* 2003). The tissue selective actions of tibolone differ from SERMs (Ederveen and Kloosterboer, 1999). Although the mechanisms of action of SERMs are incompletely understood, it is clear that they bind to ER in all estrogen target tissues and behave as mixed estrogen agonists/antagonists (Evans *et al.,* 1994; Harris, Tan *et al.,* 1995; Gustafsson,

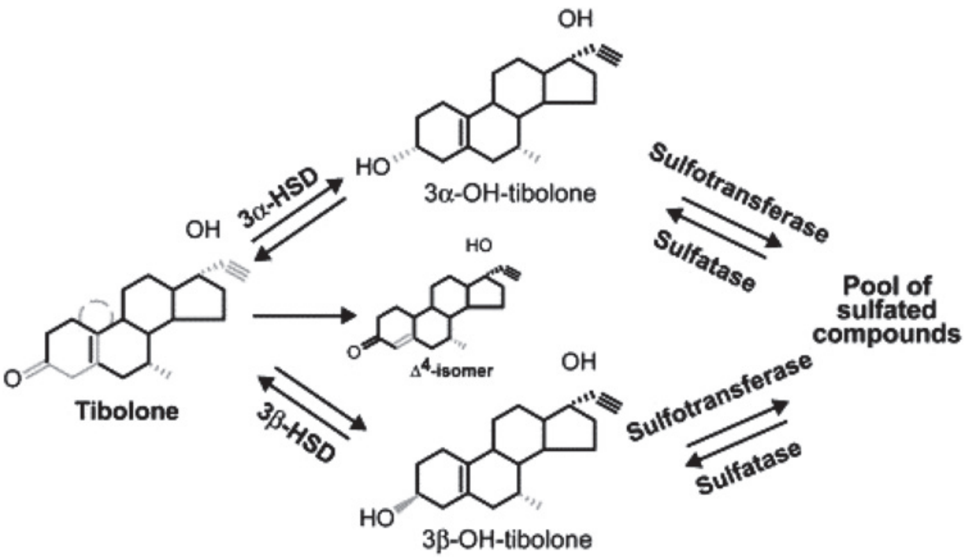

FIGURE 4 Tibolone is a prodrug that is metabolized to compounds that bind to ER, PR, and androgen receptors in a tissue selective manner. The tissue selective distribution of enzymes that metabolize estrogen plays an important role in the physiological actions of the hormone.

1998; Lonard and Smith, 2002). The relative level of agonism is tissue-specific and depends upon several factors, including the recruitment of tissue-specific coactivators and corepressors (Smith *et al.*, 1997). In contrast, the tissue selective action of tibolone depends upon its differential metabolism and enzyme regulation in individual estrogen target tissues (Yoshitake *et al.*, 1999; Kloosterboer, 2004; Reed *et al.*, 2004).

As previously mentioned, environmental or dietary compounds that bind to ER can act as endocrine disruptors. Similarly, chemicals that modify estrogen metabolism can influence the skeleton. Moderate alcohol consumption, for example, is thought to increase the levels of estrogen whereas abusive alcohol consumption patterns may reduce estrogen levels. Increased estrogen may be, in part, responsible for the protective effect of moderate alcohol consumption on bone mass in postmenopausal women, whereas the reduced estrogen levels may contribute to alcohol-induced bone loss in heavy drinkers.

Even though postmenopausal women lose bone at different rates, the level of serum estrogen does not differ greatly among them (Riis *et al.*, 1995). However, serum levels of adrenal androgens and the conversion of these androgens and estradiol to other estrogen metabolites do differ among individuals and ethnic groups and may play a role in maintaining bone mass in certain individuals.

The predominant postmenopausal estrogen, estrone (E_1): is primarily metabolized through two pathways (Fishman *et al.*, 1984; Martucci and Fishman, 1993). The catalytic conversions of E_1 by 2-hydroxylase or 16α-hydroxylase enzymes results in the formation of either 2-hydroxyestrone (2-OHE$_1$) or 16α-hydroxyestrone (16α-OHE$_1$) respectively. The 16α-OHE$_1$ has been shown to bind covalently and noncovalently to ER, but with reduced affinity compared to estradiol. However, its lack of binding to the serum-binding globulin makes it more available for estrogen target tissues (Fishman and Martucci, 1980; Swaneck and Fishman, 1988). In addition, 16α-OHE$_1$ serves as an estrogen agonist in reproductive tissues (Fishman and Martucci, 1980; Schneider *et al.*, 1984). In contrast, 2-OHE$_1$ binds very weakly (even less so than 16α-OHE$_1$) to the ER (Fishman and Martucci, 1980) and has been shown to have no estrogenic activity and, in some cases, to act as an estrogen antagonist (Schneider *et al.*, 1984; Vandewalle and Lefebvre, 1989).

Lim *et al.* (1997) reported that urinary levels of 16α-OHE$_1$ were lower and 2-OHE$_1$ levels were higher in postmenopausal osteopenic than in nonosteopenic individuals. Furthermore, the 16α-OHE$_1$/2-OHE$_1$ ratio was correlated positively with bone mineral density. African American women, who are at lower risk for developing osteoporosis, reportedly have increased 16α-hydroxylation and therefore have a higher 16α-OHE$_1$/2-OHE$_1$ ratio compared to Caucasian women (Coker *et al.*, 1997). When ovariectomized rats were treated with 16α-OHE$_1$, cancellous bone turnover associated with ovariectomy was prevented (Westerlind *et al.*, 1998). However, 2-OHE$_1$ displayed no estrogenic or antiestrogenic activities on cancellous bone turnover in ovariectomized rats. In agreement with these findings, 16α-OHE$_1$ but not 2-OHE$_1$, mimicked the effects of estradiol on the *in vitro* regulation of alkaline phosphatase activity and osteocalcin secretion in an estrogen-responsive osteoblast cell line stably expressing ERα (Robinson *et al.*, 2000).

Another naturally occurring estrogen metabolite is 2ME, produced primarily by the liver. This metabolite has very low affinity for ER. As a consequence, its physiological effects are thought not to be mediated through the ER pathway (the nonclassical pathways of action of 2ME were discussed earlier). 2ME has antitumorigenic activity and may act as a physiological tumor suppressor (Zhu and Conney, 1998). Tumor cell proliferation is inhibited by molecular pathways that are specific to the lineage of tumor cell origin, and angiogenesis is reduced via increased senescence and apoptosis of endothelial cells by 2ME (Fotsis *et al.*, 1994). Administration of high concentrations of 2ME to rapidly growing rats inhibits longitudinal bone growth but has no effect on either radial bone growth or cancellous bone turnover (Turner and Evans, 2000). Thus, excessive 2ME reduces proliferation and/or stimulates apoptosis of rapidly dividing growth plate chondrocytes.

Phytoestrogens

Phytoestrogens are plant compounds that bind to and activate estrogen receptors. There are three main classes of phytoestrogens: isoflavones, lignans, and coumestans. A single dietary source often contains more than one class of phytoestrogens. Using a competition-binding assay, Kuiper *et al.* (1998) found that although the binding affinity of phytoestrogens for either ERα or ERβ was lower than that of estradiol, the relative binding affinity of phytoestrogens for ERβ was significantly greater than that for ERα. In contrast to phytoestrogens, the relative binding affinity of the SERMs, tamoxifen, and raloxifene, was greater for ERα than for ERβ. Using a reporter gene construct, the phytoestrogens tested activated both ER subtypes, although the overall potency of these compounds was approximately 1/100th to 1/1000th that of estradiol (Kuiper *et al.*, 1998).

Soy is especially rich in phytoestrogens and has been intensively studied. The phytoestrogens in soy consist primarily of isoflavones, predominantly genistein and diadzein. However, not all of the skeletal effects of soy are attributable to isoflavones. For example, soy protein is reported to be less calciuretic than casein. Soy protein would therefore be expected to improve calcium retention, which in turn has the potential to reduce parathyroid hormone levels and bone resorption rates (Breslau *et al.*, 1988; Arjmandi *et al.*, 1998).

Numerous studies have addressed the role of soy in preserving bone mass and ameliorating the effects of postmenopausal and age-related bone loss (Brynin, 2002; Balk *et al.*, 2005). Constituents of soy have been reported as having beneficial effects on the skeleton in postmenopausal women (Potter *et al.*, 1998). However, conclusions regarding the effects of soy as a food staple, or its specific components, on the musculoskeletal system are mixed with some studies showing positive effects, while others showing either no effects or detrimental effects. Importantly, there have been no placebo-controlled studies demonstrating the efficacy of soy in reducing bone fractures (Potter *et al.*, 1998). In lieu of well-controlled clinical studies, the most convincing evidence for soy effects on bone comes from laboratory animal studies. Several studies have examined the effects of phytoestrogens on bone loss models with generally positive findings. Both genistein in mice and coumestrol in rats reportedly prevented ovariectomy-induced osteopenia by reducing bone resorption and osteoclast number in a manner at least superficially similar to estradiol (Draper *et al.*, 1997; Ishimi *et al.*, 1999). Other studies have reported that the reduction in bone loss following ovariectomy in rats with genistein and soy protein was associated with unchanged indices of bone resorption but increased bone formation (Arjmandi *et al.*, 1998; Fanti *et al.*, 1998; Harrison *et al.*, 1998). These latter data suggest that there may be important differences in bone effects of phytoestrogens and estrogen. Animal studies to date, however, have serious limitations that contraindicate extrapolation of results based on animal models to postmenopausal and aging humans. The animal studies were largely performed in growing rodents, which are poor models for postmenopausal bone loss (Iwaniec and Turner, 2008). Other major limitations of prior research in evaluating dietary soy include one or more of the following: (1) administration of concentrated extracts and purified phytoestrogens; (2) delivery of active agents by injection rather than orally; (3) short treatment duration; (4) inadequacy of the skeletal endpoints measured; and (5) no examination of gender differences. A well-designed, 3-year-long, longitudinal study in ovariectomized monkeys failed to detect a beneficial effect of soy protein isolate containing phytoestrogens in protecting against bone loss resulting from estrogen deficiency (Register *et al.*, 2003). Thus, studies to date do not provide compelling evidence for beneficial effects of phytoestrogens on musculoskeletal health across the life span.

Many of the beneficial effects of a soy diet are believed to be mediated through phytoestrogens acting as partial estrogen agonists (Heaney *et al.*, 2005; Weaver and Cheong, 2005). Whereas estrogen receptor agonists could have beneficial effects on bone during aging, they could also have detrimental effects during growth and maturation. As discussed earlier, the sexual dimorphism of human and rat skeletons (whereby males have larger bone and muscle mass than females) is, in part, owing to the inhibitory effect of estrogen on growth (Iwaniec and Turner, 2008). Exposure of humans and rats during rapid skeletal growth to estrogens leads to premature epiphyseal closure (resulting in shorter bones) increased cancellous bone density, and a reduction in cortical bone owing to decreased periosteal growth. Muscle mass is also reduced. If the phytoestrogens act on the skeleton in the same manner as estradiol, consumption by adolescents could result in reduced peak bone and muscle mass, predisposing the skeleton to osteoporotic fractures later in life (Cutler, 1997). On the other hand, the biological effects of phytoestrogens are not well characterized. Circulating and tissue levels of phytoestrogens are in competition with much more potent endogenous estrogens for ER and may have greater effects in the aged because of gender-specific declines in bioavailable estrogens and androgens.

The bioavailability of dietary phytoestrogens, which is influenced by their absorption, systemic metabolism, and tissue level metabolism, ultimately determines the physiological effects of plant-derived estrogens. These variables have not been explored in detail with soy phytoestrogens. However, studies performed using synthetic and endogenous estrogens reveal multiple levels of complexity. As described earlier, the synthetic steroid tibolone functions as a prodrug. It can behave as an estrogen, androgen, or progestin, or have no effect on potential target cells, depending upon tissue level metabolism (Eden, 2005). A similar role for local metabolism has been demonstrated for endogenous estrogens (Belanger *et al.,* 2002; Pino *et al.,* 2006; Brueggemeier *et al.,* 2007; Yamaguchi, 2007). These examples demonstrate why it is critically important to assess the whole-life musculoskeletal response *in vivo* to a phytoestrogens-rich diet using models that accurately mimic the delivery of these agents to humans.

Phytoestrogens have the potential to act as endocrine disrupters during reproductive tract development in males and females and could interfere with reproduction in adult females (Humfrey, 1998; North and Golding, 2000). High concentrations of dietary extracts of phytoestrogens have been associated with uterine hyperplasia in humans and rats and with breast cancer in men (Dimitrakakis *et al.,* 2004; Nakai *et al.,* 2005). For the reasons discussed previously, a food-based diet may avoid the detrimental effects associated with high concentrations of purified bioactive constituents. However, the issue of detrimental side effects on the musculoskeletal and reproductive systems needs to be thoroughly addressed. Because soy proteins have not been adequately shown to lessen vasomotor symptoms of menopause, slow postmenopausal bone loss, or prevent cancer, and because adverse effects have not been adequately evaluated, the American Heart Association Nutrition Committee has concluded that the use of isoflavin supplements in food or pills to reduce cardiovascular risk is not recommended (Sacks *et al.,* 2006).

PROGESTERONE

Progesterone is often given in conjunction with estrogen during hormone replacement therapy of postmenopausal women to minimize undesirable effects of estrogen on reproductive tissues. However, the effects of progesterone on bone physiology have not been examined as extensively as those for estrogen (for a review, see Prior *et al.,* 1990). As discussed earlier, progesterone exerts its effects on cells via mechanisms analogous to estrogen by binding and activating the PR, a member of the steroid nuclear receptor transcription factor family, which subsequently binds to the regulatory regions of target genes, classically at progesterone response elements (PREs) to modulate gene transcription. Nongenomic effects of progesterone in osteoblasts have also been documented (Le Mellay *et al.,* 1999; Grosse *et al.,* 2000).

Progesterone Receptors in Bone Cells

In humans, rats, and rabbits, PR exists as two isoforms, A and B (see Fig. 5), transcribed from the same gene using different promoters (Kastner *et al.,* 1990). Both PR promoters are estrogen inducible. The PR_B isoform is generally a stronger activator of gene transcription than PR_A, and in certain conditions, PR_A can reduce the transactivation

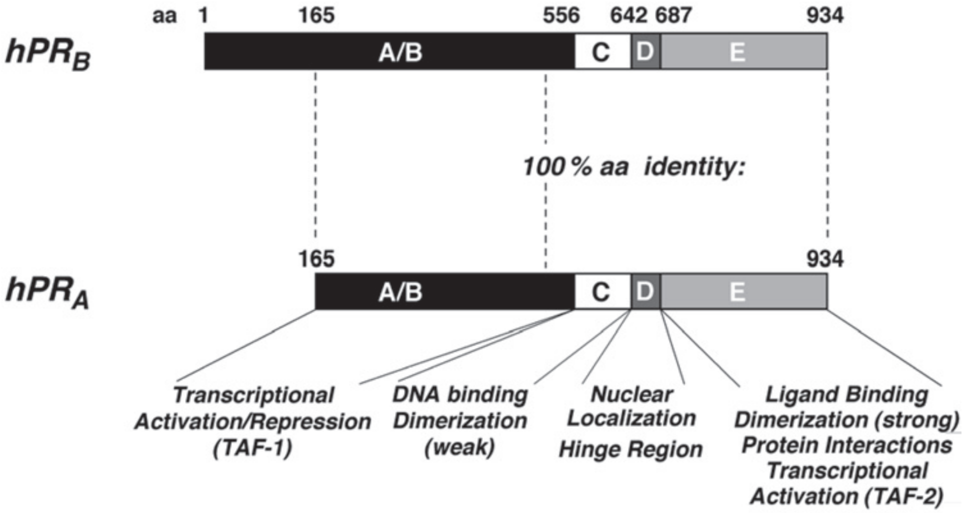
aa 1
165
556
642 687
934
hPRB
A/B
C
D
E
100 % aa identity:
165
934
hPRA
A/B
C
D
E
Transcriptional Activation/Repression (TAF-1)
DNA binding Dimerization (weak)
Nuclear Localization Hinge Region
Ligand Binding Dimerization (strong) Protein Interactions Transcriptional Activation (TAF-2)

FIGURE 5 Model of human progesterone receptor isoforms with defined domain functions and homologies.

stimulated by PR_B and other steroid receptors, including the ER (Kraus *et al.*, 1995; Giangrande and McDonnell, 1999). Because of these important functional distinctions between the two PR isoforms, it is of interest to determine their relative expression level in target cells. The presence of PR mRNA and protein has been demonstrated in primary cultures of human osteoblasts (Eriksen *et al.*, 1988) and in several osteoblast and osteosarcoma cell lines (HOS TE85, MG-63, and SaOS-2) (Wei *et al.*, 1993; MacNamara *et al.*, 1995; MacNamara *et al.*, 1998). In some cases estrogen increases the level of PR expression (Eriksen *et al.*, 1988; Harris, Enger *et al.*, 1995; MacNamara *et al.*, 1998). Consequently, some of the effects on bone metabolism that have been attributed to estrogen may in fact be mediated by progesterone.

Effects of Progesterone on the Skeleton

Progesterone reportedly affects osteoblast and osteoblast precursor cell proliferation and differentiated functions, but some data are conflicting. Canalis and Raisz (1978) reported that progesterone inhibited proliferation and collagen synthesis by fetal rat calvarial cells. In contrast, Slootweg *et al.* (1992) found that progesterone had no effect on proliferation of SaOS-2 cells but did stimulate cell growth synergistically with estrogen. In contrast, progesterone stimulated the proliferation of TE85 osteosarcoma cells and normal human osteoblasts (Tremollieres *et al.*, 1992). Other studies have also shown stimulatory effects of progesterone on proliferation of normal and transformed osteoblastic cells (Scheven *et al.*, 1992; Manzi *et al.*, 1994; Verhaar *et al.*, 1994). Progesterone increased the number of alkaline phosphatase positive colonies – and hence osteogenic development– of a subclass of osteoprogenitor cells isolated from rat vertebral bone explants (Ishida and Heersche, 1997; Ishida and Heersche, 1999). Additionally, progesterone treatment of primary human osteoblast and TE85 osteosarcoma cells increased IGF-II secretion (Tremollieres *et al.*, 1992) but inhibited IL-6 secretion in primary human osteoblasts and mouse bone marrow stromal cells (Girasole *et al.*, 1992). Progesterone may regulate components of the IGF system in human osteosarcoma cells by stimulating expression of IGFBP-5 by a transcription-dependent mechanism (Boonyaratanakornkit *et al.*, 1999). IGFBP-5 is unique among the various IGFBPs becasue it enhances the mitogenic activity of both IGF-I and IGF-II in osteoblasts *in vitro*. Taken together, these studies suggest that progesterone is capable of direct regulation of osteoblast metabolism.

A number of experimental animal studies have reported significant effects of progesterone on bone metabolism; progesterone stimulated mineralization of newly induced bone in rats (Burnett and Reddi, 1983) and increased cortical bone formation in spayed Beagle dams (Snow and Anderson, 1985). Moreover, progesterone prevented ovariectomy-induced bone loss in rats by reducing resorption and increasing bone formation (Barengolts *et al.*, 1990; Barengolts *et al.*, 1996). In addition, high circulating levels of progesterone in pseudopregnant rats were associated with preservation of bone mass despite estradiol levels comparable to ovariectomized rats (Bowman and Miller, 1996). Progesterone was also reported to have a significant stimulatory effect on bone formation and to act synergistically with estrogen to inhibit bone resorption in ovariectomized growing rats (Schmidt *et al.*, 2000) However, these generally positive results contrast with studies that failed to detect an action of progesterone on cancellous bone with or without co-administration of estradiol (Kalu *et al.*, 1991; Fujimaki *et al.*, 1995). Also, the PR antagonist RU486 was reported to reduce bone loss in ovariectomized rats (Barengolts *et al.*, 1995). This unexpected finding contrasts with the bone loss induced by estrogen antagonists. The effects of RU486 on bone may have been mediated by the drug's antiglucocorticoid effect. Long-term mifepristone (RU486) therapy was used to treat a patient with Cushing's syndrome and morbid osteoporosis. Although inducing benign endometrial hyperplasia, the treatment prevented further bone loss (Newfield *et al.*, 2001).

Skeletal Phenotype of Mice with Disruption of PR Genes

Mice have been generated with a homozygous mutation of the PR locus in which expression of both A and B isoforms of PR is prevented. The PR_B has been shown to be the dominant player in gene regulation in progesterone target cells (Giangrande and McDonnell, 1999). PR knockout mice (i.e., $PR_{A/B}$ knockouts) exhibit multiple reproductive abnormalities and homozygous females are infertile (Lydon *et al.*, 1995). Female PR knockout mice have been analyzed histomorphometrically for possible skeletal defects. Although preliminary studies suggested that PR knockout animals had peak cortical and cancellous bone mineral densities that were not significantly different from age-matched wild-type animals (Bain *et al.*, 1997) subtle alterations were evident at earlier ages. Specifically, young postpubertal PR knockout mice exhibited increased cortical bone thickness with no changes in cancellous bone (Rickard *et al.*, 1999). This initial finding has been verified using high-resolution μCT (Rickard *et al.*, 2008). Longitudinal bone growth was unaffected at all ages examined. These findings suggest that progesterone may be involved in the early growth and sexual dimorphism of the female skeleton. However, a more detailed analysis of the skeleton of PR knockout mice suggests that they are resistant to early onset age-related cancellous bone loss (Rickard *et al.*, 2008). If confirmed, this finding would suggest that PR signaling plays a role in mineral homeostasis during pregnancy and lactation, periods where the circulating levels of progesterone are elevated. Studies on PR_A knockout female mice showed that progesterone had a larger effect in reproductive tissue compared to wild-type mice (Mulac-Jericevic *et al.,* 2000). This suggests that PR_A may play a dominant-negative role in the actions of PR_B. Future studies should include examining the skeleton of PR_A knockout female mice.

Humans

Despite some ongoing controversy (Waller *et al.,* 1996; De Souza *et al.,* 1997) the majority of evidence from human studies suggest a role for progesterone in bone metabolism. For example, women with ovulatory disturbances related to luteal phase defects have been shown to lose more bone mass over one year compared to women with normal menstrual cycles (Prior *et al.,* 1990) and to have increased bone mass in response to progesterone therapy during the luteal phase (Prior *et al.,* 1994). Several studies have found that progesterone treatment of postmenopausal women protects against bone loss, particularly at cortical sites (Gallagher *et al.,* 1991; McNeeley *et al.,* 1991; Grey *et al.,* 1996). Progesterone replacement therapy reduces postmenopausal bone loss in part by suppressing bone resorption (Mandel *et al.,* 1982; Lobo *et al.,* 1984). Although some studies have shown that treatment of postmenopausal women with either progesterone alone or combination therapy of estrogen with progesterone is as effective as estrogen alone in the prevention of bone loss (McNeeley *et al.,* 1991; Grey *et al.,* 1996) other trials have demonstrated a lesser effect of progesterone (PEPI, 1996; Prior *et al.,* 1997).

CONCLUSIONS

Numerous major advances have been made in recent years in the area of estrogen action on bone. It is now evident that estrogen affects bone at all levels of cellular regulation: progenitor cell proliferation, differentiation, activity, and life span. Consequently, estrogen regulation of bone growth and remodeling at the tissue level is recognized as extremely complex. How estrogen elicits its effects within the cell, as well as the range of possible responses, is also more diverse than previously believed. The latter is because, in part, of the identification of the second ER isoform, ERβ, exhibiting an activity and expression distinct from that of ERα. In addition, the presence of tissue specific metabolism, steroid receptor coregulators, and nongenomic pathways of estrogen regulation adds to the diverse responses. The differential expression and activities of the ER isoforms and the receptor coregulators provide explanations for the cell- and tissue-selective actions of SERMs.

Answers to several important questions remain incomplete. For example, what is the physiological significance of nongenomic signaling by estrogens? Do the two ER isoforms regulate the same target genes? Are the two isoforms coexpressed in osteoblasts and osteoclasts? Does the relative ratio of ERα to ERβ change during differentiation and, if so, is estrogen responsiveness affected? Is estrogen responsiveness in osteoblasts and osteoclasts also determined by alterations in the relative levels of the steroid receptor coactivators and corepressors? Further analysis of the various ER mutant mice should provide answers as to whether the effects of estrogen at particular skeletal sites (such as trabecular surfaces, periosteum, and growth plate) are mediated primarily by one or both ER isoforms. In addition, the generation of bone cell lines from each of the various ER-deficient genotypes should prove valuable in addressing many of the questions just posed.

Advances in our understanding of the actions and importance of progesterone in bone growth and remodeling have, unfortunately, been more modest. The availability of PR-deficient mice will make it possible to examine the interaction between estrogen and progesterone in bone, including the potential involvement of progesterone and PR as regulators of certain skeletal effects of estrogen. Lastly, and in analogy to studies with ERs, investigation into possible exclusive roles of the A and B isoforms of PR may reveal heretofore unrecognized effects of progesterone on bone.

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Chapter 41

The Pharmacology of Selective Estrogen Receptor Modulators

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The widespread distribution of estrogen receptors (ERs), and their critical role in normal physiology and various pathophysiological states when estrogen levels decline indicate the importance of ER-targeted therapies for use in postmenopausal women. Controversy and concern over the use of estrogen replacement therapies creates the opportunity to design molecules to selectively modulate estrogen action in those tissues where estrogen agonism is the desired goal while simultaneously producing an estrogen-neutral or-antagonistic effect in tissues where estrogen-related side effects are a concern. Selective estrogen receptor modulators, or SERMs, are currently in clinical use for the treatment and prevention of osteoporosis (raloxifene), breast cancer prevention (tamoxifen and raloxifene) and treatment (tamoxifen and toremifene), and the induction of ovulation (clomiphene). To date, seven different SERMs have reached advanced clinical evaluation for postmenopausal osteoporosis with three molecules (droloxifene, idoxifene, and levormeloxifene) withdrawn for unfavorable risk/benefit profiles and three additional molecules (lasofoxifene, bazedoxifene, and arzoxifene) currently in phase 3 status or under regulatory review. Experience with these molecules reveals several key themes for chronic use of SERMs. (1) Each SERM generates a unique complex with the ER that influences cofactor recruitment in estrogen-target tissues responsible for the tissue-selective pharmacological profile, which translates to each SERM generating potentially an entirely unique overall safety and efficacy profile, indicating the need for thorough evaluation of each individual SERM across multiple tissue types for efficacy and safety determination. (2) Uterine safety historically has been the critical safety feature for chronic SERM use in osteoporosis therapy and careful assessment of the potential for uterine stimulation is a key element in the consideration of new molecules in this class. (3) The pharmacokinetic and distribution properties of SERMs offer an additional aspect influencing the magnitude of the overall biological response by either improving systemic bioavailability or altering uptake into important estrogen-responsive tissues.

INTRODUCTION

Research in the ER field has attracted considerable attention over the past 15 years with significant events ranging from very basic research discoveries, such as resolution of the liganded ER crystal structure and identification of a second ER form (ERβ), to important clinical observations regarding estrogen use in postmenopausal women from the Women's Health Initiative or WHI trial. Estrogen exhibits a "Jekyl and Hyde" therapeutic profile, as hormone replacement (estrogen + progestin) is associated with distinct benefits on the menopausal syndrome, including reductions in vasomotor symptoms and fracture incidence, as well as other benefits such as a reduction in colon cancer. However, these benefits are offset by significant increases in risk for coronary events (myocardial infarction and stroke) and breast and uterine cancer. In the early 1990s, research around the concept of SERMs—molecules that simultaneously agonized or antagonized estrogen action in different tissue types—offered a new way of looking at ER pharmacology and served to trigger the renewed interest in estrogen-related research in the mid-1990s.

Prior to the development of the "SERM-concept," ER ligands were generally thought of as falling into either the category of full agonists, partial agonists, or full antagonists across all tissue types (i.e., uterus, mammary, and bone). For example, steroidal hormones such as 17β-estradiol were known to behave as full agonists both *in vitro* and *in vivo* across multiple tissue types, whereas compounds such as fulvestrant (ICI-182,780) were known to be complete ER antagonists that bound tightly to the ER, but lacked intrinsic activity and, therefore, completely blocked the action of full

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ER agonists like 17β-estradiol. These “pharmacotypes” are depicted in Figure 1(A and B), respectively. Conversely, compounds such as tamoxifen were known to, in the presence of estrogen, block estrogen action in estrogen-responsive tissues (i.e., breast cancer cells) but, in the absence of estrogen, mimic estrogen in bone and uterus in estrogen-deficient animals and women, thus exhibiting a classical partial agonist profile (see Fig. 1C). Although this profile held some attractive features for use in ER-dependent breast cancer, the profile was prohibitive for chronic use in postmenopausal women for noncancer indications (like osteoporosis) where even the potentially less robust uterine stimulation induced by tamoxifen’s partial agonist action produced untenable side effects that created a risk/benefit ratio that was unfavorable for use in diseases like osteoporosis (Kalu *et al.*, 1991). As a result, virtually no work was being done in the pharmaceutical industry developing novel ER ligands for osteoporosis, because the prevailing medical opinion at the time was that any molecule with sufficient estrogen agonism capable of producing a benefit in bone would also generate sufficient agonism (even if a partial agonist) in uterine tissue to create a risk that would unfavorably offset the bone benefit (Feldman *et al.*, 1989).

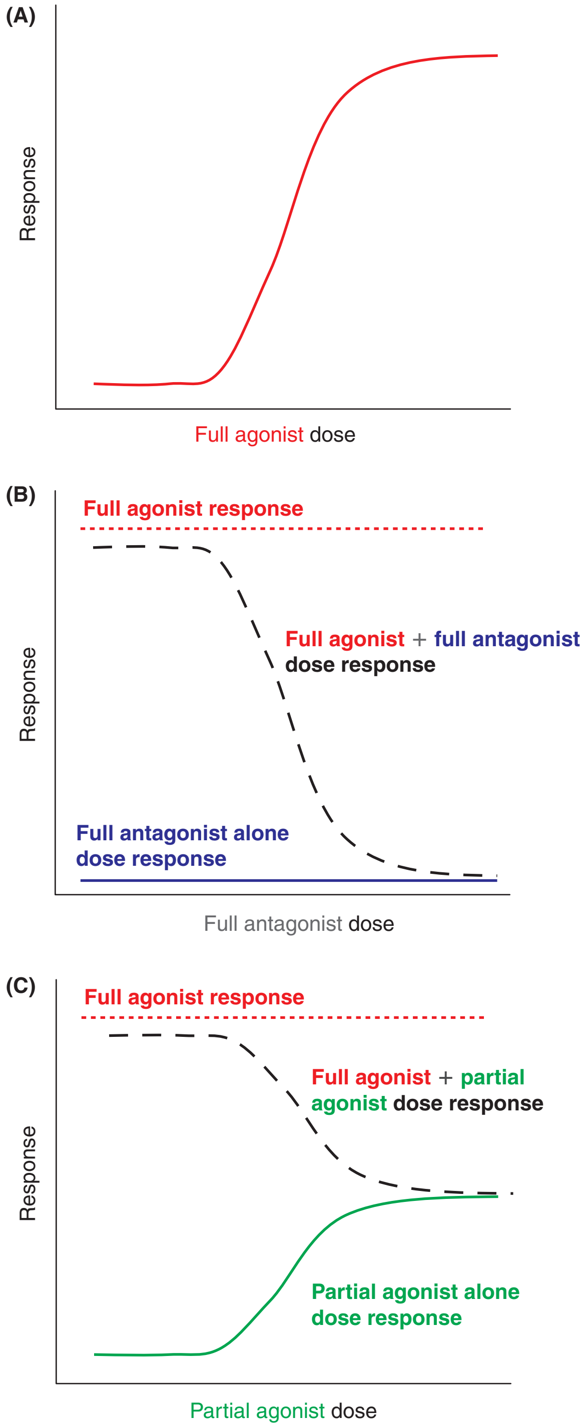


FIGURE 1 Potential pharmacotypes resulting from various ER–ligand interactions in the form of hypothetical dose–response curves for a full agonist (**A**), a full antagonist (**B**), and a partial agonist (**C**). (See plate section)

With the first preclinical and clinical descriptions of the unique profile of raloxifene in estrogen-deficient animals and postmenopausal women (Black *et al.*, 1994; Draper *et al.*, 1996), the concept of a SERM was born, radically shifting thought around use of ER-based ligands in postmenopausal women and opening the door for use in chronic diseases like osteoporosis. Accordingly, the initial goals of a SERM-based therapy for osteoporosis required the molecule to have estrogen-like efficacy on bone and concomitant fracture reduction without estrogen-like stimulatory effects on uterus or mammary tissue. As of the writing of this chapter, only four molecules with SERM-like profiles have achieved clinical use (Table I) and only one, raloxifene, has attained approval for use in the treatment and prevention of osteoporosis. However, other molecules have been evaluated clinically, or are currently under clinical evaluation, for postmenopausal osteoporosis and will be reviewed here as well as some other SERM applications that might benefit other diseases or disorders in postmenopausal women in the future.

SERM MECHANISM

The effects of SERMs on biological systems are predominately mediated by specific, high-affinity interactions with ERs that are primarily located in target cell nuclei (Nilsson *et al.*, 2001). Their nuclear hormonal action involves the complex interplay of a number of protein and genomic elements that allow SERMs to regulate gene transcription and subsequent protein production by the cell. Three key elements of the SERM mechanism are depicted in Figure 2, which include: (1) high-affinity interaction with the ER, (2) ER-ligand dimerization and the association with a tissue-specific set of coregulatory proteins, and (3) binding of the ER/adaptor protein complex to specific DNA response elements located in the promoter regions of nuclear target genes and ensuing regulation of gene transcription. Depending on the cellular

TABLE I SERMs Currently Approved for Human Use

SERM	Trade name	Approved indications	Daily dose
Clomiphene	Clomid®	Induction of ovulation	50–100 mg
Raloxifene	Evista®	• Osteoporosis prevention • Osteoporosis treatment • Breast cancer risk reduction in postmenopausal osteoporotic women and in high-risk postmenopausal women	60 mg
Tamoxifen	Nolvadex®	• Metastatic breast cancer treatment • Adjuvant breast cancer treatment • Ductal carcinoma *in situ* • Breast cancer risk reduction in high-risk women	20–40 mg
Toremifene*	Fareston®	Metastatic breast cancer treatment	60 mg

**Toremifene (Fareston®) is currently not approved in the United States, but is approved for metastatic breast cancer treatment in Europe.*

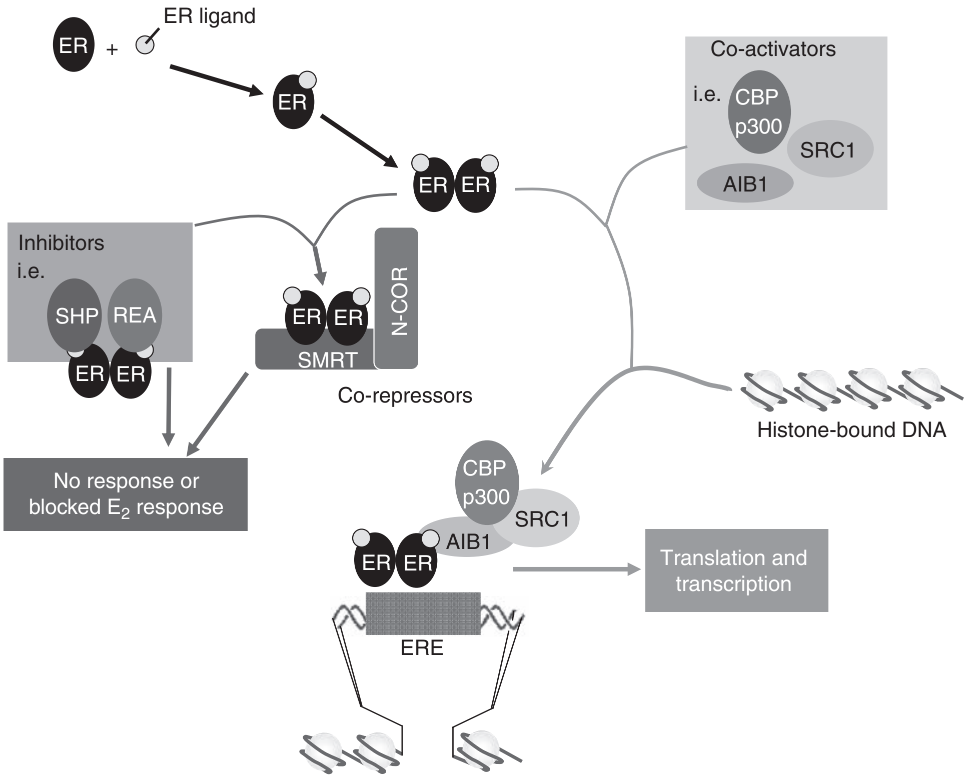

ER ligand
ER
+
ER
ER ER
Co-activators
i.e.
CBP p300
SRC1
AIB1
Inhibitors i.e.
SHP
REA
ER ER
ER ER
N-COR
SMRT
Co-repressors
Histone-bound DNA
No response or blocked E$_2$ response
CBP p300
SRC1
AIB1
ER ER
Translation and transcription
ERE

FIGURE 2 SERM mechanism of action.

and promoter context, the DNA-bound receptor can induce or inhibit the transcription of specific genes within the tissue.

The ability to specifically bind to the ER is perhaps the single most important feature of all molecules with a SERM profile. The affinities of several of the more extensively studied SERMs are provided in Table II. An important determinant of the ultimate pharmacological response is the shape of the ligand–ER complex, which is unique with each individual ligand (McDonnell *et al.*, 1995), with different SERMs leading to specific orientation of subunits of the ER (i.e., raloxifene; Brzozowski *et al.*, 1997). This conformation or shape of the ligand–ER complex

TABLE II Affinities of Various SERMs for Human Estrogen Receptor (ER)-α and ER-β

ER ligand	ERα(IC_{50})	ERβ(IC_{50})
17α-Estradiol	0.3–0.8*	0.9–2.5*
Tamoxifen	72–138	173–1204
4-Hydroxytamoxifen	0.22–0.98	1.5–2.46
Raloxifene	0.4–1.31	5.6–13.0
Fulvestrant (ICI-182,780)	0.8–1.0	1.12–3.6

**All values are in nM. As binding data often can vary from laboratory to laboratory, and various approaches can be taken to attaining binding affinities, ranges for ERα and ERβ obtained from representative references are presented (Sun et al., 1999; Brady et al., 2001; de Boer et al., 2004; He et al., 2005; Leblanc et al., 2007)).*

then determines the subsequent protein–protein interactions that ensue. Herein lays the basis for the wide array of different pharmacological profiles produced by different SERMs, because the confirmation of the ER–SERM complex is distinct for each molecule (McDonnell *et al.*, 1995). It is important to recognize that a second form of the ER is known to exist, ERβ (Kuiper *et al.*, 1996). ERα and ERβ display unique patterns of tissue distribution typically with expression levels of one subtype dominating (Saunders *et al.*, 1997), although it should be noted that most tissues contain at least small amounts of both subtypes, and with the role of putative α:β-heterodimers unknown, it is possible that a low-expression subtype may be a key rate-limiting step in ultimate nuclear activity. To date, all of the SERMs that have reached advanced clinical evaluation show high affinity for both ERα and ERβ with sufficient circulating and tissue exposure to ensure binding of both subtypes, indicating that, for these molecules at least, differential ERα or ERβ activation does not explain the tissue-selective pharmacological effects.

In addition to the ERs themselves, a number of other coregulatory proteins, such as coactivators (which enhance transcription) and corepressors (which reduce transcription), play an essential role in determining the ultimate response of an individual cell to liganded ER. A number of various coactivators and corepressors that interact with the ligand-bound ER have been identified and are reviewed thoroughly elsewhere (Smith *et al.*, 1997).

The relative tissue expression of the different cofactors and the ability of the ER–ligand complex to interact with those cofactors determine the tissue-selective agonist/antagonist profile of the various SERM molecules. Cofactor tissue expression can also be altered with various pathophysiological states, such as breast cancer (Bautista *et al.*, 1998). The important nature of the tissue-relevant cofactor context was demonstrated by Shang and Brown (2002), who compared the effects of two SERMs, tamoxifen and raloxifene, to estrogen in two tissue contexts: a breast cancer cell line and a uterine endometrial carcinoma cell line. In the mammary cells that proliferate in response to estrogen, 17β-estradiol recruited coactivators leading to increased gene expression. In these same cells, where tamoxifen and raloxifene both display estrogen antagonist pharmacology, the ligand–SERM complex with both molecules recruited corepressors and not the coactivators observed with 17β-estradiol on ER-mediated transcription. However, in a uterine cell line where tamoxifen exhibits estrogen agonist pharmacology and raloxifene is a complete antagonist, tamoxifen was associated with the recruitment of a coactivator protein complex that is expressed at higher levels in uterine cells. The coactivator requirements for estrogen-stimulated gene expression in uterine cells were distinct from those for tamoxifen, indicating multiple signaling mechanisms even for the agonist response. Conversely, raloxifene failed to recruit a coactivator construct, rather it induced a corepressor construct in uterine cells (Shang and Brown, 2002). Thus, the relative abundance of ER-associated coactivators and corepressors are an important factor in the tissue-specific pharmacology of SERMs.

In addition to the layers of complexity provided by multiple ER–SERM conformations and tissue-selective cofactor recruitment, the mechanism of tissue selectivity of SERMs is further complicated by the existence of multiple DNA response elements. Many estrogen-responsive genes contain the classical estrogen response element (ERE), and a number of DNA response elements, such as activator protein-1 and steroidogenic factor-1 response element (Vanacker *et al.*, 1999). The mechanism for SERM activation (or inactivation) of ER-mediated function is further complicated by the presence of novel DNA response elements that are more apparent following formation of the ER–SERM cofactor complex.

Although there has been considerable strides in understanding the molecular biology of SERM action in a general sense, with the critical role of the ER, specific cofactors that are recruited to the transcriptional complex and the specific DNA response elements activated, it is important to recognize that each SERM has the potential to produce a unique fingerprint of pharmacological activity at the whole-organism level. Contributing to the eventual profile are the molecular mechanisms reviewed earlier, as well as other factors, such as absorption, distribution, excretion, and metabolism of the SERM, that add another layer of complexity for the ultimate pharmacological response, creating the need to fully characterize the tissue-specific effects of each individual SERM in an *in vivo* paradigm.

SERM CHEMISTRY

A key element in determining the pharmacological profile of each distinct SERM is influenced heavily by the chemical makeup of the SERM, including the basic scaffold, the placement of the hydroxyl moieties, and the positioning and nature of the basic side chain. Crystal structures of various ligands bound to the ER indicate that small molecules can induce a spectrum of receptor conformations. As described previously, the specific SERM–ER conformation has tremendous impact on cofactor recruitment and ultimate genomic activation or inhibition by the SERM. Chemical scaffolds that have produced SERMs in current clinical use, or at least that have reached phase 3 clinical evaluation in humans, are depicted in Figure 3 and include triphenylethylenes (i.e., tamoxifen, droloxifene, idoxifene, clomiphene, and toremifene), benzothiophenes (raloxifene and arzoxifene), tetrahydronaphthylenes (lasofoxifene and nafoxidine), indoles (bazedoxifene), and benzopyrans (acolbifene and levormeloxifene). Key structural features of these molecules, which are indicated in Figure 4 for raloxifene versus 17β-estradiol, are typical for the entire class with the most important features being the hydroxyl moieties and the basic side chain, as described thoroughly elsewhere (Grese *et al.*, 1997).

SERM PHARMACOLOGY

Given the wide distribution of ER and the pleiotropic nature of estrogen and its multiple metabolites, SERMs should be expected to likewise affect multiple organ systems, and this is indeed the case. Because the focus of this volume is on skeletal pharmacology, emphasis here will be placed on the pharmacologic effects of SERMs on bone and on other tissues of relevance to safety in the clinical setting. Accordingly, emphasis will be placed on those SERMs where osteoporosis and bone has been the primary focus of research. SERMs primarily used for other indications (present or with future potential) will be briefly summarized as well.

Skeletal System

Preclinical Studies

Much as in postmenopausal women, estrogen deficiency in ovariectomized (OVX) animals leads to a rapid increase in

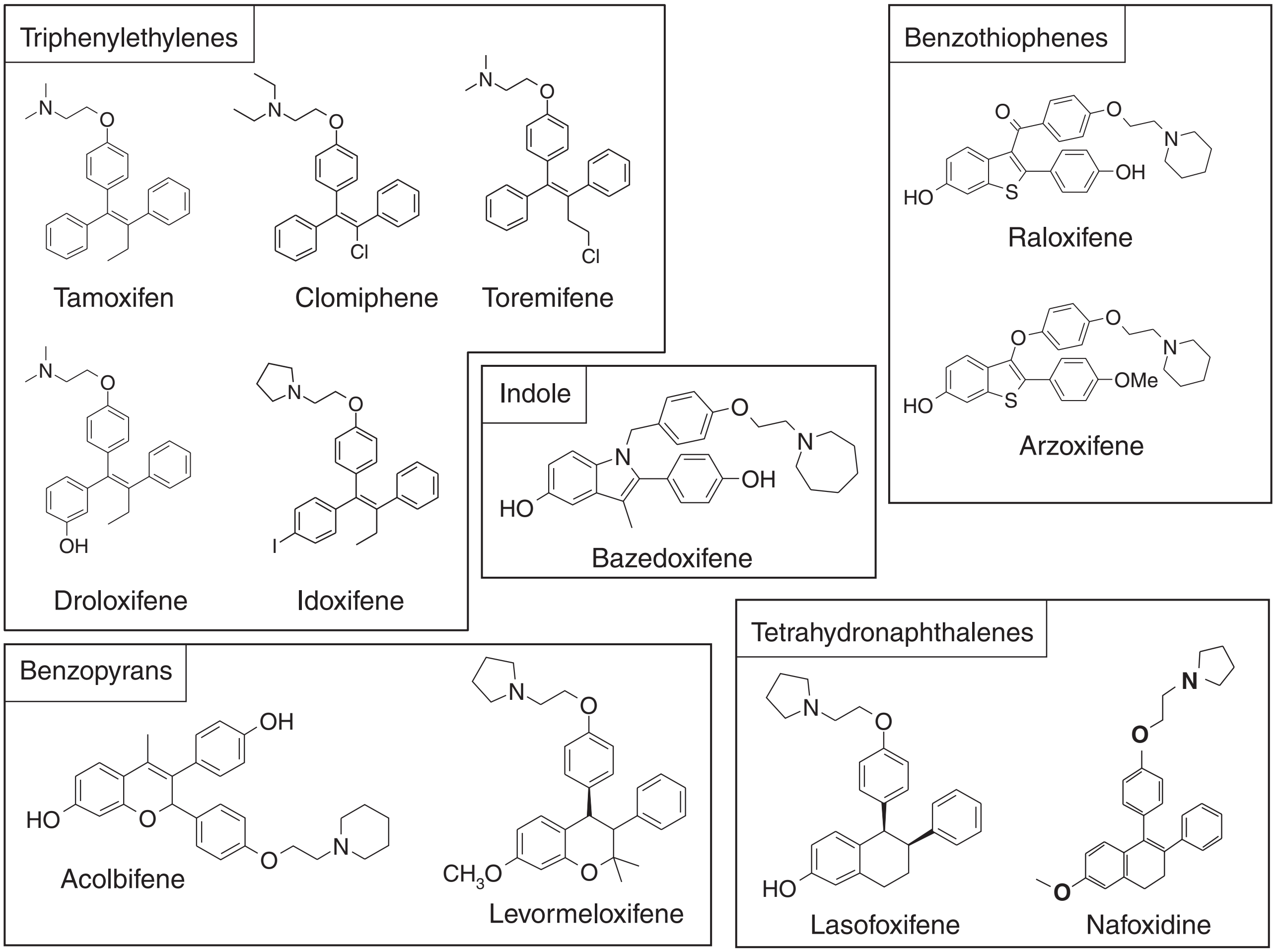
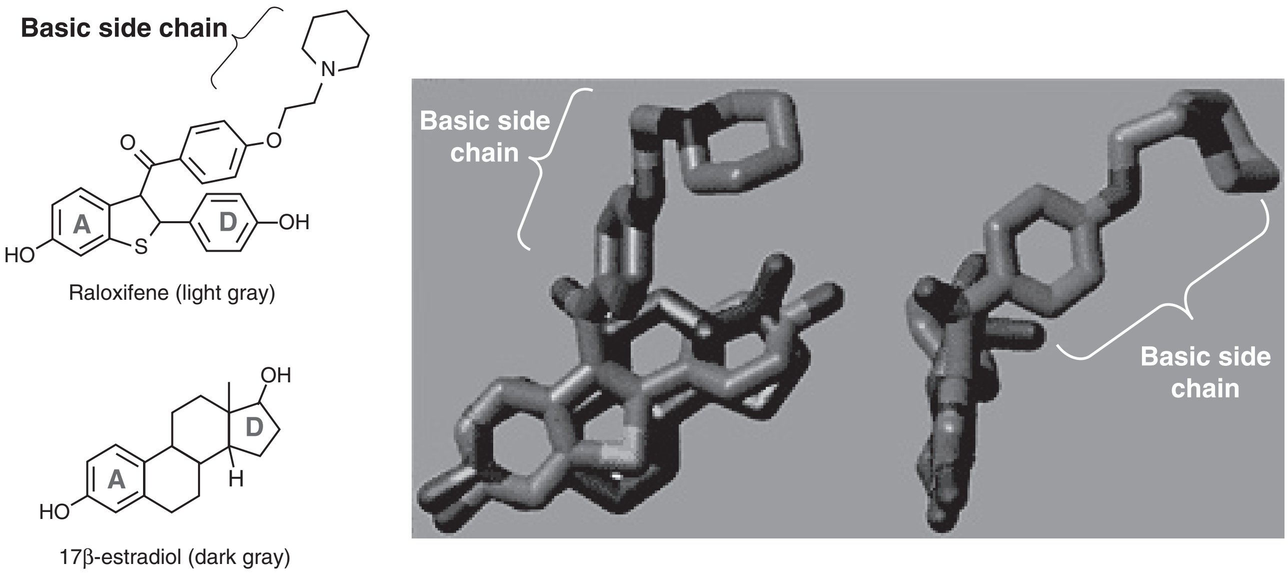


FIGURE 3 Structures and chemical class of SERMS currently in clinical use or that have reached phase 3 clinical trials in women.



Raloxifene (light gray)

17β-estradiol (dark gray)

FIGURE 4 Key structural features of raloxifene versus 17β-estradiol.

bone turnover, where excessive osteoclast resorptive activity results in a marked decline in trabecular bone mass and strength, with concomitant increase in fractures. In rats, ovariectomy produces a rapid osteopenic response, which can be discerned within 5 weeks. All of the SERMs depicted in Figure 3 have been evaluated in the OVX rat, and demonstrate estrogen-like protection from bone loss induced by estrogen deficiency. In the OVX rat model, SERMs like raloxifene (Black *et al.*, 1994), arzoxifene (Sato *et al.*, 1998), tamoxifen (Sato *et al.*, 1996), droloxifene (Ke *et al.*, 1995), idoxifene (Nuttall *et al.*, 1998), clomiphene (Jiminez *et al.*, 1997), bazedoxifene (Komm *et al.*, 2005), lasofoxifene (Ke *et al.*, 1998), levormeloxifene (Galbiati *et al.*, 2002), toremifene (Qu *et al.*, 2000), and acolbifene (Martel *et al.*, 2000) prevent the loss of bone in vertebrae, distal femur, and proximal tibia, all trabecular-rich bone sites. In addition to maintaining bone mass, SERMs also preserve bone strength through improvements in bone microarchitecture (i.e., raloxifene; Turner *et al.*, 1994). For both bone mass and bone strength, the absolute magnitude of the effects of most SERMs in OVX rats are indistinguishable from those of estrogen and can approach values attained for sham-surgery controls, when the SERM is administered in a preventative mode. However, differences in potency for these bone-protective effects can occur, with third-generation SERMs like arzoxifene, bazedoxifene, and lasofoxifene producing equivalent efficacy to raloxifene in OVX rat trabecular BMD responses at approximately 10% of the dose (Ke *et al.*, 1998; Sato *et al.*, 1998). There have been preclinical hints for improved efficacy of some of these latter agents as well, for example, with arzoxifene, which improves cortical bone strength to a greater degree than observed with maximally effective doses of either estrogen or raloxifene (Sato *et al.*, 1998). Similarly with bazedoxifene, improved biomechanical properties in trabecular bone were observed relative to estrogen after 1 year of treatment in OVX rats (Komm *et al.*, 2007).

As with estrogen, the primary activity of SERMs responsible for the beneficial effect on bone is antiresorptive. Mechanistic studies *in vitro* demonstrated that raloxifene and estrogen exert their antiresorptive effects primarily as inhibitors of osteoclast differentiation, rather than as direct inhibitors of activated osteoclasts. Raloxifene acts to suppress mediators of osteoclast differentiation, such as the receptor activator of nuclear factor-κB (RANK), RANK-ligand (Bashir *et al.*, 2005) and increase endogenous antiresorptive factors such as osteoprotogerin (Viereck *et al.*, 2003). Biochemical markers of bone turnover (i.e., serum osteocalcin, urinary collagen cross-links) are suppressed in OVX rats in a manner similar to that observed with estrogen (Frolik *et al.*, 1996). Histomorphometric analysis of bone from raloxifene-treated, OVX, rats confirmed the antiresorptive mechanism of action for raloxifene (Evans *et al.*, 1994). Similar studies with the other SERMs discussed here indicate the same antiresorptive mechanism for bone protection. Of likely importance with respect to long-term safety in the skeleton is the finding that SERMs, like raloxifene, produce their inhibitory action on bone resorption with minimal suppressive effects on bone formation, leaving bone formation rates at levels comparable to sham-operated control animals (Evans *et al.*, 1994). The molecular fingerprint of SERMs in estrogen-deficient rat trabecular bone, as assessed by DNA microarray, is unique for each SERM, although it is clear that some SERMs are less suppressive of bone formation. For example, in OVX rats raloxifene returned a cluster of genes associated with bone formation to ovary-intact control levels, as opposed to a bisphosphonate (alendronate), estrogen, or even another SERM (acolbifene), which exhibited a greater suppressive effect on bone formation-associated genes (Helvering *et al.*, 2005). The overall SERM profile on bone then represents a sharp distinction from the marked suppression of bone formation that occurs with other bone antiresorptives, such as the bisphosphonates. A final bone cell type that may be of relevance to the skeletal-protective effect of SERMs is the osteocyte. Whereas relatively less work has been done with this bone cell owing to technical challenges, work in MLO-Y4 cells (an osteocyte-like cell line) has indicated that raloxifene prevents oxidative stress-induced apoptosis and inhibits generation of reactive oxygen species with oxidative insults, such as hydrogen peroxide (Mann *et al.*, 2007).

Clinical Studies

The abundance of preclinical information on the effects of SERMs on bone has easily been matched by a plethora of long-term clinical trials that have been conducted on a number of different SERM molecules, either as the primary element of registration trials for postmenopausal osteoporosis or as part of the safety assessment for use in breast cancer. Certainly, the most extensively studied SERM on the human skeleton has been raloxifene, which has been investigated in nearly 40,000 clinical trial subjects enrolled in prospective, randomized trials (placebo or active comparator) that have ranged in duration from 1 to 8 years. In postmenopausal women raloxifene (60 mg/day) exhibits an antiresorptive action as evidenced by reductions in the accelerated bone turnover as measured by biochemical markers of bone resorption (Ettinger *et al.*, 1999), while only modestly suppressing bone formation. In calcium tracer kinetic studies in postmenopausal women, Heaney and Draper (1997) showed suppression of bone resorption with raloxifene whereas bone formation was not affected after 31 weeks of treatment. The observation of resorption inhibition with minimal formation suppression by raloxifene was confirmed by histomorphometric analysis of iliac crest bone biopsies (Ott *et al.*, 2002). This antiresorptive activity is associated with an approximately 2.5% increase in vertebral BMD, relative to placebo-treated controls. This increase in spine BMD that occurs following raloxifene treatment in postmenopausal women is less marked than observed with a bisphosphonate (alendronate; Johnell *et al.*, 2002). However, this magnitude of BMD improvement in the spine underestimates the mechanical improvement produced by raloxifene, as evidenced by the 55% reduction in new vertebral fractures in women with prevalent fractures (Ettinger *et al.*, 1999), a rate comparable to that produced by bisphosphonates. This observation has led to an increased attentiveness to potential effects of raloxifene (and putatively other SERMs as well in the future) on bone quality. The eventual resistance of bone to fracture is the result both of the content, or mass of the material (i.e., BMD), and the quality of that material, which is likely the result of bone microarchitecture and the nature of the mineralized matrix itself. In animal models, increased trabecular thickness and maintenance of plate-like trabecular structures (versus rod-like), both of which correlate with improved biomechanical strength of bone, were observed in OVX mice (Cano *et al.*, 2008). Although BMD is a noninvasive, easily quantifiable, parameter in clinical trials, bone quality remains a more difficult to quantify, because it is primarily revealed by the eventual incidence of fracture. A number of efforts have targeted better understanding, and quantifying, of bone quality where raloxifene has shown some benefits over other antiresorptive therapies such as histomorphometric analyses of trabecular bone architecture and microcrack frequency in bone (Allen *et al.*, 2000; Li *et al.*, 2005). One area where some aspect of bone quality is beginning to be elucidated is the proximal femur, where imaging technologies have been applied to postmenopausal clinical trial subjects to show an increase in resistance to axial and bending stresses in raloxifene-treated women (Uusi-Rasi *et al.*, 2006), indicating improved structural components of bone strength and stability with the SERM. Raloxifene produces positive effects on hip BMD, which increased 2.1% versus placebo after 3 years in postmenopausal women (Ettinger *et al.*, 1999), although without a significant effect on nonvertebral fracture rates (Ettinger *et al.*, 1999). However, an interesting trend was noted in a subset of women who entered the trials with severe vertebral fractures. In this subset of more severely osteoporotic women, raloxifene produced a 50% reduction in nonvertebral fractures (Delmas *et al.*, 2003). Finally, in addition to reduction of vertebral fracture in osteoporotic women, raloxifene also provides fracture risk protection to osteopenic women (Kanis *et al.*, 2003).

A number of other SERMs have attempted unsuccessfully to register for an osteoporosis prevention/treatment indication, are currently in phase 3 clinical trials, or are awaiting regulatory approval. Those molecules that have failed to achieve regulatory approval for osteoporosis failed primarily on the basis of safety and risk/benefit analysis, as each demonstrated some level of improvement on skeletal parameters in earlier clinical trials. Prior to discontinuation of the levormeloxifene phase 3 clinical trials because of gynecological-associated adverse events, phase 2 clinical trials demonstrated positive effects of this SERM on BMD and bone turnover (Alexandersen *et al.*, 2001). Idoxifene, a triphenylethylene also discontinued in phase 3 for uterine adverse events, produced clinically relevant increases in BMD in osteopenic postmenopausal women (Chestnut *et al.*, 1998). The most recent SERMs to report advanced clinical testing results for osteoporosis are the third-generation molecules, lasofoxifene and bazedoxifene, both very potent SERMs with relatively high bioavailability (Gardner *et al.*, 2006; Patat *et al.*, 2003). In a 2-year trial in 410 postmenopausal women lasofoxifene at 0.25 or 1 mg/day suppressed bone turnover similarly to raloxifene, but lasofoxifene increased lumbar spine BMD by 3.6% and 3.9%, respectively, which outpaced the increase observed with raloxifene (McClung *et al.*, 2006). A 2-year BMD trial and 3-year fracture prevention trial demonstrated the skeletal protective effects of bazedoxifene relative to raloxifene. In the 3-year trial, nearly 7500 women were treated with 20 or 40 mg/day bazedoxifene, placebo, or raloxifene at 60 mg/day. In this trial, the bazedoxifene produced a significant reduction in the relative risk reduction for new vertebral fractures of 37% for the higher dose and 42% for the lower dose, with raloxifene producing a comparable 42% in relative risk of new vertebral fractures (Silverman *et al.*, 2007). Mean lumbar spine BMD was significantly improved, relative to placebo, by bazedoxifene with a magnitude of response comparable to raloxifene, and biochemical markers of bone turnover were also significantly lowered with bazedoxifene (Miller *et al.*, 2007).

A number of clinical trials have focused on the bone-sparing effects of two triphenylethylene SERMs used for breast cancer treatment: tamoxifen and toremifene. Although most studies demonstrate a skeletal benefit for these two agents, trials have typically been small and not placebo controlled in design. There is a consistent benefit observed with tamoxifen and toremifene primarily at trabecular bone sites, which is consistent with observations made with raloxifene in postmenopausal women. After 3 years of use, tamoxifen or toremifene in breast cancer patients led to a less-than-expected decline in vertebral BMD (Tiitinen *et al.*, 2004). In shorter trials (1 year), similar effects were observed with the effect of tamoxifen somewhat stronger than that of toremifene (2% higher BMD with tamoxifen versus toremifene, which basically prevented age-related decline over the 1-year trial; Marttenun *et al.*, 1998). Although many studies have reported similar benefits, particularly with tamoxifen, on BMD in postmenopausal breast cancer patients (e.g., Love *et al.*, 1992), there is at least one indication that use of tamoxifen in normal premenopausal women is associated with a reduction in bone mineral density (Powles *et al.*, 1996).

Reproductive System

Uterus

Atrophy of the uterus accompanies estrogen deficiency in humans and most animal species, and cessation of menses is a hallmark feature of the menopause in women. A major side effect of most current ER-based therapies is stimulation of the uterus resulting, in part, from estrogen-induced proliferation of uterine endometrial tissue. The cancer concern associated with this proliferative effect and the resumption of menses (when combined with progestin regimens as hormonal replacement therapy) are major limitations to estrogen replacement therapeutic approaches These uterine side effects of estrogen are often the primary deterrent in the risk/benefit decision for postmenopausal women to utilize or remain compliant with, hormonal estrogen therapies for chronic use with diseases such as osteoporosis. A major and significant advantage of SERMs like raloxifene, for postmenopausal women, over hormonal estrogen therapies, is the lack of uterine stimulation with the SERM. However, not all SERMs share the same degree of uterine safety that is observed with raloxifene and, thus, effects on the uterus also serve as an important distinguishing feature among various SERMs. To this regard, those SERMs that have failed to date in phase 3 clinical trials have done so primarily because of an untenable degree of uterine stimulation or uterus-related adverse events. The accumulated clinical experience with uterine safety for a number of SERMs can help one determine which preclinical models and parameters provide the optimal predictive value for uterine safety. The use of *in vitro* systems, estrogen-depleted animals, and estrogen-replete animals to assess antagonist potential form a triangulated approach for uterine safety assessment of SERMs.

Estrogen Agonism in the Uterus

Initial indication of the uterine estrogenic potential of SERMs can be demonstrated using Ishikawa cells, a human endometrial cancer cell line. Ishikawa cell proliferation is stimulated by 17β-estradiol, tamoxifen, or 4-hydroxytamoxifen (active metabolite of tamoxifen), but not by uterine-sparing SERMs such as raloxifene (Koda *et al.*, 2004). More subtle changes among various SERMs can be detected by evaluating their effects on ER-mediated alkaline phosphatase production, creatine kinase production, or expression of progesterone receptor. The more uterine stimulatory SERMs produce greater induction of alkaline phosphatase and progesterone receptor expression, and are less effective antagonists of 17β-estradiol-stimulated responses in Ishikawa cells (Bramlett *et al.*, 2002). Of note, whereas uterine-safe SERMs like raloxifene fail to stimulate creatine kinase production in Ishikawa cells, raloxifene induces this activity in cell lines with an osteoblast background, consistent with the "SERM" activity profile (Koda *et al.*, 2004).

Lack of biologically meaningful stimulation of the uterus in the estrogen-depleted state (e.g., postmenopausal women or OVX animals) is the crux of SERM uterine safety evaluation. The uterine effects of tamoxifen and raloxifene have been thoroughly evaluated in numerous clinical settings, as well as in a variety of preclinical models. Raloxifene does not produce estrogen-like stimulatory effects in the uterus of OVX rats (Black *et al.*, 1994). In the OVX rat model, a slight, non-dose-related elevation of uterine weight is frequently observed. However, this phenomenon contrasts markedly with the robust, dose-related elevation of uterine weight produced by estrogen in these animals. Raloxifene fails to stimulate other estrogen-sensitive markers in the uterus of OVX rats, such as uterine eosinophilia, or uterine epithelial cell height (Black *et al.*, 1994). In large-scale clinical trials after 8 years of chronic use in postmenopausal women, extensive uterine safety evaluation revealed no significant uterine-stimulatory effects of raloxifene in humans (Delmas *et al.*, 1997). Indeed, a significant reduction in endometrial cancer of the uterus has been noted in postmenopausal women using raloxifene (Delmas *et al.*, 1997).

As indicated, not all SERMs exhibit the uterine safety profile demonstrated with raloxifene. This is perhaps most evident in the extensive work done with tamoxifen. Studies in the uterus of OVX rats demonstrate robust, dose-related stimulatory effects of tamoxifen on uterine weight, uterine epithelial cell height, and uterine eosinophilia (Adrian *et al.*, 1996). Clinical experience with tamoxifen is consistent with these observations, as uterine bleeding and significant elevation of endometrial cancer has been observed in women exposed to tamoxifen in long-term studies (Fisher *et al.*, 1994).

Thus, raloxifene and tamoxifen serve as bookend profiles for uterine safety for chronic SERM use in postmenopausal women where molecules can be assessed for either a "raloxifene-like" profile of little or no uterine stimulation, or a more estrogenic, "tamoxifen-like" profile. Several SERMs have advanced to clinical research and have corroborated the preclinical observations for those molecules. For example, droloxifene, idoxifene, and levormeloxifene all produced dose-related elevation of uterine weight, uterine epithelial height, and uterine eosinophilia in the OVX rat (Adrian *et al.*, 1996), and were eventually halted in clinical development on the basis of uterine liabilities such as increased bleeding, increased endometrial thickness, and polyps that developed in phase 3 studies for osteoporosis (Silfen *et al.*, 1999). The other triphenylethylenes used clinically today, clomiphene and toremifene, also exhibit an overall uterine stimulatory profile in OVX rats (Turner *et al.*, 1998; Carthew, 1999), although uterine safety is less of a concern with the acute therapeutic use of clomiphene, and the risk/benefit profile for toremifene use in breast cancer treatment has a different weighting of risk. Mixed effects have been observed with lasofoxifene, which in some preclinical reports produced significant, dose-related stimulation of uterine epithelial cell height and uterine eosinophilia (Cole *et al.*, 1997), but not in others (Ke *et al.*, 1998). The outcome of clinical trials with lasofoxifene at this time is incomplete, although initial observations indicate statistically significant uterine stimulation with approximately an 80% increase in endometrial thickness relative to placebo-treated controls and an increased incidence of leukorrhea with lasofoxifene (McClung *et al.*, 2006). On the other hand, bazedoxifene, arzoxifene, and acolbifene produced very little stimulation of the uterus in OVX rats (Komm *et al.*, 2005; Sato *et al.*, 1998; Martel *et al.*, 2000) and, at least with bazedoxifene, studies in postmenopausal women have corroborated the animal work (Adachi *et al.*, 2007).

A significant uterine-related adverse event, which emerged in a number of clinical trials, is relaxation of the pelvic floor and typically associated prolapse of the uterus. Estrogen therapy was traditionally thought to improve structural integrity of pelvic tissue with expected favorable effects on symptoms such as urinary incontinence. However, data from both the WHI Trial and the Heart and Estrogen/progestin Replacement Study (HERS) provided evidence for increased incidence of urinary incontinence in association with estrogen use (Hendrix *et al.*, 2005). Related to this, pelvic organ prolapse was associated with both levormeloxifene and idoxifene within one year of therapy (Fleischer *et al.*, 1999, Goldstein *et al.*, 2002). These observations, taken together, suggest that these uterine wall-associated adverse events with idoxifene and levormeloxifene might also be because of their more estrogen-like uterine activity. The mechanism by which estrogen and some SERMs compromise the uterine wall in postmenopausal women may be linked to an increase in collagen-degrading enzymes, such as metalloproteinase (MMP)-2 based on OVX rat studies. In OVX rats, uterine MMP-2 levels were reduced relative to ovary-intact animals. Estrogen, as well as levormeloxifene, produced marked increases in uterine MMP-2 expression (Helvering *et al.*, 2005). Not all SERMs, however, have been associated with pelvic floor problems, because no increase in this adverse event has been related to chronic use of tamoxifen, toremifene, bazedoxifene, or lasofoxifene (Fisher *et al.*, 1994, Maenpaa *et al.*, 1997, McClung *et al.*, 2006, Adachi *et al.*, 2007). Consistent with its uterine-safe overall profile, the extensive uterine safety evaluations with raloxifene show no increase in the incidence of problems associated with pelvic floor relaxation. Rather, in at least one report, raloxifene produced benefit with significant reduction in the frequency of surgery for pelvic floor relaxation in a population of postmenopausal women (Goldstein *et al.*, 2001).

Estrogen Antagonism in the Uterus

The final important component in assessment of the uterine safety profile of SERMS relies primarily on preclinical data: the ability of SERMs to antagonize the uterine stimulatory effects of estrogen. Further insight into the uterine activity profile of SERMs is gleaned from effects on the uterus in the presence of estrogen, because this allows assessment of the uterine estrogen antagonist potential for these compounds. As in the estrogen-deficient state, the various SERMs also produce one of two general activity profiles in estrogen-replete animals: that of either a complete estrogen antagonist or that of a partial agonist at the ER. In the uterus of either estrogen-treated immature or adult-OVX rats, SERMs producing desirable uterine safety profiles in postmenopausal women like raloxifene, arzoxifene, and bazedoxifene produce a complete estrogen antagonistic effect (Adrian *et al.*, 1996, Sato *et al.*, 1998, Komm *et al.*, 2005). This effect is most clearly demonstrated in the estrogen-treated immature rat, a model classically used to determine uterine liability of ligands for the ER. In this model, raloxifene blocks the uterotrophic effects of estrogen with an ED_{50} of 0.3 mg/kg (Silfen *et al.*, 1999), whereas arzoxifene and bazedoxifene completely antagonizes the estrogen response with greater potency (Sato *et al.*, 1998; Komm *et al.*, 2005). A key feature of this antagonistic effect with SERMs like raloxifene in the immature rat uterus is the complete antagonism of the uterotrophic effect of estrogen. That is, uteri from estrogen-treated immature rats given doses of raloxifene exceeding 3 mg/kg are indistinguishable from those of non-estrogen-treated immature rats (Adrian *et al.*, 1996). This is in dramatic contrast to other SERMs, best exemplified by tamoxifen, which behaves as a classical partial agonist in the uterus. That is, tamoxifen does significantly antagonize the effects of estrogen in the immature rat uterus. However, in the case of tamoxifen, the maximal degree of this antagonism is only approximately 50% (Adrian *et al.*, 1996). The primary reason for this incomplete antagonistic effect of tamoxifen is that, at higher doses, the inherent uterine stimulatory capacity of tamoxifen limits further suppression of the estrogen-induced uterotrophic response. Other triphenylethylene SERMs, as well as levormeloxifene (Adrian *et al.*, 1996), produce partial estrogen agonist profiles in estrogen-stimulated immature rats.

In addition to usefulness of this parameter for SERM uterine safety assessment, the complete estrogen antagonistic effects of SERMs like raloxifene have been utilized successfully in a variety of preclinical models of estrogen-stimulated uterine pathologies with some limited success in clinical studies. For example, *in vitro* and *in vivo* models of uterine leiomyoma (fibroids) demonstrated favorable responses to raloxifene (Porter *et al.*, 1998) with some reports of successful use of high-dose raloxifene to prevent the progression of uterine leiomyomas in small groups of premenopausal and postmenopausal women, either alone or in combination with a GnRH agonist (Jirecek *et al.*, 2004; Palomaba *et al.*, 2002b, 2005) via a combined antiproliferative/proapoptotic effect. However, other small clinical trials have failed to detect a benefit of raloxifene on uterine leiomyoma (Palomba *et al.*, 2002a), indicating the need for randomized, well-controlled clinical trials for this indication, which would be necessary to balance benefit on leiomyoma status versus the additional risk of SERM use in premenopausal women of ovarian stimulation (discussed later; note: raloxifene is not currently indicated for any uses in premenopausal women). In a rat model of another estrogen-dependent, uterine-related pathology, endometriosis, raloxifene inhibited the estrogen-dependent growth of peritoneal uterine explants (Swisher *et al.*, 1995). In monkeys with spontaneous endometrial lesions, raloxifene eliminated the occurrence of peritoneal endometriosis lesions (Fanning *et al.*, 1997).

The uterine estrogen antagonist effect of SERMs is also of potential benefit in the treatment of endometrial cancer, the most common neoplasm of the female urogenital tract. In many cases, endometrial cancer is susceptible to hormonal-targeted therapies involving both progestin-based and estrogen-depletion strategies. Given the role of estrogens, both directly and indirectly via regulation of progesterone receptor, SERM approaches have been explored. Tamoxifen was evaluated in a small cohort of advanced or recurrent endometrial carcinoma patients with limited success, dissuading the use of tamoxifen as a single agent for this tumor type (Thigpen *et al.*, 2001). Promising efficacy in endometrial cancer treatment was reported with the third-generation SERM, arzoxifene. In early studies in a small number of treatment-refractory endometrial cancer patients, arzoxifene produced a favorable clinical response rate and stabilized the disease in a substantial number of women (Burke and Walker, 2003). In a phase 2 open-label study, ER-positive and progesterone receptor-positive patients with recurrent/advanced endometrial cancer also exhibited a high response rate and duration of response with a favorable side effect profile after administration of arzoxifene (McMeekin *et al.*, 2003).

Mammary

The effects of estrogens on mammary tissue in normal adult animals or women are not typically as clear and robust as those effects observed in the uterus. However, clearly, a majority of mammary tumors are ER positive, and respond favorably to estrogen antagonism (Wakeling *et al.*, 1987). The role of estrogen replacement in the risk of breast cancer is a topic of some controversy and, clearly, the concomitant use of progestins in most hormonal replacement therapy paradigms is confounding. In the WHI Trial, a significant increase in risk of breast cancer in postmenopausal women was a key finding (Writing Group for WHI Investigators, 2002), contributing to dramatic reductions in the use of estrogen replacement strategies for various postmenopausal indications. The role of the progestin in this observation was suggested by a follow-up study in hysterectomized women using unopposed conjugated equine estrogens for more than 7 years, where no increase in breast cancer risk was observed (Stefanick *et al.*, 2006). Although some controversy remains over the exact potential for risk of breast cancer with estrogen therapies, it is clear that concern over this risk has limited patient compliance with steroidal estrogen therapies. A major advantage of the SERM class of molecules is the lack of this cancer concern with respect to mammary tissue. Indeed, SERMs as a class have demonstrated a benefit in either treating or preventing breast cancer in animal models and women. The effects of SERMs in normal mammary tissue, as breast cancer therapies and preventatives, will be summarized here.

In normal mammary tissue, the effects of SERMs are largely unnoticeable. Extensive use of tamoxifen in both premenopausal and postmenopausal women for the use of breast cancer risk reduction has not been associated with untoward effects on mammary tissue, although in developing mammary glands in mice, tamoxifen impaired growth of mammary ducts and increased mammary alveolar development (Hovey *et al.* (2004)). A cautionary note in interpreting animal data with respect to SERMs on mammary tissue is worth mentioning. The rat is unique in that male mammary tissue exhibits a layering of epithelial cells that has a somewhat hypertrophic appearance, which is an androgen-dependent phenotype in rats. SERMs with highly effective estrogen antagonism in mammary tissue can sufficiently block estrogen influence on mammary epithelia in rats permitting the low ambient level of androgen in the female rat to predominate and convert mammary histological phenotype to resemble that of the male rat (Rudmann *et al.*, 2005), a phenomenon known not to exist in humans. Caution should be taken not to confuse this female/male phenotype conversion in the rat with hypertrophy of mammary tissue.

The antitumor effects of SERMs can be demonstrated readily in mammary tumor cell lines (i.e., MCF-7, T-47D, ZR-75-1) *in vitro*. Each of the SERMs depicted in Figure 3 are estrogen antagonists in one or all of these cell lines (Short *et al.*, 1996, Simard *et al.*, 1997, Komm *et al.*, 2005). The MCF-7 human mammary tumor cell line is an excellent, estrogen-dependent, *in vitro* system for determining antagonism of estrogen-induced proliferative activity of compounds. Most SERMs inhibit estrogen-stimulated proliferation in the 0.2 to 1 n*M* range in this assay, although for those triphenylethylene SERMs requiring formation of active metabolites, such as tamoxifen and toremifene, one must evaluate the active metabolite to see this level of potency. For example, the IC_{50} for tamoxifen in MCF-7 cells is 200 n*M* vs. 1.2 n*M* for 4-hydroxytamoxifen (Suh *et al.*, 2001). Des-methylarzoxifene, a likely active metabolite of arzoxifene, is the most potent inhibitor of MCF-7 proliferation with an IC_{50} value of 0.05 n*M* (vs. 0.4 n*M* IC_{50} for arzoxifene in this cell line; Suh *et al.*, 2001). Also of relevance in the MCF-7 tumor cell line, raloxifene fails to induce proliferation of these cells in the absence of exogenous estrogen, contrasting raloxifene with other SERMs, such as tamoxifen, which produce a low level of MCF-7 proliferation in estrogen-deficient cell culture media (Sato *et al.*, 1995).

Consistent with the mammary tumor cell culture work is the estrogen antagonist effects of SERMs in animal models of estrogen-dependent breast cancer. Various animal models have shown the ability of SERMs such as tamoxifen, toremifene, and raloxifene to blunt the growth of established mammary tumors induced by carcinogens such as dimethylbenzanthrocene (Clemens *et al.*, 1983; Robinson *et al.*, 1988) or in breast cancer tumor cell line xenografts in athymic mice (Fuchs-Young *et al.*, 1997; Qu *et al.*, 2000). Tamoxifen, raloxifene, and lasofoxifene are also effective in preventing mammary tumors induced by other chemical carcinogens, such as nitrosomethylurea (Gottardis *et al.*, 1987; Anzano *et al.*, 1996; Cohen *et al.*, 2001). Of great interest is the apparent increase in mammary tumor efficacy that has been demonstrated preclinically for some of the more recently developed SERMs. For example, arzoxifene, a third-generation SERM molecule, produced a significantly improved efficacy in preventing mammary tumor growth *in vivo* (Suh *et al.*, 2001).

With respect to treatment of human breast cancer, numerous options are in current use that employ endocrine-based strategies with some patients considered suitable for estrogen reduction or antagonism strategies alone (i.e., estrogen-positive tumors) and others using endocrine manipulation approaches as an adjunct to traditional tumor chemotherapy. Two SERMs, tamoxifen and toremifene, are currently approved for chemo/endocrine treatment in the management of postmenopausal women with node-positive breast cancer. With respect to breast cancer, tamoxifen has been in use the longest of any SERM, with greater than 20 years of clinical use. When used as an adjunct, tamoxifen reduces the risk of recurrent cancer and also decreases the risk of new tumors arising in the other breast. Both tamoxifen and toremifene show similar efficacy in terms of 5-year overall survival and disease-free survival rates (the disease-free rate for tamoxifen is 69%, and for toremifene is 72%) for early-stage breast cancer and also demonstrate similar efficacy as first line therapy for metastatic breast cancer in postmenopausal women (International Breast Cancer Study Group, 2004). The only other SERM in late-stage clinical development with clinical assessment of breast cancer treatment potential is arzoxifene, which has been evaluated in several phase 2 trials in advanced breast cancer patients, where some benefit in terms of time to progressive disease and clinical benefit rate were observed (Baselga *et al.*, 2003).

The other significant current application of SERMs is in breast cancer prevention. A number of environmental and genetic factors are associated with increased risk of developing breast cancer in women, including advanced age, family history of breast cancer, and a greater lifetime estrogen exposure (assessed via surrogate indicators such as estradiol levels, use of estrogen therapy, age at menopause, and body mass index). The best current tool for overall assessment of breast cancer risk is the Gail model, where a risk factor of greater than or equal to 1.67 defines a woman at risk (Costantino *et al.*, 1989). Tamoxifen was the first SERM to show reduced risk of breast cancer through a number of large, placebo-controlled trials. In the Breast Cancer Prevention Trial, tamoxifen was evaluated in a cohort of 13,388 women at increased risk of breast cancer and produced a 49% reduction in the relative risk of invasive breast cancer, with a 69% reduced risk of ER-positive mammary tumors (Fisher *et al.*, 1998). However, despite this substantial reduction in risk, and inclusion of breast cancer risk reduction as an approved use for tamoxifen, the clinical use of tamoxifen for this indication has been rather lackluster—primarily owing to a side effect profile that tilts the risk/benefit ratio in a negative direction in the mind of most physicians and women. The side effects profiles will be reviewed in subsequent sections, but for tamoxifen include endometrial cancer, uterine sarcoma, stroke, venous thrombus events, and cataracts. The increase in endometrial cancer in postmenopausal women likely stems from the uterine stimulatory properties of tamoxifen and represents one area for improvement in other SERMs. In this regard, raloxifene has recently received approval for reducing the risk of invasive breast cancer in postmenopausal women with osteoporosis and in postmenopausal women at high risk for invasive breast cancer. After 8 years of monitoring 4,011 postmenopausal women with osteoporosis, a 66% reduction in the incidence of invasive breast cancer was observed with raloxifene use (Martino *et al.*, 2004). In the Study of Tamoxifen and Raloxifene (STAR) Trial, a head-to-head comparison of the two SERMs was conducted in 19,000 postmenopausal women at high risk of breast cancer, where tamoxifen and raloxifene were found to produce similar reductions in the incidence of invasive breast cancer (Vogel *et al.*, 2006), with the primary benefit being a reduced risk of ER-positive invasive breast cancers (Barrett-Conner *et al.*, 2006). The most significant differences between raloxifene and tamoxifen in the STAR trial were significantly fewer uterine-associated adverse events with raloxifene (most notably the lack of endometrial cancer), whereas tamoxifen appeared to have a greater effect on noninvasive breast cancer incidence than raloxifene (Vogel *et al.*, 2006). These differences between tamoxifen and raloxifene, although subtle, indicate a difference from preclinical and even early clinical indicators and, as such, demonstrate the need for thorough clinical evaluation before accurate therapeutic risk/benefit assessment and approval of indications can be made for human use. In this regard, several SERMs in development, such as acolbifene and bazedoxifene (Labrie *et al.*, 2004; Adachi *et al.*, 2007), have preclinical and early clinical profiles that are promising for potential use in reduction of risk for breast cancer, but until sufficient clinical evaluation has been completed, it is too early to predict the ultimate utility of these molecules in this regard.

The mechanism by which SERMs such as tamoxifen, raloxifene, and toremifene inhibit breast cancer development or progression is likely the result of multiple beneficial effects. Direct antagonism of estrogen action at ER in target cells in breast tissue, as previously described, is a key component of the action of the SERMs, given the strong positive linkage of estrogen exposure to relative risk for developing breast cancer and the fact that SERMs are much more effective versus ER-positive breast cancers. However, it is also likely that antitumor effects to reduce estrogen bioavailability as well as effects independent of estrogen contribute to the ultimate anti-breast cancer effect. Raloxifene is known to elevate levels of sex hormone-binding globulin (Reindollar *et al.*, 2002), which would be expected to reduce bioavailable estrogen levels and thereby further reduce the risk of estrogen-associated breast cancer. Other beneficial indirect SERM effects include: (1) modification of signaling proteins with a role in tumor cell biology, as is observed with tamoxifen on protein kinase C, TGFβ, calmodulin, ceramide, MAP-kinases (Mandlekar and Kong, 2001); (2) induction of apoptosis in mammary tumor cell lines, as with tamoxifen, raloxifene, and toremifene (Mandlekar and Kong, 2001; Diel *et al.*, 2002; Houvinen *et al.*, 1993); and/or (3) dampening of growth factor systems known to play a role in tumor progression. The latter of these indirect mechanisms may be the most important, because the contribution of growth factors in the pathogenesis of breast cancer is attracting considerable attention. The insulin-like growth factor (IGF) system, inclusive of signaling factors, IGF-receptors, and IGF-binding proteins, has strong connections to the malignant transformation of normal breast epithelium and thus is implicated in the development and progression of breast cancer (Ward *et al.*, 1994). The IGF system protects cancer cells from apoptosis, thus promoting their survival. Beneficial effects of SERMs on the IGF system were demonstrated in postmenopausal women after 2 years of raloxifene treatment. Raloxifene reduced circulating IGF-1 levels and increased IGF-binding protein-3 levels, which would be expected to be associated with reductions in bioavailable IGF-1 (Lasco *et al.*, 2006).

Mammographic density of the breast depends on the contributions of the predominant cell types in the breast: stromal, epithelial (both of which are higher-density tissue types), and fat tissue (which is relatively radiolucent by standard mammography). Although the role of breast density in prediction of relative breast cancer risk remains a topic of debate, most investigators agree that breast cancer risk is higher in women with higher-density breast tissue (Boyd *et al.*, 1995). Whether this is related to a protective effect of increased abundance of fatty tissue, or simply impedance in detecting small amounts of cancerous tissue in higher-density breast is not clear. However, it is clear that estrogen use is associated with a significant increase in mammographic density (Breendale *et al.*, 1999). Conversely, SERMs, specifically raloxifene and arzoxifene, were associated with a reduction of breast density in postmenopausal women (Lasko *et al.*, 2006; Kimler *et al.*, 2006).

Other

In addition to the uterus and mammary tissue, other components of the female reproductive system are under the direct or indirect control of estrogen and, thus, are also susceptible to the effects of SERMs. Depending on the patient, those SERM effects may be desirable, undesirable, or neutral.

Ovarian Effects

The ovary is an important regulator of cyclicity in the female reproductive system and the predominant source of circulating estrogen, and it is indirectly regulated via estrogen action on the hypothalamic-pituitary-ovarian (HPO) axis. In mice, 15- to 24-month exposure to tamoxifen, toremifene, or raloxifene is associated with ovarian tumors. However, there is no evidence of increased ovarian cancer risk with these agents in women. Careful evaluation of multiple randomized, placebo-controlled studies indicates that raloxifene did not increase ovarian cancer in postmenopausal women compared with placebo (Neven *et al.*, 2002). Thus, the murine observations may be owing to species differences in ovarian responses in terms of tumor development between mice and humans. The endocrine system plays a key role in the production of ovarian tumors in mice, particularly when gonadotropins are sustained at elevated levels for extended periods of time (Murphy *et al.*, 1973). In this regard, raloxifene produces a sustained, dose-related increase in serum luteinizing hormone (LH) levels and inhibition of ovarian follicle maturation in mice, both effects being reversible upon discontinuation of the SERM (Cohen *et al.*, 2000). *In vitro*, raloxifene is an antagonist of 17β-estradiol in pituitary gonadotrophs (Ortmann *et al.*, 1988), suggesting the elevation of LH with SERMs is related to blockade of the feedback inhibitory properties of estrogen on the HPO axis.

Hormonal Effects

In women, the most striking effects of SERMs on ovarian function are primarily through hormonal effects on the HPO axis. Because ovarian function is obviously a major difference between the premenopausal and postmenopausal state, the effects of SERMs are likewise different with each state of ovarian function. The HPO axis is a complex endocrine system for which normal physiology requires the interplay of a number of steroid and peptidyl hormones, including GnRH from the hypothalamus, follicle-stimulating hormone (FSH), prolactin and LH from the anterior pituitary, and estradiol as a hormone primarily originating from the ovary (although multiple tissue types possess the necessary enzymes to interconvert estrogen and testosterone, as well as generate multiple estrogen metabolites).

In premenopausal women, SERMs predominately exert a stimulatory effect on the HPO axis and ovulation, with stimulatory effects on GnRH, FSH, and LH, and marked increases in serum estradiol levels (Adashi *et al.*, 1996). Depending on the situation, this may be a desired therapeutic goal or an undesirable side effect. This effect has been taken advantage of for more than 40 years with the widespread use of clomiphene for induction of ovulation in women with ovulatory dysfunction who desire pregnancy. For this indication, clomiphene is used acutely to induce ovulation and increase the number of follicles produced in a given cycle. Use of clomiphene for more than twelve cycles in women who failed to become pregnant has been associated with an increase in ovarian cancer, leading to the recommendation to limit use of this agent to no more than six cycles if a pregnancy does not occur (Rossing *et al.*, 1994). The other two SERMs currently approved for use in premenopausal women are tamoxifen and toremifene, used in the management of breast cancer. In these women, chronic treatment with the SERM results in ovarian-associated adverse effects. Of note is the induction of ovarian cysts and high circulating levels of estradiol that frequently occur with tamoxifen in reproductive age women (Cook *et al.*, 1995). Although these ovarian cysts can cause discomfort, they rarely require surgical intervention.

By far, the predominant use of SERMs is in postmenopausal women, either for osteoporosis treatment or prevention (raloxifene), breast cancer treatment (tamoxifen or toremifene), or breast cancer risk reduction (tamoxifen or raloxifene). After the menopause or in estrogen-deficient OVX animals, estrogen levels drop and the lack of negative feedback provided by estrogen on the hypothalamus-anterior pituitary leads to an increase in FSH and LH levels or pulses. In general, SERMs exhibit a partial agonist effect on the HPO axis in postmenopausal women. Raloxifene, tamoxifen, and toremifene are associated with reductions in LH and FSH levels in postmenopausal women (Cheng *et al.* 2004, Ellmen *et al.*, 2003). HPO axis-related adverse effects, such as ovarian cysts, are not typically seen in postmenopausal women and have been reported only occasionally with tamoxifen use (Shushan *et al.*, 1996).

Another important estrogen-regulated hormonal product of the anterior pituitary is prolactin. In premenopausal women, raloxifene failed to alter circulating prolactin levels (Faupel-Badger *et al.*, 2006). In postmenopausal women, raloxifene (Cheng *et al.*, 2004) and clomiphene (Garas *et al.*, 2006) reduced serum prolactin, operating either directly on lactotropes in the anterior pituitary, or via increasing opiatergic tone in the hypothalamus (Lasco *et al.*, 2002).

Vaginal Effects

The drop in circulating estradiol levels associated with menopause is responsible for various vaginal-related symptoms, including itching, dryness, and dyspareunia. Various forms of estrogen or hormone replacement, via both systemic and local delivery routes, have been widely used to provide relief for postmenopausal women who have these symptoms. SERMs demonstrate a range of profiles on vaginal symptoms of menopause. Most reports in postmenopausal women indicate estrogen-like maturation of vaginal epithelial cells with tamoxifen and toremifene (Ellmen *et al.*, 2003; Friedrich *et al.*, 1998). Conversely, there is some indication for antagonism of estrogen influence on vaginal tissue, because both toremifene and tamoxifen produce a fourfold increase in vaginal dryness in postmenopausal women (Marttunen *et al.*, 2001), and toremifene partially antagonized the effect of estrogen on vaginal epithelium in postmenopausal women (Homesley *et al.*, 1993). The background estrogen status of the individual may be an important determinant, because, in women with a high estrogenic activity (with respect to hormonal cytology), tamoxifen produced no vaginal estrogen-like effects, whereas women with lower estrogen levels saw an increase in vaginal tissue maturation index (Shiota *et al.*, 2002). Even with these modest estrogen-like effects produced by toremifene or tamoxifen on the vagina, no beneficial or untoward urogenital effects of either agent have been observed in postmenopausal women (Marttunen *et al.*, 2001). Raloxifene use in postmenopausal women is associated with a more neutral profile on vaginal tissue in postmenopausal women, because it failed to affect vaginal epithelium in this population (Komi *et al.*, 2005) and is not associated with adverse vaginal symptoms (Davies *et al.*, 1999). Raloxifene also did not antagonize the beneficial effect of vaginal estrogen cream or an estradiol-releasing ring on vaginal atrophy or sexual function in postmenopausal women (Kessel *et al.*, 2003). One SERM that has been extensively studied for potential beneficial effects on postmenopausal vaginal-associated symptoms in phase 3 clinical trials is lasofoxifene. In clinical trials, lasofoxifene improved the vaginal epithelium maturation index (as was observed with tamoxifen and toremifene), but also reduced vaginal pH and reduced the incidence of dyspareunia (Portman *et al.*, 2004; Bachmann *et al.*, 2004). These unique effects of lasofoxifene might be related to increased vaginal mucus formation, which was observed in vaginal tissue from OVX rats treated with lasofoxifene (Wang *et al.*, 2006)—effects not observed with raloxifene or tamoxifen.

Cardiovascular System

Menopause is associated with a dramatic increase in cardiovascular disease and characteristic changes in a number of heart disease-associated risk factors, such as an increase in serum cholesterol and specifically LDL-cholesterol, with a decline in the cardioprotective HDL-cholesterol, elevated levels of lipoprotein(a) [Lp(a)], increased insulin resistance and fat mass resulting in a classic "metabolic syndrome" profile for heart disease risk (Spencer *et al.*, 1997). Unfavorable markers of vascular endothelial damage, such as elevations in homocysteine levels and C-reactive protein (CRP) also accompany the declining ovarian function during menopause (Hak *et al.*, 2000). The fact that women enjoy a relatively "cardioprotected" status prior to menopause relative to their male counterparts historically presented the impression that estrogen replacement should be beneficial for reducing cardiovascular disease in postmenopausal women. Certainly, in both estrogen-deficient animal models as well as in postmenopausal women, estrogen produces a number of effects that would be associated with an improvement in risk for cardiovascular disease, such as reduction of total circulating cholesterol levels, LDL-cholesterol, in particular, and a concomitant increase in HDL-cholesterol. Accordingly, observational clinical trials suggested a significant reduction of cardiovascular disease in women who used hormone replacement therapy after menopause (i.e., Stampfer and Colditz, 1991). Thus, it was an unanticipated result when randomized clinical trials, which were conducted to confirm the beneficial effects of hormone replacement therapy on the cardiovascular system, indicated the opposite effect: an increase in cardiovascular disease associated with estrogen/progestin use. The first trial to indicate this was the HERS trial, where no reduction in coronary heart disease was detected, but an increase in cardiovascular-related deaths in the initial year of therapy was observed (Hulley *et al.*, 1998). Similarly, the WHI Trial failed to demonstrate any beneficial effects of estrogen use, but again an increased risk of adverse cardiovascular outcomes, including increased incidence of myocardial infarction and stroke (Manson *et al.*, 2003). Finally, the Women's Estrogen for Stroke Trial (WEST) indicated an early increase in risk of fatal stroke during the first year of estrogen use in women with preexisting cerebrovascular disease (Viscoli *et al.*, 2001). Both observational and randomized, placebo-controlled studies in postmenopausal women have indicated a significant increase in risk of deep venous thrombosis and pulmonary embolism in association with hormone replacement (Manson *et al.*, 2003).

Thus, it is clear with SERMs that one needs to address two important considerations. First, and foremost, the cardiovascular safety profile of SERMs in postmenopausal women must be thoroughly evaluated based on the risk ascribed to hormone replacement therapy by trials such as HERS, WHI, and WEST. The second consideration revolves around potential beneficial effects on cardiovascular risk factors, which might be instructive in determining additional benefit of these molecules. However, if we are to learn a lesson from the hormone replacement field, one needs to be careful to rely on randomized, well-controlled trials to make this assessment. The cardiovascular safety of SERMs in clinical use and their potential cardiovascular benefits are discussed here.

Cardiovascular Safety of SERMs

The primary adverse event associated with every chronically used ER ligand is the occurrence of venous thrombolicevents (VTEs), typically as deep vein thromboses or pulmonary emboli. The relative frequency of VTEs with SERMs is typically 2- to 3-fold greater than placebo when assessed in randomized clinical trials, a rate that is comparable to that observed with use of oral estrogen replacement therapy (Cosman and Lindsay, 1999). Such an increase in venous thromboses has been described with tamoxifen or toremifene use in breast cancer patients (Cuzick *et al.*, 2003, Harvey *et al.*, 2006) and raloxifene use in osteoporotic patients (Duvernoy *et al.*, 2005). Comparable rates of venous thromboses have also been described in the 2- and 3-year phase 3 clinical trials for lasofoxifene and bazedoxifene (Adachi *et al.*, 2007; McClung *et al.*, 2006). The incident rate of VTEs is elevated in postmenopausal women subjected to prolonged periods of inactivity (i.e., extended bed stay during invasive surgical procedures) and, as such, SERMs used chronically carry a recommendation to discontinue drug therapy during periods of anticipated immobility of several hours or more (Cuzick *et al.*, 2002; Seeman, 2001). The mechanism for increased incidence of VTEs with SERMs is not clear, although a number of clinical trials have noted procoagulant changes with estrogen replacement, tamoxifen, or raloxifene along with impairment of anticoagulant factors with these three regimens. Although there is some variability in the results of different studies with respect to specific factor changes, such as fibrinogen that is increased by raloxifene in some reports (Sgarabotto *et al.*, 2007) but decreased in others (Walsh *et al.*, 1998), some clear trends have emerged. In a randomized, placebo-controlled trial in healthy postmenopausal women (Cosman *et al.*, 2005), estrogen replacement increased the coagulation factor VII and reduced the anticoagulation factors antithrombin and plasminogen activator inhibitor-1 (PAI-1). Tamoxifen generated an overall procoagulant profile, although via an increase in clotting factor VIII, factor IX, and von Willebrand factor on the coagulant side, and decreases in antithrombin, protein C, and PAI-1 on the anticoagulant side. Raloxifene produced a different pattern of changes, with some similarity to that of tamoxifen, although without elevation in clotting factor IX or reduction of protein C (Cosman *et al.*, 2005). In a separate study, Dahm *et al.* (2006) demonstrated that estrogen replacement, tamoxifen, and raloxifene all acted to reduce human endothelial production of tissue factor pathway inhibitor-1 (TFPI), an anticoagulant factor. Thus, some clear trends have emerged as the most critical determinants for VTE occurrence with estrogen or SERMs, being reduction of important factors such as antithrombin, PAI-1 and TFPI, all anticoagulant functional proteins. However, this remains more of a correlative hypothesis as, to date no chronically used SERM has avoided the 2% to 3% rate of VTE occurrence. No preclinical models or predictors are available to predict the relative likelihood for VTEs in humans with SERMs or estrogens.

With respect to the more severe cardiovascular adverse events observed with hormone replacement in the HERS, WHI, and WEST clinical trials, there are mixed reports with respect to incidence of stroke or myocardial infarction with various SERMs. Although a coronary heart disease neutral profile was reported initially with tamoxifen in the breast cancer prevention trial, an increased risk for stroke with tamoxifen was eventually observed (Reis *et al.*, 2001). The long-term clinical trials that supported raloxifene approval for osteoporosis indications found no change in the incidence of myocardial infarction relative to the placebo (Martino *et al.*, 2005). Analysis on a per year basis showed no increase in cardiovascular events in the first year of raloxifene use, which contrasts with the pattern reported for estrogen replacement in the HERS and WHI trials (Keech *et al.*, 2005). Cardiovascular events were the primary outcome of the Raloxifene Use for The Heart or RUTH trial, where no increase in coronary events or stroke incidence was observed, but a statistically significant increase in stroke-associated mortality was reported (Barrett-Conner *et al.*, 2006).

Potential Cardiovascular Benefit of SERMs

Although the large, randomized, placebo-controlled trials conducted with SERMs have not shown a significant reduction in cardiovascular events, smaller trials or subsets of the larger placebo-controlled trials have demonstrated some favorable trends, leaving open the possibility that a cardioprotective SERM is a possibility. With tamoxifen, one clinical study concluded a slight reduction in cardiac death and reduced risk for myocardial infarction (Rutqvist *et al.*, 1993). In the raloxifene osteoporosis registration clinical trial, a positive effect on the incidence of cardiovascular events was observed in a subset of women at high risk for heart disease (Barrett-Conner *et al.*, 2002). Despite the lack of verifiable cardiovascular outcomes with SERMs, most SERMs evaluated in both the clinical and preclinical settings show largely favorable effects on most cardiovascular risk factors for heart disease, including lipid metabolism, clotting factors, and vessel wall factors. However, the "fingerprint" of each SERM on the wide array of cardiovascular surrogates is strikingly distinct, suggesting each SERM needs to be carefully evaluated clinically before a complete assessment of cardiovascular benefit or risk can be ascribed.

The most clear and robust effect that can be observed with SERMs on cardiovascular relevant parameters in preclinical models is the reduction of serum cholesterol levels (i.e., raloxifene, Black *et al.*, 1994; tamoxifen, Sato *et al.*, 1996; clomiphene, Turner *et al.*, 1998; toremiphene, Qu *et al.*, 2000; acolbifene, Martel *et al.*, 2000; lasofoxifene, Ke *et al.*, 1998; bazedoxifene, Komm *et al.*, 2005; arzoxifene, Palkowitz *et al.*, 1997). As with the skeletal responses in OVX rats, all of the SERMs depicted in Figure 3 are capable of reducing serum cholesterol to roughly the same magnitude, thus mimicking the response to estrogen in this animal model, although differences in potency are evident. This hypocholesterolemic effect of SERMs is mediated by the ER, as demonstrated in the case of raloxifene by a very close correlation of ER-binding affinity and cholesterol lowering *in vivo* for a series of raloxifene analogues (Kauffman *et al.*, 1997).

In clinical trials, most of the SERMs depicted in Figure 3 reduced total serum cholesterol and LDL-cholesterol (Reid *et al.*, 2004; Joensuu *et al.*, 2000; McClung *et al.*, 2006) even in hypertriglyceridemic women (Dayspring *et al.*, 2006), with raloxifene and tamoxifen also reducing Lp(a) (Love *et al.*, 1994; Mijatovic *et al.*, 1999)—all effects that are cardioprotective with respect to cardiovascular disease risk factors. Neither tamoxifen nor raloxifene elevated HDL-cholesterol (Love *et al.*, 1994; Walsh *et al.*, 1998), a cardiovascular beneficial effect of estrogen replacement. Triglyceride elevation, an undesired effect often associated with estrogen replacement, is increased in most clinical trials with tamoxifen, although a triglyceride-neutral profile was observed with raloxifene or toremifene (Walsh *et al.*, 1998; Kusama *et al.*, 2004).

Preclinical models focused on the vessel wall have produced beneficial effects after SERM administration. In rats, neointimal thickening following aortic denudation injury was reduced by either raloxifene or tamoxifen, where both of these SERMs were shown to regulate vascular smooth muscle cell function, indicating a potential benefit against restinosis following percutaneous transluminal coronary angioplasty (Savolainen-Peltonen *et al.*, 2004). Even though SERMs are linked to increases in VTEs in clinical studies, preclinical work has indicated a beneficial effect of raloxifene in a model of carotid artery thrombosis. In OVX mice, estrogen deficiency amplifies thrombosis following carotid photochemical injury. In this model system, raloxifene, as well as estrogen, significantly reduces intra-arterial thrombosis prolonging time to occlusion, likely via a mechanism that involves reduced platelet adhesion and increased expression of COX-2 (Abu-Fanne *et al.*, 2008). Vascular anti-inflammatory effects of raloxifene were also linked to vasorelaxant properties (Pinna *et al.*, 2006).

Cardiovascular disease surrogates associated with vessel wall function and inflammation are also generally improved in clinical studies following raloxifene and tamoxifen. CRP, a marker that is linked to vascular injury and reflects inflammatory activity in the vascular wall is elevated with estrogen replacement (Cushman *et al.*, 1999) but is reduced with raloxifene and tamoxifen (Cushman *et al.*, 2001; Walsh *et al.*, 2000), as is homocysteine (Anker *et al.*, 1995; De Leo *et al.*, 2001), a circulating factor linked to toxicity of vascular endothelial cells. Improved endothelial function with tamoxifen (Stamatelopoulos *et al.*, 2004) and reduction in endothelin-1 (Saitta *et al.*, 2001), an endogenous vasoconstrictor, has also been described.

Clearly other factors, in addition to circulating lipids, influence the ultimate potential cardiovascular benefit provided by compounds like the SERMs. In this regard, direct effects of SERMs on cardiovascular tissue has been the subject of extensive investigation in recent years and agents such as raloxifene produce a number of effects of potential cardiovascular benefit. For example, raloxifene produces an antioxidant effect on serum lipoproteins (Zuckerman and Bryan, 1996), inhibits vascular smooth muscle migration (Wiernicki *et al.*, 1996), and elevates vascular endothelial cell nitric oxide production (Saitta *et al.*, 2001). Preclinical studies in models of atherogenesis have provided mixed results. Raloxifene failed to prevent the reduction of the coronary artery intimal area in cholesterol-fed, OVX monkeys (Clarkson *et al.*, 1998). However, six-month exposure to raloxifene did reduce aortic atherogenesis in cholesterol-fed, OVX rabbits (Bjarnason *et al.*, 1997) and improved the coronary artery intimal area in OVX sheep (Gaynor *et al.*, 2000). Given that nearly all of the direct cardiovascular studies with SERMs in postmenopausal women over the past few years have been conducted with raloxifene, it is difficult to know at this point whether these particular effects can be generalized across the SERM class, or whether, as in the uterus, distinct cardiovascular SERM profiles will emerge.

Central Nervous System (CNS)

The mixed biological results observed with estrogen in the cardiovascular system are paralleled by the profile of estrogen activity in the CNS. Extensive *in vitro* and animal studies suggest neuroprotective and other beneficial effects of estrogen on central processes ranging from cognition to fine motor control and mood. However, translational work to human neurodegenerative disease has failed to corroborate the preclinical data and, as in the cardiovascular system, suggests potential untoward effects of estrogen on the human CNS.

Clearly the ER is broadly distributed throughout the brain. Original thoughts were that ER was predominately restricted to the hypothalamus and associated with well-known functions such as regulation of reproductive hormones in the periventricular nucleus and thermoregulation in the lateral hypothalamus. The discovery of the ERβ subtype and improved antibodies for detection of ERα, however, led to reconstruction of ER distribution neuroanatomical charts to include higher brain regions such as the cortex and hippocampus, specifically on neurons associated with learning and memory, such as pyramidal cells throughout the CA1 and CA3 regions of the hippocampus and cortex (Shugrue and Merchenthaler, 2001).

Estrogen produces a number of beneficial effects *in vitro* and in animal models. In neuronal cell culture, 17β-estradiol has neurotrophic effects and inhibits neuronal damage induced by neurotoxicants (O'Neill *et al.*, 2004). In OVX rats, estrogen increases hippocampal choline acetyltransferase activity, which leads to increased acetylcholine levels, a neurotransmitter associated with cognition (Wu *et al.*, 1999). Estrogen also reduces neural damage after experimental forebrain ischemia (Simpkins *et al.*, 1997) and produces beneficial effects in animal models of Parkinson 's disease (Gomez-Mancilla and Bedard, 1992). Estrogen also affects central serotonergic neurotransmission in animal models, leading to increased serotonin production and firing and reduced degradation (Bethea *et al.*, 2002). Dopaminergic neurotransmission in the striatum is influenced by estrogen (Landry *et al.*, 2002) as are glutamate receptors (Cyr *et al.*, 2001).

The preclinical data are consistent indicating a beneficial effect of estrogen on CNS function. However, clinical data have not supported this conclusion. Certainly, early observational studies suggested up to a 30% reduction in risk of dementia with estrogen use (Yaffe *et al.*, 1998). However, because these trials were not well-controlled prospective studies, there is considerable room for group-related artifacts (e.g., education status), which may affect data interpretation. In this regard, in large, randomized, placebo-controlled trials, estrogen not only failed to reduce the incidence of dementia, but also was associated with an increased risk of dementia and stroke (Shumaker *et al.*, 2003). Additional data are clearly needed, because the role of progestin in studies where hormone replacement is employed is a complicating factor, although even with estrogen only use of a similar profile was observed (Shumaker *et al.*, 2004). Other potential effects of estrogen on the brain, e.g., on mood, are equally controversial at this time. Clearly, in consideration of SERMs, careful attention must be paid not only to potential beneficial effects, but also to CNS safety.

CNS Safety of SERMs

No outwardly neurotoxic effects of tamoxifen, raloxifene, or any of the SERMs depicted in Figure 3 have been reported in either neuronal cell culture or in animal studies. Some differences among various SERMs have been reported with respect to their ability to antagonize estrogen effects in the brain. For example, tamoxifen blocks the effect of estrogen on serotonin 2 A receptor expression in the forebrain or dorsal raphe of rats (Sumner *et al.*, 1999), whereas raloxifene fails to show an antagonist profile on this receptor subtype in these brain regions (Cyr *et al.*, 2000).

In clinical studies, use of tamoxifen for 5 years in women for breast cancer therapy did not alter performance on a series of cognitive tests compared with breast cancer patients who had never used tamoxifen, although an increase in physician visits for memory problems was noted (Paganini-Hill and Clark, 2000b). The confounding variable of the ongoing disease state in these women must be factored into consideration though, because studies conducted in elderly women found no differences in mental functional tests or speed of response with tamoxifen use (Ernst *et al.*, 2002). In pilot studies conducted to evaluate the safety of raloxifene on cognitive function in postmenopausal women with osteoporosis, no negative effects on memory function were detected as determined by a number of mental acuity tests (Nickelsen *et al.*, 1999). Follow-up work in more than 7700 postmenopausal women with osteoporosis confirmed the initial observation of no negative effects of raloxifene on cognitive performance (Yaffe *et al.*, 2001). Consistent with the latter observation, no untoward effects of raloxifene have been observed on mood, sexual behavior, or sleep in postmenopausal women.

CNS Efficacy of SERMs

Much as with estrogen, SERMs are largely associated with preclinical profiles that suggest neuroprotection and an overall positive CNS profile. Although certain subtle differences can be observed among different SERMs, further indicating the need to thoroughly evaluate each specific SERM molecule. Positive effects of SERMs can be demonstrated *in vitro*; as in a neural cell line, raloxifene increased neurite outgrowth in culture (Nilsen *et al.*, 1998). Raloxifene and tamoxifen were neuroprotective in a neuroepithelial cell line by conferring resistance to β-amyloid-induced toxicity via an elevation of seladin-1 (Benvenuti *et al.*, 2005), a factor known to be downregulated in brain regions affected by Alzheimer's disease (Greeve *et al.*, 2001).

Neurotransmitter-related changes within the CNS by the various SERMs can be similar for some transmitters in some brain regions, but can differ in other neurotransmitter systems. Much as with estrogen, raloxifene and tamoxifen increase hippocampal choline acetyltransferase activity (Wu *et al.*, 1999) indicating comparable effect of these two SERMs on acetylcholine neurotransmission, a neurotransmitter associated with cognition. Raloxifene and tamoxifen, however, produce different overall profiles on serotonin neurotransmission in the brain. In both rats and monkeys, raloxifene produces a spectrum of changes in the forebrain that are favorable for serotonin neurotransmission, such as increased tryptophan hydroxylase, increased serotonin 2A receptor expression, and reduced serotonin transporter (Cyr *et al.*, 2000; Smith *et al.*, 2004). In contrast, tamoxifen does not affect serotonin transporter and reduces tryptophan hydroxylase activity in the forebrain (Sumner *et al.*, 1999), a profile unfavorable for serotonin neurotransmission. Of note, the SERM arzoxifene produces a pattern of effects on forebrain serotonin neurotransmission that parallels that produced by raloxifene (Bethea *et al.*, 2002). Other differences on neurotransmitter profiles can be observed with dopaminergic systems, as in the lateral striatum dopamine receptor expression is increased in response to raloxifene, but not affected by tamoxifen (Landry *et al.*, 2002).

Distinct SERM efficacy profiles can also be detected in various CNS pathology animal models. Hippocampal neurodegeneration can be induced in rats by systemic injection of kainic acid, which leads to induction of inflammatory astroglia and neural loss that is protected by pretreatment with estrogen. In OVX rats injected with kainic acid, tamoxifen, raloxifene, and bazedoxifene all prevented hippocampal neural loss without affecting the reactive gliosis component (Ciriza *et al.*, 2004), a profile distinct from that of 17β-estradiol, which was both neuroprotecive and anti-inflammatory in this model. Again, not all SERMs behaved the same in this model system as lasofoxifene failed to exhibit a neuroprotective profile (Ciriza *et al.*, 2004). Neuroprotective effects of SERMs in other animal models have also been observed. In separate studies, tamoxifen and arzoxifene reduced neural damage following occlusion of the middle cerebral artery in OVX rats, an experimental model for stroke (Mehta *et al.*, 2003; Rossberg *et al.*, 2000). The mechanism for SERM protection from focal cerebral ischemia in these animal models remains unclear, because raloxifene induced a relaxation of rat cerebral arteries *in vitro* via an inhibition of L-type calcium channels (Tsang *et al.*, 2004), although direct effects on cerebral blood flow were ruled out in one study (Rossberg, 2000). Attenuation of excitatory amino acid release and putative antioxidant effects have also been demonstrated as potentially contributing to the neuroprotective effect of tamoxifen and raloxifene (Osuka *et al.*, 2001; Siefer *et al.*, 1994). Finally, neuroprotective effects of tamoxifen and raloxifene were observed in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopamine depletion in a Parkinsonian model (Obata and Kubota, 2001; Grandbois *et al.*, 2000).

A number of metabolic-based imaging and cognitive performance clinical trials suggest potential CNS benefits with some SERMs. Proton magnetic resonance spectroscopy in elderly women who had taken tamoxifen for at least 2 years for breast cancer treatment demonstrated a reduction in myo-inositol, a glial marker that reflects glial proliferation in response to brain injury (Ernst *et al.*, 2002), suggesting a neuroprotective effect of tamoxifen. Although the disease status of these women complicates interpretation of these data, it is interesting to note that in a comparable population of women taking estrogen replacement therapy, but without breast cancer, a similar effect on myo-inositol was observed (Ernst *et al.*, 2002). Assessment of adverse CNS events in the randomized, placebo-controlled osteoporosis registration studies for raloxifene revealed no negative effects during the course of safety assessment, these studies did suggest some interesting trends for raloxifene related improvement in cognitive performance, specifically, higher verbal memory and attention scores, putative harbingers of mild cognitive impairment and Alzheimer's disease (Nickelsen *et al.*, 1999; Yaffe *et al.*, 2001). Women over the age of 70, in particular, experienced smaller declines in memory and attention on raloxifene (Yaffe *et al.*, 2005). In studies focused on the risk for Alzheimer's disease in postmenopausal women with osteoporosis, Yaffe and colleagues (2005) reported one-third risk reduction of mild cognitive impairment with a trend for reduced risk of Alzheimer's disease. In both studies where cognitive improvement was suggested with raloxifene, it is worth noting that the benefit was primarily observed in women receiving 120 mg/day of raloxifene. These benefits of raloxifene were not observed in similar women taking 60 mg/day of raloxifene, the standard and approved daily dose for osteoporosis prevention and treatment and breast cancer risk reduction. The potential requirement for a greater dose of raloxifene to generate meaningful CNS benefits is consistent with preclinical literature, such as the increase in hippocampal choline acetyltransferase activity, which also requires higher doses of raloxifene than are necessary for the bone effects in OVX rats (Wu *et al.*, 1999), and the recognition that raloxifene has a poor penetration of the blood-brain barrier (Bryant *et al.*, 1997). The relative estrogen background may be a complicating factor, because women with undetectable circulating estrogen levels prior to administration of raloxifene show a greater benefit for mild cognitive impairment risk than women with higher circulating estrogen levels at baseline (Yaffe *et al.*, 2005). The precise process of cognitive function that the SERMs affect remains an area of research. Although executive function/decision making is the ultimate output of cognitive networks, these activities depend on more basic processes, such as alertness and arousal, to be operational. To this regard, it is interesting to note that in a study conducted in a small cohort of elderly men using functional magnetic resonance imaging, raloxifene improved memory function via increasing arousal during initial encoding of information, likely via a neurogenic effect (Goekoop *et al.*, 2005).

The important principle of drug exposure for the SERMs will be reviewed in the next section, but is particularly germane to the brain, as a central pharmacological tenant, is that for agents to exert their effect on a receptor, they must be available to the receptor. The limited brain exposure with raloxifene predicted by the preclinical models is certainly an important factor. ER-binding studies also suggest reduced exposure of tamoxifen in the brain relative to peripheral tissues, such as the uterus (Bowman *et al.*, 1982). Clearly the functional studies with various SERMs in animal models with raloxifene and tamoxifen argue strongly that there is sufficient exposure to exert a biological response to these SERMs in the brain. However, critical factors such as dose, duration, and agonist/antagonist potential may be severely or subtly affected by the ability of the molecule to penetrate the brain. As a result, dose–response relationships that are expected based on peripheral tissues may not be monitored for effects within the CNS.

General Safety Profile and Other Pharmacological Considerations

Other Safety

In addition to the adverse events already reviewed (i.e., VTEs observed with all SERMs and uterine stimulation observed with some SERMs), SERMs are associated with other untoward effects that should be considered in the risk/benefit decision for each patient. Of these other adverse events observed in clinical trials, leg cramps and the induction of hot flushes are observed in women, to at least some extent, with all of the SERM molecules depicted in Figure 3. Hot flushes, or vasomotor symptoms, are a hallmark indicator of the menopausal transition, occurring in up to 70% of U.S. women. The incidence of hot flushes is likely reflective of declining, or changing, estrogen status and typically abates when circulating estrogen levels reach their postmenopausal, steady-state concentration. However, in a small percentage of women, vasomotor symptoms can be severe and extend well into the menopause. Estrogen replacement clearly is effective in relieving postmenopausal hot flushes. With SERM use, however, it is likely that an estrogen withdrawal-like response is initiated producing a state similar to that experienced by women who are estrogen depleted with subsequent hot flushes. Although it is unclear as to whether this phenomenon is caused by estrogen antagonist or agonist properties of the SERM at ER in hypothalamic thermoregulatory centers, SERM-induced hot flushes are transient in nature, because, with continued use in most cases, this side effect subsides, typically within 6 months, likely as the thermoregulatory setpoint reestablishes (Tataryn *et al.*, 1980). Consistent with this proposed mechanism, proximity to the climacteric state may influence the incident rate, and severity, of SERM-induced hot flushes. In postmenopausal women over the age of 55, significantly fewer hot flushes are observed in response to raloxifene compared with younger postmenopausal women. In a similar context, tamoxifen-induced hot flushes tend to be more severe in premenopausal breast cancer patients than in postmenopausal patients.

One problem in the assessment of the incident rate of hot flush induction following SERM administration is the relatively high placebo response rate in the postmenopausal population. In observational studies performed on randomized, placebo-controlled trials, the rate of reported hot flushes in postmenopausal women receiving placebo was 21% over a 30-month trial period (Cohen and Lu, 2000). In this study, the incidence of hot flushes in postmenopausal women using raloxifene was 28%. Others have confirmed an approximate 7% increase in hot flush incidence as a side effect of raloxifene use (Davies *et al.*, 1999). Of note, the hot flushes induced by raloxifene are in the mild to moderate category in terms of severity, as severe hot flushes in postmenopausal women using raloxifene occur at a rate indistinguishable from that of placebo controls. Finally, the increase in hot flush incidence with raloxifene is transient, because no differences relative to placebo controls are observed after 6 months (Davies *et al.*, 1999).

The observation of hot flush incidence is typical for other SERM molecules as well. Tamoxifen use in both premenopausal and postmenopausal women for breast cancer treatment has long been associated with hot flushes as a side effect in 10% to 20% of patients, and is more common in women with higher estrogen levels (Legha, 1988). Toremifene also induces hot flushes at rates equivalent to or slightly greater than tamoxifen (Hays *et al.*, 1995). Clomiphene use for induction of ovulation increases hot flushes, as well (Derman and Adashi, 1994). Levormeloxifene (Alexanderson *et al.*, 2001), lasofoxifene (McClung *et al.*, 2006), and bazedoxifene (Adachi *et al.*, 2007) also increase hot flush incidence in postmenopausal women.

An interesting note of relevance to hot flushes and SERMs is the potential application of SERMs in combination with estrogen use for the treatment of hot flushes. In a small study of postmenopausal women, who were using 17β-estradiol in combination with raloxifene, a significant decrease in hot flushes was observed (compared with raloxifene treatment alone), although signs of endometrial stimulation were detected as well with this combination (Stoval *et al.*, 2007). Large, randomized, placebo-controlled clinical trials have evaluated the combination of bazedoxifene and conjugated equine estrogens as a potential alternative for treatment of hot flushes (and potentially other menopausal symptoms) without the need to include a progestin for maintenance of uterine safety. Uterine assessment at 1- and 2-year intervals demonstrated the lack of endometrial hyperplasia for the bazedoxifene/conjugated equine estrogens combination (Pickar *et al.*, 2007), indicating that the SERM had effectively blocked the potent stimulatory action of the estrogenic component of the combination. The combination also produced an increase in lumbar spine BMD in postmenopausal women that was superior to both placebo and raloxifene (Lindsay *et al.*, 2007). Full assessment of the risk/benefit ratio of the bazedoxifene/conjugated equine estrogens combination for use in relief of menopausal symptoms will require full assessment of hot flush and other symptom efficacy, as well as effects on other adverse events, such as VTEs.

Other adverse events not already discussed that are associated with some of the triphenylethylene SERMs are those associated with the eye. These ocular-related untoward effects include retinopathy, macular crystal formation, corneal keratopathies, and cataracts. Eye pathologies have been primarily associated with the use of tamoxifen and toremiphene with comparable incidence of 7% to 10% (Hays *et al.*, 1995). Visual disturbances have also been noted with clomiphene use (Asch and Greenblat, 1976), but are not increased with non-triphenylethylene SERMs, such as with raloxifene use in postmenopausal women (Cohen and Lu, 2000). Length of therapy increases the risk of cataract development with tamoxifen use (Paganini-Hill and Clark, 2000a). One mechanism that has been proposed for cataract formation with tamoxifen is the blockade of chloride channels in the lens of the eye, which are important for maintenance of lens hydration (Zhang *et al.*, 1994). Of importance, this effect of tamoxifen on chloride channels in the lens seems to be independent of interaction with ER, and as such, is likely off-target toxicity for certain triphenylethylene SERMs.

Pharmacokinetics

Pharmacokinetic properties of the SERMs are an important consideration in the overall effects of these molecules and have served as a focal point for development of novel and improved agents. For example, the third-generation SERMs arzoxifene, bazedoxifene, and lasofoxifene all have pharmacokinetic properties that represent improvements over raloxifene and tamoxifen. The ultimate advantage of these improvements to patients remains to be seen, as these molecules have yet to achieve registration approval. More information as to this impact will certainly be revealed in the upcoming years.

As previously indicated, tamoxifen generates active metabolite(s) with greater affinity and efficacy at the ER (Coezy *et al.*, 1982). After a single oral dose of tamoxifen, maximal plasma levels are reached within several hours with an elimination half-life of 5 to 7 days (Fromson *et al.*, 1973). Steady-state concentrations are attained within 3 to 4 weeks of chronic dosing (Adam *et al.*, 1980). The potential for preferential tissue distribution to some tissues was suggested in animal studies where greater levels of radioactive tamoxifen were detected in mammary gland, uterus, and liver than in blood (Furr and Jordan, 1984). In humans, elevated uterine levels of tamoxifen, with respect to circulating concentrations, were observed with endometrial levels twice that of myometrial or cervical concentrations (Fromson and Sharp, 1974). The primary route of elimination of tamoxifen follows hepatic metabolism, primarily glucuronidation, and subsequent biliary excretion with fairly little of the parent molecule being excreted in the urine and the potential for enterohepatic recirculation suggested in animal studies (Furr and Jordan, 1984).

Toremifene also is a triphenylethylene SERM that generates active metabolites (i.e., deaminohydroxytoremifene; DeGregorio *et al.*, 2000). Peak plasma concentrations of toremifene occur within 2 to 4 hours after a single oral dose, with nearly complete absorption, and plasma levels are linear with dose over a fairly wide dose range (Anttila *et al.*, 1990). The elimination half-life of toremifene is 5 days, and steady-state circulating concentrations are reached within 2 to 4 weeks of chronic dosing. There is evidence in animal models for relatively increased tissue distribution of toremifene in some tissues, with mammary gland uptake similar to tamoxifen (Kargas *et al.*, 1989). Toremifene is extensively metabolized in the liver via demethylation, hydroxylation, and side-chain oxidation modifications (Anttila *et al.*, 1990) and the primary route of elimination is fecal elimination following enterohepatic recirculation (Anttilla *et al.*, 1990). Hepatic impairment significantly increases the half-life of toremifene, nearly doubling it (Anttila *et al.*, 1995).

Clomiphene is the only triphenylethylene SERM in current clinical use for which there are no known active metabolites. Clomiphene is a racemic mixture of *cis*-(zuclomiphene) and *trans*- (enclomiphene) isomers that is rapidly absorbed following oral administration. Peak plasma concentrations are achieved in approximately 6 hours, with a half-life of approximately 5 days (Dickey and Holtkamp, 1996), although metabolites have been detected up to 6-weeks after a single dose, suggesting likely enterohepatic recirculation (Kausta *et al.*, 1997). Clomiphene is hepatically metabolized and fecally excreted (Adashi, 1996).

The pharmacokinetics of raloxifene, a benzothiophene SERM, have some features similar to the triphenylethylenes but also some considerable differences. Like tamoxifen and its relatives, raloxifene is rapidly absorbed from the gastrointestinal tract after oral administration, with peak blood levels attained in approximately 6 hours and 60% of the oral dose absorbed (Heringa, 2003). Raloxifene is also highly bound to plasma proteins (approximately 95%; Heringa, 2003). However, in contrast to the triphenylethylenes, the elimination half-life of raloxifene is considerably shorter at 28 hours and there are no known active metabolites of raloxifene in humans or rodents. Although there is virtually no P450 metabolism of raloxifene in the liver, it is extensively metabolized by first-pass hepatic glucuronidation, yielding an absolute oral bioavailability of only approximately 2% in humans (Snyder *et al.*, 2000). Raloxifene is widely distributed, and as with the triphenylethylenes, very little is excreted in the urine with the bulk of clearance through biliary excretion and loss in the feces (Knadler *et al.*, 1995).

Relatively less information is available on pharmacokinetic profiles of the third-generation SERMs, arzoxifene, bazedoxifene, and lasofoxifene. Lasofoxifene demonstrates improved bioavailability because the molecule was designed to resist intestinal wall glucuronidation (Gennari *et al.*, 2006). The elimination half-life of lasofoxifene is 165 hours, and there is a linear relationship between plasma concentrations and dose (Gardner *et al.*, 2006). The primary metabolic route for lasofoxifene is hepatic oxidation and subsequent conjugation (Branson *et al.*, 2006). Bazedoxifene produces an absolute bioavailability of 6.2% following an oral dose, which is about threefold greater than that produced by raloxifene. Bazedoxifene demonstrates kinetic linearity at dose levels of 5 to 40 mg

(Ermer *et al.*, 2003). Maximal circulating concentrations of bazedoxifene are achieved within 1 to 2 hours after oral exposure and the elimination half-life is approximately 28 hours with steady-state circulating levels attained in 7 days (Ermer *et al.*, 2003). As with raloxifene, there is very little P450-mediated metabolism of bazedoxifene, and glucuronidation is the major metabolic route. The primary route of elimination is the feces, with evidence for enterohepatic recirculation (Chandrasekaran *et al.*, 2003). Arzoxifene also exhibits pharmacokinetic advantages over raloxifene. Over a dosage range of 10 to 100 mg, blood levels of arzoxifene increase linearly with respect to dose, with maximal levels attained at 2 to 6 hours and an elimination half-life of 30 to 35 hours over this dose range (Munster *et al.*, 2001). Arzoxifene is metabolized (demethylated) to desmethylarzoxifene, which is an active metabolite with a high binding affinity for the ER (Rash and Knadler, 1997).

FUTURE DIRECTIONS WITH SERMS

In addition to the use of SERMs for osteoporosis, breast cancer, or the induction of ovulation, the ability of ER-activity-modulating agents to favorably impact other diseases and syndromes, both in women and men, is under active discovery and early clinical development efforts. The overall scope of these drug discovery efforts is broad and beyond the focus of this review, and thus, only a few examples of potential new SERM agents or novel applications for existing SERMs are briefly discussed here.

SERMs for Hot Flushes

As previously mentioned, estrogen replacement is a highly effective therapeutic tool for relief from hot flushes induced by estrogen deficiency. However, concerns around estrogen use often limits patient willingness to initiate estrogen replacement therapy, particularly in women with a history of breast cancer. The lack of sufficiently effective alternatives for hot flushes creates an undressed medical need for new hot flush treatment alternatives. Currently available SERMs do not offer benefit for hot flushes; indeed, they induce vasomotor symptoms as a side effect in some women as previously indicated. Preclinical drug discovery efforts have focused on identifying molecules with a favorable SERM-profile on bone, mammary, and uterus that also produce an estrogen-like attenuation of thermoregulatory vasomotor responses. A spiroindane SERM was identified by Watanabe *et al.* (2003) that produces estrogen-like effects on thermoregulation and bone in OVX rats, with only a stimulation of the uterus and modest stimulatory effects on mammary tumor cell proliferation. A more favorable preclinical profile was reported for a benzopyran SERM, with a clear estrogen-like effect on elevated tail skin temperature in an OVX rat hot flush model and prevention of bone loss on OVX rats. This benzopyran produced a more complete estrogen antagonist profile with minimal uterine stimulatory activity and minimal stimulation of MCF-7 cells *in vitro* (Wallace *et al.*, 2006). A third, chromane-derived SERM also shows efficacy in a rat model of hot flushes with minimal stimulation of Ishikawa or MCF-7 tumor cell lines (Jain *et al.*, 2006). A key point of emphasis is that, because animals do not experience clinical "hot flushes," contrived models typically use induced withdrawal in morphine-dependent rats as the gold standard for assessment of thermoregulatory modulation. None of these models have reported clinical results as yet, and as such, remain in the proof-of-concept phase of inquiry.

SERMs for Vaginal Atrophy/Dryness

Vaginal atrophy and dryness are also common features of estrogen deficiency that respond favorably to estrogen replacement, delivered systemically or locally. As with vasomotor symptoms, SERMs that may produce beneficial vaginal effects are currently under clinical investigation. As previously discussed, lasofoxifene was evaluated in phase 3 clinical trials for potential vaginal benefits. Currently, ospemifene, a triphenylethylene SERM, is in early clinical development for vaginal atrophy. Ospemifene, a metabolite of toremifene, prevents bone loss and lowers serum cholesterol in OVX rats and inhibits dimethylbenzanthrocene-induced mammary tumors in rats (Qu *et al.*, 2000). In postmenopausal women, administration of ospemifene for 3 months produced an estrogen-like effect to suppress FSH levels, and also produced a clear estrogenic effect on vaginal epithelium, with neither effect observed in a raloxifene comparator arm. Over this short treatment period, ospemifene produced no apparent uterine endometrial stimulation (Komi *et al.*, 2005). In postmenopausal women, ospemifene reduced bone turnover markers with a magnitude, and in a time frame, that was comparable to that observed with raloxifene (Komi *et al.*, 2006). Currently under preclinical evaluation for this indication is the chromane SERM described under the "hot flushes" subheading earlier. In addition to the hot flush-reducing activity this molecule also increased the amount of vaginal fluid in OVX rats (Jain *et al.*, 2006), providing preclinical indications for possible additional benefits of this SERM on postmenopausal vaginal symptoms.

SERMs for Gynecological Indications

A number of gynecological disorders, such as endometriosis and leiomyoma (uterine fibroids) are frequently estrogen-driven pathologies. Given their ability to antagonize estrogen action in the uterine environment, SERMs are a potential option for these diseases. Clearly, estrogen deprivation, such as with chronic GnRH administration, is efficacious for endometriosis and leiomyoma, although bone loss induced by ovarian shutdown produced by these regimens limits the time of therapy, with symptoms typically returning upon discontinuation of the GnRH agonist. As previously mentioned, SERMs like raloxifene produce some benefit versus these gynecological disorders. However, because the primary treatment population for endometriosis and leiomyoma is premenopausal women, the concern over ovarian stimulation and ovarian cyst formation, as has been observed with tamoxifen in these women (Cook *et al.*, 1995), has limited application of currently available SERMs for therapy of estrogen-associated gynecological disorders in ovulating women. In preclinical studies a naphthalene-SERM, with minimal CNS penetration (Richardson *et al.*, 2007), was identified. This molecule exhibits a unique profile among SERMs in preclinical testing with potent and complete uterine antagonism in the uterus of estrogen-replete animals, lack of uterine stimulation in estrogen-deficient animals, and a prevention of bone loss in OVX animals without stimulation of the HPO axis with subsequent elevation of ovarian-derived estrogen and ovarian cyst production in ovary-intact rats (Geiser *et al.*, 2005).

SERMs for Osteoarthritis

A loose connection between estrogen status and osteoarthritis has long been known, first identified in 1925 by Cecil and Archer and later confirmed by multiple epidemiological studies (Wluka *et al.*, 2000). Although there has been some controversy over the relative benefit in osteoarthritis provided by estrogen replacement, there is a growing body of evidence suggesting significant benefit, ranging from reduced risk of radiographic osteoarthritis in postmenopausal women who use estrogen (Nevitt *et al.*, 1996) to a reduction in hip and knee joint replacement in women using unopposed estrogen (Cirillo *et al.*, 2006). The presence of ER in articular cartilage in both animals (Tsai and Liu, 1992) and humans where both ERα and ERβ are expressed (Ushiyama *et al.*, 1999) and beneficial effects of estrogen on cartilage in animal models of osteoarthritis (Turner *et al.*, 1997), suggests that a SERM approach could provide a safe and effective therapeutic alternative for osteoarthritis disease modification. Indeed, some beneficial effects of SERMs have been established in osteoporosis clinical trials that are suggestive of a benefit in the prevention or treatment of osteoarthritis. Levormeloxifene reduced cartilage degradation as determined by collagen type II degradation products (CTX-II) in postmenopausal women (Christgau *et al.*, 2004), and raloxifene was associated with a reduction in musculoskeletal pain in an observational study of postmenopausal women, as well as a reduction in consumption of analgesics (Scharla *et al.*, 2006). In preclinical development, a chromane-SERM both reduced CTX-II levels in OVX rats, as well as reduced articular cartilage degradation and erosion in the knee of OVX rats with minimal uterine stimulation (Hoegh-Andersen *et al.*, 2004), suggesting the potential for development of ER-selective disease-modifying agents for osteoarthritis.

SERMs for Use in Males

A few SERMs have been studied in men for various potential therapeutic applications. For example, clomiphene was evaluated for the management of male infertility, where some beneficial effects on seminal vesicle hypofunction owing to high sperm chromatin stability were identified (Gonzalez *et al.*, 1998). Consistent stimulation of FSH and LH can also be attained in men with clomiphene, which can be associated with reversal of infertility in a subset of hypogonadotrophic men (Whitten *et al.*, 2006). Similar data have been generated with toremifene. Administration of toremifene for 3 months to men with idiopathic oligozoospermia increased sperm count and motility and improved sperm morphology, most likely owing to increased FSH secretion (Farmakiotis *et al.*, 2007).

Applications of SERMs to various endocrine tumors have met with some success in men as well. High-dose estrogen has long been recognized as beneficial for certain forms of prostate cancer (Huggins and Hodges, 1941), via reduction of circulating androgens and likely direct antiproliferative effects on the prostate (Ferro *et al.*, 1989). These effects of estrogen are likely ER-mediated, which is present in the prostate epithelium (ERβ) and stroma (ERα; Lau *et al.*, 2000). SERMs are quite effective antimetastatic agents in animal models of prostate cancer. For example, in the PAIII rat prostatic adenocarcinoma model, raloxifene markedly reduces tumor metastases to lymphatic and pulmonary beds and increases survivability (Neubauer *et al.*, 1995). Accordingly, some stabilization of androgen-independent prostate cancer was observed with raloxifene in small pilot clinical trials (Shazer *et al.*, 2006). Although, in other phase 2 clinical trials with fairly high doses of toremiphene, no benefit was observed in androgen-independent prostate cancer patients (Stein *et al.*, 2001). Whether this reflects a difference among various SERMs for this particular indication or simply insufficient clinical trial experience for this indication remains an uncertainty. Another important consideration is the dose, because in more recent work, considerably lower dose levels of toremifene (doses comparable to those used in women for breast cancer therapy) led to a reduction in the incidence of prostate cancer in a high-risk population of men, suggesting that toremifene might be effective at prevention of prostate cancer (Price *et al.*, 2006). SERMs are also potentially valuable as an adjunct to other common forms of prostate cancer therapy, specifically the use of androgen deprivation therapies, which are frequently associated with bone loss, increased risk of cardiovascular disease, gynecomastia, as well as a number of other undesirable side effects (Sharifi *et al.*, 2005). The beneficial effect of SERMs on a number of the side effects of androgen deprivation therapy has led to their evaluation in combination with agents such as the GnRH agonists—a commonly used form of androgen deprivation therapy. In men receiving GnRH agonists, raloxifene produced a significant increase in BMD of the hip (1.1% increased hip BMD vs. 2.6% decline in hip BMD in men not receiving raloxifene; Smith *et al.*, 2004), with a trend for increase in spine BMD. Recent results with toremifene suggest a similar ability to counter untoward effects of androgen deprivation therapy, because an improvement in bone turnover biomarkers and BMD was observed (Taneja *et al.*, 2006). It is important to note, though, that BMD alone is not sufficient and full assessment of the impact of these agents on bone fracture is required.

SERMs have also been evaluated for therapeutic benefit in male breast carcinoma, a low-incidence tumor with males representing only about 1% of all breast cancer diagnoses. However, a very high percentage of male breast carcinomas are ER and progesterone receptor positive (Kinne, 1991), suggesting a possible benefit to be derived from ER blockade. Owing to the low incidence of male breast carcinoma, clinical trial sizes are small, but in 301 cases of male breast cancer, adjuvant therapy with tamoxifen for 1 year was associated with 86% reduction of ER/progesterone receptor-positive patients showing a positive result (Ribeiro, 1985). A third tumor type that is responsive to SERM therapies is desmoid tumors, the benign mesenchymal tumors that can lead to destruction of vital structures and/or organs. In clinical trials involving only small numbers of patients to date, both raloxifene (Tonelli *et al.*, 2003) and toremifene (Heidemann *et al.*, 2004) decreased desmoid tumor and mesenteric fibromatosis size and symptoms, possibly via a combined antiangiogenic and antiproliferative mechanism.

A number of other potential uses for SERMs in males have been explored in clinical trials. In adolescent boys with persistent pubertal gynecomastia, a significant reduction in breast nodule diameter with tamoxifen (41% of patients) and raloxifene (86% of patients) was observed, with no significant side effects, following 6 to 9 months of dosing (Lawrence *et al.*, 2004). In men with acromegaly, both tamoxifen and raloxifene decreased circulating IGF-1 levels (Cozzi *et al.*, 1997; Dimaraki *et al.*, 2004). In elderly men, raloxifene enhanced brain activation, with improved retrieval of information in memory function tests (Goekoop *et al.*, 2006), and in another trial, increased circulating testosterone levels (Duschek *et al.*, 2004).

SUMMARY

SERMs are a diverse class of molecules that affect a broad spectrum of biological systems with potential therapeutic benefit for a variety of diseases. Current concern over long-term use of estrogen-containing regimens has created an opportunity for application of SERMs to chronic indications such as osteoporosis treatment or prevention. The unique SERM profile also allows their use in other chronic indications of interest to postmenopausal women, most notably, breast cancer risk reduction and treatment. However, safety considerations are a very important consideration for SERM use in these chronic indications. The pleiotropic nature the ER and its role in numerous physiological systems raise the importance of considering potential SERM benefits and/or adverse events in the cardiovascular system and other tissues.

As reviewed here, several central themes are key considerations for use and development of SERMs in postmenopausal women. These include:

1. A central feature of all SERMs and a central aspect of their mechanism of action, is high-affinity interaction with ERα and ERβ (for SERMs currently used in humans). Each SERM produces a unique conformation of the SERM:ER complex, which dictates which cofactor proteins bind to activate or repress gene transcription in a given cell.
2. A wide variety of chemical scaffolds can be used to generate molecules with SERM-like properties. Key structural features of the molecular backbone of SERMs are the location of hydroxyl moieties that interact with specific residues within the binding pocket of the ER and the presence of a basic side chain that, in some cases, reorients specific domains of the ligand-bound ER.
3. Bone efficacy in estrogen-deficient states, both in animal models and postmenopausal women, are features demonstrated by most SERMs. Although the skeletal response in terms of BMD may not be as robust with some SERMs as is observed with other skeletal agents (such as the bisphosphonates), SERMs produce comparable rates of fracture reduction. This latter observation suggests likely important beneficial effects of SERMs on bone quality.
4. SERMs in clinical use today share certain common adverse events in some women, with induction of hot flushes and VTEs being the most common. However, the most important safety parameter SERMs used for chronic indications, such as osteoporosis, revolves around uterine safety. SERMs that have failed in clinical trials for osteoporosis have done so primarily because of excessive risk for uterine side effects, as predicted by excessive stimulation of uterine endometrial thickness or induction of uterine prolapse.
5. The widespread distribution of the ER in cardiovascular, central nervous system, and reproductive tissue create the possibility of either additional potential benefits of SERMs in these tissues,

or the possibility of adverse events. Clearly these then represent important tissues in the toxicological and safety assessment of SERMs. Simply put, no two SERMs are necessarily equal in terms of overall tissue activity profiles. Careful preclinical and clinical evaluation is necessary to fully understand the risk/benefit ratio of novel SERM agents across multiple tissue types.

6. Pharmacokinetics play an important role in determining the magnitude and duration of SERM efficacy responses. Third-generation SERM molecules are emerging for osteoporosis with decidedly improved bioavailability and linear kinetics for dose and exposure.

A wide variety of new SERM agents are beginning to enter early-stage clinical development for use in multiple indications in women, and even men, that could significantly expand the armory of SERM-like molecules available to clinicians for the treatment of a myriad of human diseases.

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Chapter 42

Mechanisms of Estrogen Action in Bone

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INTRODUCTION

Postmenopausal osteoporosis is a heterogeneous disorder characterized by a progressive loss of bone tissue that begins after natural or surgical menopause and leads to fracture within 15 to 20 years from the cessation of the ovarian function. Although suboptimal skeletal development ("low peak bone mass") and age-related bone loss may be contributing factors, a hormone-dependent increase in bone resorption and accelerated loss of bone mass in the first 5 or 10 years after menopause appears to be the main pathogenetic factor (Riggs and Melton, 1986a; Riggs and Melton, 1986b) of this condition. The ability of bones to withstand mechanical loading depends upon the establishment and the maintenance of proper bone mass and architecture. In women, the rapid loss of bone associated with menopause alters bone structure with consequent increase in bone fragility, although how estrogen influences the relationship between bone mass and bone loading remains largely unknown (Lanyon *et al.,* 2004).

That estrogen deficiency plays a major role in postmenopausal bone loss is strongly supported by the higher prevalence of osteoporosis in women than in men (Nilas and Christiansen, 1987), the increase in the rate of bone mineral loss detectable by bone densitometry after artificial or natural menopause (Genant *et al.,* 1982; Riggs *et al.,* 1981; Slemenda *et al.,* 1987), the existence of a relationship between circulating estrogen and rates of bone loss (Johnston *et al.,* 1985; Ohta *et al.,* 1992), and the protective effect of estrogen replacement with respect to both bone mass loss and fracture incidence (Ettinger *et al.,* 1985; Lindsay *et al.,* 1980). Both a decreased ovarian production of sex steroids and an increase in follicle-stimulating hormone (FSH) production secondary to estrogen deficiency contribute to postmenopausal bone loss (Iqbal *et al.,* 2006; Sun *et al.,* 2006).

Menopause also alters the expression of estrogen receptors (ER) in bone. A reduction in ERα expression after estrogen withdrawal has been demonstrated and results in a less osteogenic response to loading (Armstrong *et al.,* 2007; Zaman *et al.,* 2006). Thus, the bone loss associated with estrogen deficiency could be regarded, in part, as a consequence of reduction in ERα expression in bone cells that reduces the anabolic response of bone cells to strain.

The potential fracture risk for any postmenopausal female depends on the degree of bone turnover, the rate and extent of bone loss, associated disease processes that induce bone loss, age of menarche and menopause, and the bone mass content achieved at skeletal maturity. The latter depends on the extent of estrogen exposure, habitual physical activity, quantity of calcium intake, and genetic predisposition.

The bone-sparing effect of estrogen is mainly related to its ability to block bone resorption (Weitzmann and Pacifici, 2005), although stimulation of bone formation is likely to play a contributory role (Manolagas *et al.,* 2002). Estrogen-dependent inhibition of bone resorption is, in turn, owing to both decreased osteoclastogenesis and diminished resorptive activity of mature osteoclasts. However, inhibition of osteoclast formation is currently regarded as the main mechanisms by which estradiol (E_2) prevents bone loss (Weitzmann and Pacifici, 2005).

CELLS AND CYTOKINES THAT REGULATE OSTEOCLAST FORMATION

The dominant acute effect of estrogen is the blockade of new osteoclast formation. Osteoclasts arise by cytokine-driven proliferation and differentiation of monocyte precursors that circulate within the hematopoietic cell pool (Teitelbaum, 2000). This process is facilitated by bone marrow stromal cells, which provide physical support for nascent osteoclasts and produce soluble and membrane-associated factors essential for the proliferation and differentiation of osteoclast precursors (Fig. 1).

The minimal essential cytokines required for osteoclast formation under basal conditions are receptor activator of NFκB ligands (RANKL) and macrophage colony-stimulating factors (M-CSF). These factors are produced primarily by bone marrow stromal cells, osteoblasts, and activated T cells (Khosla, 2001). RANKL is a tumor

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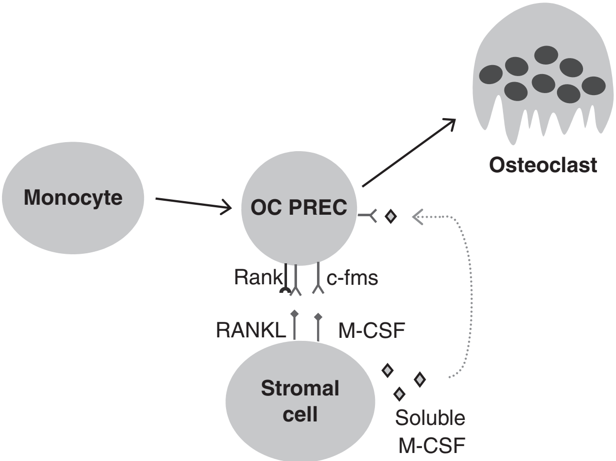

FIGURE 1 Cells and cytokines responsible for physiological osteoclast formation. Osteoclast precursors may be differentiated from the monocyte/macrophage population, among which they circulate, by virtue of their expression of the receptor RANK. When RANKL binds to its receptor (RANK) in the presence of the trophic factor M-CSF, which in turn binds to its receptor, colony-stimulating factor receptor 1 (c-Fms), osteoclast precursors differentiate and fuse together to form mature multinucleated bone resorbing osteoclasts. Under physiological conditions the dominant source of RANKL and M-CSF in the bone marrow microenvironment is from the bone forming cells, the osteoblasts, and their stromal cell precursors.

necrosis factor (TNF) superfamily member that exists in membrane-bound and soluble forms. RANKL binds to the transmembrane receptor RANK expressed on the surface of osteoclasts and osteoclast precursors. RANKL also binds to osteoprotegerin (OPG), a soluble decoy receptor produced by numerous hematopoietic cells. Thus, OPG, by sequestering RANKL and preventing its binding to RANK, functions as a potent anti-osteoclastogenic cytokine (Khosla, 2001). RANKL promotes the differentiation of osteoclast precursors from an early stage of maturation into fully mature multinucleated osteoclasts. RANKL is also capable of activating mature osteoclasts, thus stimulating the capacity of these cells to resorb bone. M-CSF induces the proliferation of early osteoclast precursors, the differentiation of more mature osteoclasts, the fusion of mononucleated preosteoclasts, and increases the survival of mature osteoclasts.

Although RANKL and M-CSF are essential for physiologic osteoclast renewal, additional cytokines are responsible for the upregulation of osteoclast formation observed in a variety of conditions such as inflammation, hyperparathyroidism, and estrogen deficiency (Grey *et al.,* 1999; Pacifici, 1998). One such factor is TNF, a cytokine that enhances osteoclast formation by upregulating the stromal cell production of RANKL and M-CSF (Hofbauer *et al.,* 1999; Sherman *et al.,* 1990), and by augmenting the responsiveness of osteoclast precursors to RANKL (Cenci *et al.,* 2000; Lam *et al.,* 2000). The ability of TNF to increase the osteoclastogenic activity of RANKL is because of synergistic interactions at the level of NFκB and AP-1 signaling (Lam *et al.,* 2000). In addition, TNF and RANKL synergistically upregulate RANK expression in osteoclast precursors (Zhang *et al.,* 2001). Furthermore, TNF stimulates osteoclast activity (Fuller *et al.,* 2002) thus further driving an imbalance between bone formation and bone resorption. Earlier studies have suggested that TNF inhibits osteoblastogenesis (Nanes, 2003), but recently it has been proposed that ascorbic acid masks the capacity of TNF to stimulate bone formation. Therefore in ascorbate-free culture systems TNF stimulates rather than inhibiting bone formation (Iqbal *et al.,* 2006). Additional studies will be required to define the net effect of TNF on bone formation *in vivo*.

Like TNF, interleukin-1 (IL-1) promotes RANKL expression by BM stromal cells and osteoblasts and stimulates osteoclast life span and activity. IL-1 directly targets osteoclast precursors and promotes osteoclast differentiation in the presence of permissive levels of RANKL. Furthermore, IL-1 mediates, in part, the osteoclastogenic effect of TNF by enhancing stromal cell expression of RANKL and by directly stimulating differentiation of osteoclast precursors (Wei *et al.,* 2005). TNF and IL-1 have potent anti-apoptotic effects in osteoclasts prolonging osteoclast life span and contributing toward accelerated bone resorption (Kwan Tat *et al.,* 2004).

Another cytokine relevant for osteoclast formation is IL-7 (Ross, 2003). IL-7 is a powerful lymphopoietic cytokine that has previously been recognized as a potent inducer of bone destruction *in vivo* (Miyaura *et al.,* 1997). How IL-7 leads to bone loss is controversial, and its mechanisms of action are only now beginning to be elucidated. IL-7 is a stimulator of both B- and T-cell lineages, and it has been suggested that IL-7 induces bone loss by a mechanism involving the expansion of cells of the B lineage, in particular $B220^+IgM^-$ B cell precursors (Masuzawa *et al.,* 1994; Miyaura *et al.,* 1997; Onoe *et al.,* 2000; Sato *et al.,* 2001), as estrogen deficiency has been reported to potently induce the expansion of these cells (Masuzawa *et al.,* 1994; Miyaura *et al.,* 1997). How B-lineage cells may lead to bone destruction is not presently understood but may involve overexpression of RANKL, a property of activated B cells (Manabe *et al.,* 2001). Alternatively, early $B220^+IgM^-$ precursor cells have been found to be capable of differentiating into osteoclasts in response to M-CSF and/or RANKL *in vitro* (Lee *et al.,* 2003; Sato *et al.,* 2001; Toraldo *et al.,* 2003) and hence IL-7 may increase the pool of early osteoclast precursors. However, other studies have reported that IL-7 inhibits the differentiation of $B220^+$ cells into osteoclasts *in vitro* in the presence of saturating concentrations of RANKL and M-CSF (Lee *et al.,* 2003). IL-7 is also established to regulate multiple stages of T-cell metabolism (Fry and Mackall, 2001). IL-7-/- mice are severely lymphopenic (von Freeden-Jeffry *et al.,* 1995) and IL-7 receptor -/- mice have been reported to display increased bone volume and bone mineral density (Miyaura *et al.,* 1997). In contrast, IL-7 transgenic mice have expanded BM cavities with focal osteolysis of cortical bone and eroded bone surfaces (Valenzona *et al.,* 1996). These data suggest that IL-7 may induce bone loss by a T cell mediated mechanism. Indeed, IL-7 has been reported to induce production of RANKL by human T cells (Weitzmann *et al.,* 2000), and injection of IL-7 into mice *in vivo* induces bone destruction (Miyaura *et al.,* 1997; Toraldo *et al.,* 2003) by eliciting the secretion by T cells of the key osteoclastogenic cytokines RANKL and TNF (Toraldo *et al.,* 2003). In addition, levels of IL-7 are significantly elevated following ovariectomy. Attesting to the key role of IL-7 in the bone destruction associated with estrogen deficiency, *in vivo* IL-7 blockade, using neutralizing antibodies, is effective in preventing ovariectomy induced bone destruction (Weitzmann *et al.,* 2002). Furthermore, IL-7 induced osteoclastogenesis and bone loss is compounded by suppression of bone formation leading to uncoupling of bone formation from resorption.

An important, yet controversial, osteoclast-regulating factor is IFNγ. This factor was initially described as an anti-osteoclastogenic cytokine because it is a potent inhibitor of osteoclastogenesis *in vitro* (Takayanagi *et al.,* 2000). The notion that IFNγ is an inhibitor of bone resorption was reinforced by the finding that silencing of IFNγR-/- signaling leads to a more rapid onset of collagen-induced arthritis and bone resorption (Vermeire *et al.,* 1997) as compared to wild-type controls, and by the report that IFNγ decreases serum calcium and osteoclastic bone resorption in nude mice (Sato *et al.,* 1992; Tohkin *et al.,* 1994).

However, observations in humans and in experimental models of disease indicate that IFNγ promotes bone resorption and causes bone loss in a variety of conditions. Studies with IFN -/- and IFNR -/- mice have revealed that among these conditions are estrogen deficiency and endotoxin-induced bone disease (Cenci *et al.,* 2003; Gao *et al.,* 2007). Mice lacking IFNγ production are also protected against infection-induced alveolar bone loss (Baker *et al.,* 1999), whereas in erosive tubercoloid leprosy and psoriatic arthritis IFNγ production correlates positively with tissue destruction (Arnoldi *et al.,* 1990; Firestein *et al.,* 1990). In addition, randomized controlled trials have shown that IFNγ does not prevent bone loss in patients with rheumatoid arthritis (Cannon *et al.,* 1989; Veys *et al.,* 1997), nor the bone-wasting effect of cyclosporin A (Mann *et al.,* 1994). Furthermore, IFNγ has been reported to be efficacious in the treatment of osteopetrosis through restoration of bone resorption, both in humans (Key *et al.,* 1995) and rodents (Rodriguiz *et al.,* 1993). These latter findings conclusively demonstrate that in some conditions, including estrogen deficiency, the net effect of IFNγ *in vivo* is that of stimulating osteoclastic bone resorption.

The complex effects of IFNγ can be explained by the fact that IFNγ influences osteoclast formation both via direct and indirect effects (Gao *et al.,* 2007). IFNγ directly blocks osteoclast formation through targeting of maturing osteoclast (Takayanagi *et al.,* 2002). This effect is best observed *in vitro* (Fox and Chambers, 2000; Takayanagi *et al.,* 2000). However, IFNγ is also a potent inducer of antigen presentation and thus of T-cell activation. Therefore, when IFNγ levels are increased *in vivo*, activated T cells secrete pro-osteoclastogenic factors and this activity offsets the anti-osteoclastogenic effect of IFNγ.

A factor that plays a key, complex role in osteoclastogenesis is TGFβ, a pleiotropic growth factor with wide-ranging effects (Attisano *et al.,* 1994; Massague, 1990; Shi and Massague, 2003). Three isoforms (TGFβ1, 2, and 3) have been described in mammals and all use the same receptor complex for signaling (Massague, 1990). TGFβ1 is the predominant TGFβ isoform in lymphoid organs and is the major species in serum. Conversely, TGFβ2 and 3 are predominantly expressed in mesenchimal tissues and bone (Millan *et al.,* 1991; Pelton *et al.,* 1989; Schmid *et al.,* 1991). TGFβ is produced by a large number of cells including BM cells, osteoblasts, and stromal cells and is secreted in a latent form that must be activated to mediate its effects (Prud'homme and Piccirillo, 2000). Latent TGFβ is also abundantly present in the bone matrix. Latent TGFβ is non-covalently linked to a precursor molecule termed latency-associated peptide. Mature TGFβ can be released from the latent complex in an active form *in vitro* by a variety of treatments such as heating or acidification (Prud'homme and Piccirillo, 2000). Although several mechanisms of activation *in vivo* have been proposed, the precise mechanism is not known, though a role for extreme pH, free radicals, transglutaminase, or thrombospondin has been suggested (Derynck and Feng, 1997). The mechanism by which TGFβ is activated in the BM and lymphoid organs is presently unknown. Both *in vitro* and *in vivo* studies have shown that TGFβ1, 2, and 3 have complex effects on bone. For example, these growth factors stimulate or repress proliferation of osteoblasts and osteoclasts depending on cell lines used and culture conditions *in vitro* (Breen *et al.,* 1994; Fagenholz *et al.,* 2001; Spinella-Jaegle *et al.,* 2001; Yan *et al.,* 2001). Furthermore, mice with osteoblast-specific overexpression of TGFβ2 develop high turnover osteoporosis (Erlebacher and Derynck, 1996) while administration of TGFβ2 into the periosteum increases bone formation locally, but has no effects systemically.

TGFβ has also been implicated in the pathogenesis of ovariectomy-induced bone loss because local injection of TGFβ1 and TGFβ2 prevent bone loss at the site of the injection in ovariectomized rats (Beaudreuil *et al.,* 1995; Kalu *et al.,* 1993). Furthermore estrogen is known to upregulate the expression of TGFβ in murine osteoblasts, bone extracts, and BM cells (Finkelman *et al.,* 1992; Gray *et al.,* 1989) and long-term *in vivo* estrogen treatment has been shown to increase serum TGFβ1 and 2 levels in humans (Bord *et al.,* 2001).

T CELLS AND OVARIECTOMY-INDUCED BONE LOSS

Early work by McSheehy *et al.* and Thompson *et al.* showed that conditioned media derived from osteoblasts that were stimulated by a variety of factors increased osteoclastogenesis (McSheehy and Chambers, 1986a, 1986b; Thomson *et al.,* 1987; Thomson *et al.,* 1986). These results suggested that substances were expressed by osteoblasts that mediated differentiation of osteoclast precursors. After several inflammatory cytokines were found capable of producing this response, research focused on discerning their relative contributions in estrogen deficiency mediated bone loss. A lively debate ensued with proponents for IL-1, TNF, or IL-6 as the major players. The osteoclastogenic response to all three cytokines *in vitro* was based on sound data (Manolagas *et al.,* 1995; Pacifici, 1996). However; the use of different experimental models to evaluate cytokine production after estrogen deprivation may have yielded different results. Several studies reported increased production of TNF by cultures of mononuclear cells derived from postmenopausal women, an effect reversed by estrogen replacement (Pacifici *et al.,* 1991a; Ralston *et al.,* 1990; Rickard *et al.,* 1992). In this model, secretion of IL-1, but not always IL-6, mirrored that of TNF (Girasole *et al.,* 1992; Rickard *et al.,* 1992).

The production of TNF, interleukin-1, -4, -6, and IFNγ by blood cells can be negatively correlated with the estrogen level in premenopausal women. Interestingly, the levels of these cytokines also correlate inversely with bone density after menopause, bolstering the hypothesis that cytokines have an important role in menopausal bone loss (Zheng and Flavell, 1997).

The major cytokine responsible for augmented osteoclastogenesis during estrogen deficiency is TNF, and its relevance has been demonstrated in multiple animal models. For example, ovariectomy fails to induce bone loss in TNF knockout mice and in mice lacking the p55 TNF receptor (Roggia *et al.,* 2001). Likewise transgenic mice insensitive to TNF owing to the overexpression of a soluble TNF receptor (Ammann *et al.,* 1997), and mice treated with the TNF inhibitor TNF-binding protein (Kimble *et al.,* 1997) are protected from ovariectomy-induced bone loss. TNF neutralization has also been found to prevent the increase in bone resorption induced by E deficiency in humans (Charatcharoenwitthaya *et al.,* 2007).

The presence of increased levels of TNF in the BM of ovariectomized animals and in the conditioned media of peripheral blood cells of postmenopausal women is well documented (Pacifici *et al.,* 1991b; Ralston *et al.,* 1990; Shanker *et al.,* 1994). However, the cells responsible for this phenomenon had not been conclusively identified. Studies on highly purified BM cells have revealed that estrogen regulates the production of TNF by T cells, but not by monocytes (Cenci *et al.,* 2000), and that earlier identifications of TNF production by monocytes from estrogen-deficient donors were likely owing to T-cell contamination of monocytes purified by adherence. Thus, the ovariectomy-induced increase in TNF levels is likely to be because of, at least in part, T-cell TNF production. However, it is now clear that FSH directly stimulates TNF production from bone marrow granulocytes and macrophages (Iqbal *et al.,* 2006). Together the available evidence suggests that multiple cell lines contribute to the elevation of the BM levels of TNF observed after natural and surgical menopause. T cells are likely to be the main source of TNF under direct estrogen control, while BMMs and granulocytes may represent relevant FSH-regulated sources of TNF.

These findings in the mouse are concordant with those of others in humans, who demonstrated that adherent mononuclear blood cells contain CD3$^+$ CD56$^+$ lymphocytes, a TNF-producing subset of adherent T cells (Abrahamsen *et al.,* 1997). In that study the number of CD3$^+$ CD56$^+$ T cells was decreased by estrogen treatment and inversely correlated with bone density. These results are not surprising as T cells have the capacity to secrete a wide repertoire of cytokines, some pro-osteoclastogenic and some anti-osteoclastogenic. In the absence of strong activation signals, T cells appear to repress osteoclast formation (Grcevic *et al.,* 2000), but the relevance of this phenomenon *in vivo* has not been established. In contrast, activated T cells play a key role in the regulation of osteoclast formation through increased production of RANKL and TNF (Horwood *et al.,* 1999; Kong *et al.,* 1999; Weitzmann *et al.,* 2001). The net effect of T cells on osteoclast formation may consequently represent the prevailing balance of anti- and pro-osteoclastogenic T-cell cytokine secretion. However, it appears that during stimulated conditions such as inflammation (Kong *et al.,* 1999) and estrogen deficiency (Cenci *et al.,* 2000) pro-osteoclastogenic cytokines prevail.

Attesting to the relevance of T cells in estrogen deficiency induced bone loss *in vivo*, measurements of trabecular bone by peripheral quantitative CT and μCT revealed that athymic T-cell–deficient nude mice are completely protected against the trabecular bone loss induced by ovariectomy (Cenci *et al.,* 2000; Gao *et al.,* 2004; Roggia *et al.,* 2001) (Fig. 2). T-cell–deficient mice also fail to respond to ovariectomy with an expected increase in bone turnover (Cenci *et al.,* 2000; Gao *et al.,* 2004; Roggia *et al.,* 2001). T cells are key inducers of bone-wasting because ovariectomy increases T-cell TNF production to a level sufficient to augment RANKL-induced osteoclastogenesis (Cenci *et al.,* 2000). The specific relevance of T-cell TNF production *in vivo* was demonstrated by the finding that while reconstitution of nude recipient mice with T cells from wild-type mice restores the capacity of ovariectomy to induce bone loss, reconstitution with T cells from TNF-deficient mice does not (Roggia *et al.,* 2001). T-cell–produced TNF may

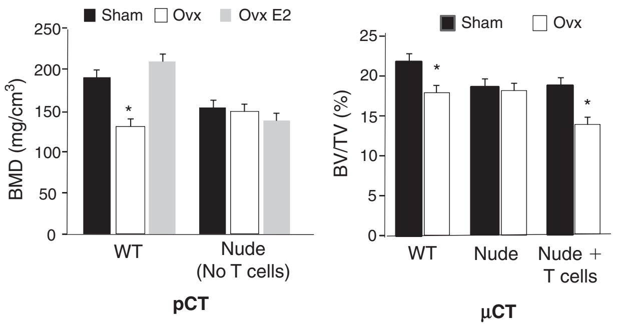

FIGURE 2 Nude mice are protected against the loss of trabecular bone induced by ovariectomy. Left panel. Trabecular BMD measurements by peripheral qCT at 4 weeks from surgery. Right panel: Trabecular BV/TV (Mean + SEM) as measured by μCT at 4 weeks from surgery. * = $p < 0.05$ compared to sham operated mice.

further augment bone loss by stimulating T-cell RANKL production.

A confirmation of the key role of T cells in ovariectomy-induced bone loss has recently been provided by the demonstration that CTLA-4 Ig, an inhibitor of T-cell costimulation approved for the treatment of rheumatoid arthritis, completely prevents the bone loss induced by ovariectomy in wild-type mice (Grassi and Pacifici, 2007). CTLA-4 Ig is a human fusion protein combining the extracellular portion of cytotoxic T lymphocyte Ag 4 with the constant-region fragment of human IgG1 (Linsley *et al.,* 1992). CTLA4-Ig binds to human and murine CD80 (and CD86) blocking their interaction with CD28, promoting anergy and T-cell apoptosis (Moreland *et al.,* 2006; Ruderman and Pope, 2005). To our knowledge, this is the first immunosuppressant shown to prevent bone loss.

While another confirmation of the key role of T cells was provided by a report by Watanabe *et al.* (2001), a study by Lee *et al.* (2006) showed that nude mice lose trabecular bone after ovariectomy, although they are protected against the loss of cortical bone. In the same investigation ovariectomy was found not to induce cortical bone loss in either the spine or the femur in TCRα -/- and RAG2 -/- mice, although it induced trabecular bone loss in both strains. The partially negative outcome of that study is likely explained by age and experimental design differences, the presence of residual T cells in some strains, and by ovariectomy-independent alterations of bone resorption induced by either the concomitant lack of B cells (Li *et al.,* 2007) or genetic compensations.

In summary, upregulated T-cell production of TNF appears to be a key mechanism by which ovariectomy induces bone loss for the following reasons: (1) ovariectomy increases T-cell TNF production in the BM; (2) TNF increases the responsiveness of osteoclast precursors to RANKL (produced by either stromal cells/OBs or T cells), while simultaneously suppressing the magnitude of the compensatory increase in bone formation; (3) mice lacking or insensitive to TNF are completely protected against ovariectomy-induced bone loss; and (4) wild-type T-cell reconstitution in nude mice restores the capacity of ovariectomy to induce bone loss while reconstitution of nude mice with TNF -/- T cells fails to do so.

MECHANISMS OF ESTROGEN REGULATION OF T-CELL TNF PRODUCTION

The BM hosts fully functional mature T cells that exhibit several distinctive features. Both in humans and mice, T cells account for 3–8% of nucleated BM cells (Di Rosa and Pabst, 2005). The percentage of activated T cells is much higher in the BM than in other secondary lymphoid organs and this feature is both cytokine (IL-7 and IL-15) and antigen driven (Clark and Normansell, 1990; Di Rosa and Santoni, 2002). As a result, the BM is the lymphoid organ with the highest percentage and number of proliferating T cells, apart from the thymus.

The BM has long been recognized as a primary lymphoid organ, but it is now clear that the BM plays a key role in the immune response by hosting and regulating adaptive immunity. The BM serves as a site for the initiation of naïve T-cell responses (Feuerer *et al.,* 2003; Tripp *et al.,* 1997) and as a reservoir of CD4 and CD8 memory T cells (Di Rosa and Pabst, 2005). Donor T cells can be found in the BM a few hours after injection into recipient mice (Berlin-Rufenach *et al.,* 1999; Koni *et al.,* 2001) but memory T cells home to BM in higher numbers than naïve T cells (Di Rosa and Santoni, 2003). This selective T-cell homing is more pronounced in recipient mice that possess a normal T-cell repertoire (Di Rosa and Santoni, 2003). The mechanism by which CD8 memory T cells are preferentially recruited in the BM has recently been described (Mazo *et al.,* 2005). Because the entry into the BM of naïve T cells is limited by space availability and competition with other T cells (Di Rosa and Santoni, 2003), senescent memory T cells accumulate in the BM with aging (Effros, 2004). These T cells produce large amounts of TNF and exhibit increased reactivity to self-peptides and foreign Ag (Di Rosa and Santoni, 2003). Furthermore, a close anatomical co-localization of T cells and osteoclasts has been demonstrated. These reasons may explain why the accumulation of aging lymphocytes correlates with an increased incidence of fractures (Effros, 2004). In response to antigenic stimulation, memory CD4 and CD8 T cells of the BM produce effector cytokines (Di Rosa and Santoni, 2003; Mazo *et al.,* 2005). BM T cells encounter Ag presented by dendritic cells and BMMs, which reside or have returned to the BM. Resident dendritic cells and BMMs capture blood-born Ag, which circulates in the BM vessels, or Ag within the BM space (Feuerer *et al.,* 2004). BM T cells may move toward Ag bearing dendritic cells in the BM or return to the BM after having encountered Ag loaded APCs outside the BM. In summary, BM T cells have specific functional characteristics that render them likely to play a pivotal role in ovariectomy-induced bone loss.

Ovariectomy upregulates T-cell TNF production primarily by increasing the number of TNF-producing T cells (Roggia *et al.,* 2001). This is the result of a complex pathway that involves the thymus and the BM (Fig. 3). In the BM, ovariectomy promotes T-cell activation, resulting in increased T-cell proliferation and life span through antigen presentation by macrophages and dendritic cells (Cenci *et al.,* 2003; Grassi and Pacifici, 2005). This process is owing to the ability of estrogen deficiency to upregulate the expression of MHCII and CD80 in macrophages and dendritic cells, respectively (Adamski *et al.,* 2004; Cenci *et al.,* 2003; Grassi and Pacifici, 2005).

Although the mechanism of T-cell activation elicited by estrogen deficiency is similar to that triggered by infections, the intensity of the events that follow estrogen withdrawal is significantly less severe and this process should be envisioned as a partial increase in T-cell autoreactivity to self-peptides resulting in a modest expansion in the pool of effector CD4$^+$ cells.

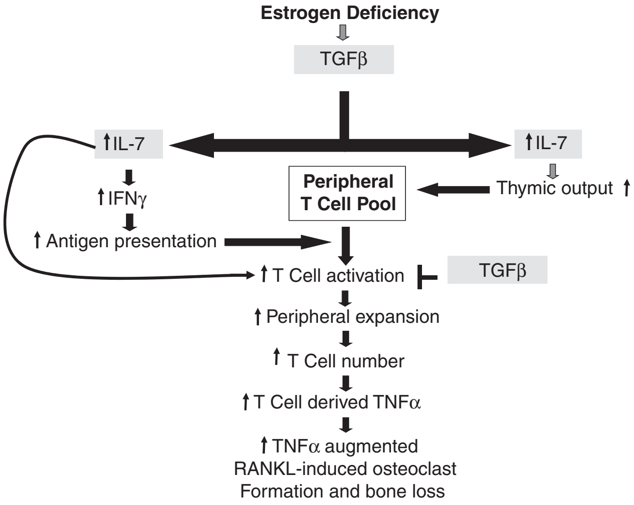

FIGURE 3 Schematic representation of the main mechanisms and feedback interactions by which estrogen deficiency leads to bone loss. The bone loss induced by estrogen deficiency is owing to a complex interplay of hormones and cytokines that converge to disrupt the process of bone remodeling. Estrogen deficiency leads to a global increase in IL-7 production in target organs such as bone, thymus, and spleen, in part through decreases in TGFβ. This leads to a first wave of T-cell activation. Activated T cells release IFNγ, which increase Ag presentation by dendritic cells and macrophages, amplifying T-cell activation and promoting release of the osteoclastogenic factors RANKL and TNF. Another mechanism by which estrogen suppresses T-cell TNF production is by regulating T-cell differentiation in the thymus. T-cell precursors originate in the bone marrow and migrate to the thymus where T-cell differentiation, selection, and expansion takes place, in large measure under the control of IL-7. Following release from the thymus (thymic export), these new T cells home to peripheral lymphoid organs including the bone marrow itself. Estrogen prevents decreases thymic output through an IL-7 dependent mechanism, blunting the size of the pool of bone marrow T cell available for activation and expansion.

The relevance of this mechanism *in vivo* was established by utilizing DO11.10 mice, a strain in which all T cells recognize a single peptide epitope of chicken albumin (ovalbumin) that is not expressed in mice. In the absence of ovalbumin antigen-presenting cells of DO11.10 mice are unable to induce T-cell activation. Therefore, if antigen-presenting cells are a relevant target of estrogen, these mice should be protected from the increased T-cell proliferation, the suppression of activation-induced T-cell death, and the bone loss that follows ovariectomy. As predicted ovariectomy fails to increase the pool of T cells and to induce bone loss in these mice (Cenci *et al.,* 2003). In addition, injection of ovalbumin, which permits the generation of the appropriate MHC-peptide antigen for these T cells, restores the capacity of ovariectomy to expand the T-cell pool by targeting proliferation and apoptosis and to induce bone loss. These data demonstrate that antigen presentation, specifically the generation of appropriate peptide-MHC complexes, is critical to the process by which ovariectomy increases T-cell proliferation and life span and leads to bone loss. Furthermore, the finding that T cells from ovariectomized mice exhibit an increased response to ovalbumin demonstrates that ovariectomy increases the reactivity of antigen-presenting cells to endogenous antigens rather than stimulating the production of a new antigen or modulating antigen levels.

The question thus arises as to the nature of the antigens. Estrogen deficiency is likely to increase T-cell reactivity to a pool of self- and foreign antigens physiologically present in healthy animals and humans. This is consistent with the fact that T-cell clones expressing T-cell receptors directed against self-antigens not expressed in the thymus, survive negative selection during T-cell maturation (Robey *et al.,* 1992). Such clones (“autoreactive” or “self-reactive” T cells) reside in peripheral lymphatic organs of adult individuals. In addition, foreign antigens of bacterial origin are physiologically absorbed in the gut. As these peptides come into contact with immune cells locally and systemically, they induce a low-grade T-cell activation (Rammensee *et al.,* 1993). Thus, a moderate immune response is constantly in place in healthy humans and rodents owing to presentation by MHCII and MHCI molecules of both self- and foreign peptides to CD4$^+$ and CD8$^+$ T cells (Grossman and Paul, 2000). This autoreactive response is thought to be essential for immune cell survival and renewal (Tanchot *et al.,* 1997).

The effects of ovariectomy on antigen presentation and the resulting changes in T-cell activation, proliferation, and life span are explained by a stimulatory effect of ovariectomy on the expression of the gene encoding class II transactivator (*CIITA*). The product of *CIITA* is a non-DNA–binding factor induced by IFNγ that functions as a transcriptional coactivator at the MHC II promoter (Boss and Jensen, 2003). Increased *CIITA* expression in macrophages derived from ovariectomized mice results from ovariectomy-mediated increases in both T-cell IFNγ production and the responsiveness of *CIITA* to IFNγ (Cenci *et al.*, 2003), an inflammatory cytokine produced by helper T cell. The relevance of IFNγ is shown by the failure of IFNγR -/- and IFNγ -/- mice to sustain bone loss in response to ovariectomy (Cenci *et al.*, 2003; Gao *et al.*, 2007).

IFNγ production by T cells is induced by either a cyclosporin-A–sensitive T-cell receptor (TCR)-dependent mechanism, mediated by T-cell activation, or by the cytokines IL-12 and IL-18 through activation of the MAP kinase p38. The increased production of IFNγ by T cells from ovariectomized mice is suppressed by *in vitro* treatment with the selective p38 inhibitor SB203580, but not by the activation inhibitor cyclosporin-A, indicating that increased IFNγ production by $CD4^+$ cells in ovariectomized mice is cytokine-driven. The expression of the IL-12 and IL-18 genes in BM monocytes is induced by NFκB and AP-1, nuclear proteins whose transcriptional activity is directly repressed by estrogen (An *et al.*, 1999; Galien and Garcia, 1997; Shevde *et al.*, 2000). Unstimulated BM monocytes such as those from estrogen-replete mice are known to express low or undetectable levels of NFκB and AP-1 (Muegge and Durum, 1990). Accordingly, BM monocytes from estrogen-replete mice express minimal levels of IL-12 and IL-18 while those from ovariectomized animals produce increased amounts of IL-12 and IL-18. Thus, one mechanism by which estrogen represses *CIITA* is by decreasing IFNγ production via an inhibitory effect on the BM monocyte production of IL-12 and IL-18.

Another mechanism by which estrogen deficiency upregulates the production of IFNγ is through TGFβ. Estrogen has a direct stimulatory effect on the production of this factor, which is mediated through direct binding of estrogen/estrogen receptor complex to an estrogen response element region in the TGFβ promoter (Yang *et al.*, 1996).

TGFβ is recognized a powerful repressor of T-cell activation. Indeed, TGFβ exerts strong immunosuppressive effects by inhibiting the activation and the proliferation of T cells and their production of proinflammatory cytokines, including IFNγ. Studies in a transgenic mouse that expresses a dominant negative form of the TGFβ receptor exclusively in T cells have allowed the significance of the repressive effects of this cytokine on T-cell function in the bone loss associated with estrogen deficiency to be established (Gao *et al.*, 2004). This strain, known as CD4dnTGFβRII, is severely osteopenic owing to increased bone resorption. More importantly, mice with T-cell–specific blockade of TGFβ signaling are completely resistant to the bone-sparing effects of estrogen (Gao *et al.*, 2004). This phenotype results from a failure of estrogen to repress IFNγ production that, in turn, leads to increased T-cell activation and T-cell TNF production. Gain of function experiments confirmed that elevation of the systemic levels of TGFβ prevents ovariectomy-induced bone loss and bone turnover (Gao *et al.*, 2004).

A third mechanism by which estrogen regulates IFNγ and TNF production is by repressing the production of IL-7. Levels of IL-7 are significantly elevated following ovariectomy (Lindberg *et al.*, 2006; Ryan *et al.*, 2005; Weitzmann *et al.*, 2002) and *in vivo* IL-7 blockade, using neutralizing antibodies, is effective in preventing ovariectomy-induced bone destruction (Weitzmann *et al.*, 2002) by suppressing T-cell expansion and TNF and IFNγ production (Ryan *et al.*, 2005). Furthermore, a recent study shows that liver-derived IGF-I is permissive for ovariectomy-induced trabecular bone loss by modulation of the number of T cells and the expression of IL-7 (Lindberg *et al.*, 2006). Indeed, the elevated BM levels of IL-7 contribute to the expansion of the T-cell population in peripheral lymphoid organs through several mechanisms. Firstly, IL-7 directly stimulates T-cell proliferation by lowering tolerance to weak self-antigens. Secondly, IL-7 increases antigen presentation by upregulating the production of IFNγ. Thirdly, IL-7 and TGFβ inversely regulate each other's production (Dubinett *et al.*, 1995; Huang *et al.*, 2002).

The reduction in TGFβ signaling, characteristic of estrogen deficiency, may serve to further stimulate IL-7 production, thus driving the cycle of osteoclastogenic cytokine production and bone wasting. New studies further implicate IL-7 as a downstream effecter of IGF-I action in ovariectomy-induced trabecular bone loss (Lindberg *et al.*, 2006).

In estrogen deficiency, IL-7 compounds bone loss by suppressing bone formation thus uncoupling bone formation from resorption. Recent studies have also identified elevated levels of IL-7 in patients suffering from multiple myeloma and in multiple myeloma-derived cell lines (Giuliani *et al.*, 2002), and have suggested a role for IL-7 in the enhanced bone resorption and suppressed bone formation associated with multiple myeloma. Increased IL-7 expression has also been implicated in the bone loss sustained by patients with rheumatoid arthritis (De Benedetti *et al.*, 1995; van Roon *et al.*, 2003).

In summary, a complex pathway links estrogen, the immune system, and the development of postmenopausal bone loss in experimental animals.

T-CELL THYMIC OUTPUT AND BONE LOSS

The thymus undergoes progressive structural and functional declines with age, coinciding with increased circulating sex-steroid levels at puberty (Haynes *et al.*, 2000). However, under severe T-cell depletion secondary to HIV infection, chemotherapy, or BM transplant, an increase in thymic output (known as thymic rebound) becomes critical for long-term restoration of T-cell homeostasis. For example, middle-aged women treated with autologous BM transplants develop thymic hypertrophy and a resurgence of thymic T-cell output that contributes to the restoration of a wide T-cell repertoire (Hakim *et al.,* 2005), although the intensity of thymic rebound declines with age.

The mechanism driving thymic rebound is not completely understood, but one factor involved is IL-7 (Mackall *et al.,* 2001). Importantly, IL-7 alone is not sufficient to enhance thymopoiesis in young mice (Chu *et al.,* 2004), but plays a more relevant role in aged mice (Alpdogan *et al.,* 2001).

Both androgens and estrogen have a profound suppressive effect on thymic function. Accordingly, castration reverses thymic atrophy and increases export of recent thymic emigrants to the periphery, (Utsuyama and Hirokawa, 1989), while the sex steroid inhibits thymus regeneration by promoting thymocyte apoptosis and an arrest of differentiation (Okasha *et al.,* 2001). Restoration of thymic function after castration occurs in young (Roden *et al.,* 2004) as well as in very old rodents (Sutherland *et al.,* 2005).

In accordance with the notion that estrogen deficiency induces a rebound in thymic function, ovariectomy expands thymic T cells and leads to the thymic export of naïve T cells (Ryan *et al.,* 2005). Indeed, stimulated thymic T-cell output accounts for ~50% of the increase in the number of T cells in the periphery, while the remaining 50% is owing to enhanced peripheral expansion. Similarly, thymectomy decreases by ~50% the bone loss induced by ovariectomy (Fig. 4), thus demonstrating that the thymus plays a previously unrecognized causal effect in ovariectomy -induced bone loss in mice. The remaining bone loss is a consequence of the peripheral expansion of naïve and memory T cells (Ryan *et al.,* 2005). This finding, which awaits confirmation in humans, suggests that estrogen deficiency-induced thymic rebound may be responsible for the exaggerated bone loss in young women undergoing surgical menopause (Hreshchyshyn *et al.,* 1988) or for the rapid bone loss characteristic of women in their first 5–7 years after natural menopause (Riggs *et al.,* 2002). Indeed, an age-related decrease in estrogen deficiency-induced thymic rebound could mitigate the stimulatory effects of sex steroid deprivation and explain why the rate of bone loss in postmenopausal women diminishes as aging progresses (Riggs *et al.,* 2002).

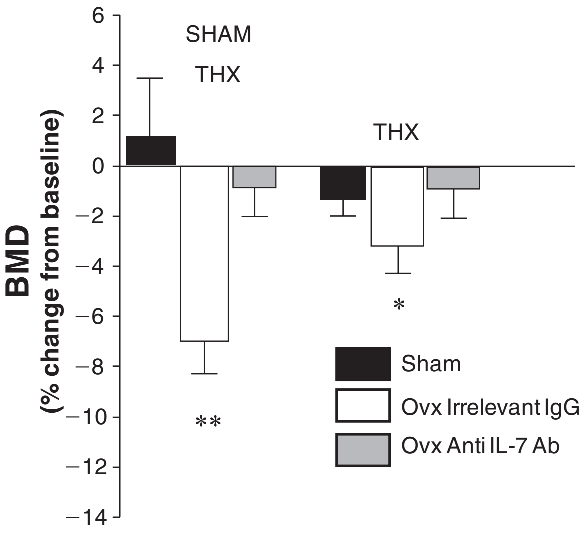

FIGURE 4 Ovariectomy causes a twofold lower bone loss in thymectomized mice than in euthymic controls. BMD (Mean + SEM) as c export), these new T cells home to peripheral lymphoid organs including the bone marrow itself. Estrogen prevents T-cell activation in the bone marrow in part by directly blunting antigen presentation, and in part via repression of IL-7 and IFNγ production. This effect is amplified by the upregulation of the IL-7 suppressor, TGFβ. The net result of these actions is a decrease in the number of TNF-producing T cells. The blunted levels of TNF diminish RANKL-induced osteoclast formation preventing bone loss.

FROM ANIMAL MODELS TO HUMAN DISEASE

Owing to the inherent difficulties associated with human experimentation, the majority of studies discussed herein represent investigations in animals. Consequently, their applicability to human disease, in particular postmenopausal osteoporosis, remains unproven. However, a number of lines of evidence suggest that pro-osteoclastogenic immunological perturbations are not uncommon in estroprevic humans and evidence is beginning to accumulate to suggest that T cells play a relevant role in regulating bone resorption in humans. It has recently been reported that RANKL expression on lymphocytes and marrow stromal cells is significantly elevated during estrogen deficiency in humans and correlates directly with increases in bone resorption markers and inversely with serum estrogen levels (Eghbali-Fatourechi *et al.,* 2003). Furthermore, in postmenopausal women, production of cytokines representative of TH1 lymphocytes are increased, an effect reversed by supplemental estrogen and one report describes a case in which a defect in T-cell immunoregulation resulted in severe osteoporosis associated with increased osteoclast activity (Rubin *et al.,* 1988).

The production of increased levels of TNF and IL-1 in the conditioned media of peripheral blood cells derived from postmenopausal women is well established (Pacifici *et al.,* 1991b), while the number of CD3+ CD56+ T cells (a TNF-producing adherent T-cell population) is reported to show a highly significant negative correlation with femoral and lumbar bone density in estroprevic postmenopausal women (Abrahamsen *et al.,* 1997).

Furthermore, animal studies of postmenopausal osteoporosis show striking similarities to autoimmune diseases. A significant body of evidence suggests that estrogen is indeed a relevant player in autoimmune disease in humans. Firstly the majority of autoimmune diseases have a gender bias toward the female population. In particular Sjögren syndrome, systemic lupus erythematosus (SLE), autoimmune thyroid disease (Hashimoto's thyroiditis as well as Graves' disease) and scleroderma, in which the patient population is >80% women, and rheumatoid arthritis, multiple sclerosis (MS), and myasthenia gravis, in which the sex distribution is 60–75% women. Secondly, puberty, menopause, and pregnancy all alter the incidence and the course of many autoimmune diseases, further suggesting a role for sex hormones in autoimmunity. These modifications of disease activity by sex steroids have been suggested to involve the generation of a hormonal environment that favors a TH2 response. In MS and rheumatoid arthritis, this environment may suppress the ongoing TH1 responses to central nervous system and joint antigens whereas in SLE a TH2 environment would enhance antibody production and possibly exacerbate disease progression (Whitacre, 2001). Evidence from a variety of sources thus implicates a role for sex hormones in modulating the incidence, course, and severity of autoimmune diseases.

CONCLUSIONS

Remarkable progress has been made in the last two decades in our understanding of the mechanisms of bone destruction during estrogen deficiency (Fig. 4). Most new data are derived from studies in mice and remain to be validated in humans, which will be essential for defining the role of inflammatory cytokines in postmenopausal bone loss, as selective inhibitors might be developed as new therapeutic agents.

The ovariectomized mouse is an excellent model to investigate the acute effects of estrogen withdrawal, although it is not suitable for studies of the long-term effects of menopause on the skeleton, as bone loss subsides within a few weeks after ovariectomy. Thus, additional animal models and long-term human studies are needed. Because critical effects of estrogen on bone involve regulation of precursor cell differentiation and signaling pathways that are few and short-lived, many pivotal effects of estrogen *in vivo* are difficult to reproduce *in vitro*. Similarly, regulatory events observed *in vitro* are often not relevant *in vivo*. It is therefore essential for *in vitro* studies to be validated *in vivo*. For example, while estrogen stimulates IFNγ production in cells in cultures (Fox *et al.,* 1991), estrogen represses it *in vivo* (Cenci *et al.,* 2003). Similarly, while IFNγ blocks osteoclast formation through direct targeting of maturing osteoclasts, IFNγ stimulates osteoclastogenesis and bone resorption in estrogen-deficient mice (Cenci *et al.,* 2003; Gao *et al.,* 2007).

In conclusion, if the findings in experimental animals are confirmed in humans, it will, perhaps, be appropriate to classify osteoporosis as an inflammatory or even an autoimmune condition and certainly new therapeutic "immune" targets will emerge.

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Chapter 43

Thyroid Hormone and Bone

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INTRODUCTION

Thyroid hormone is essential for skeletal development and also affects mature bone. Depending on the hormone concentration and stage of life, the effects of thyroid hormone can be either beneficial or deleterious to the skeleton. This chapter focuses on the relationship of the *in vitro* effects of thyroid hormone on bone cells to the observed effects of the hormone on the skeleton *in vivo* in both experimental animals and humans. Studies published since the previous edition provide information on thyroid hormone effects on additional genes of interest in osteoblasts and further assessment of the skeletal risks of excess thyroid hormone. A new paradigm related to thyroid hormone effects on bone is the proposed role of thyroid stimulating hormone (TSH) as an independent regulator of skeletal responses and a mediator of skeletal effects of thyroid hormones *in vivo*. These new aspects of the skeletal physiology are accompanied by an expansion of the section on the basic mechanisms of thyroid hormone receptor action based on recent studies in many tissues.

MECHANISM OF THYROID HORMONE ACTION ON THE SKELETON

Nuclear Receptors: Structural and Genetic Studies

Thyroid hormone receptors are members of the steroid receptor superfamily (Evans, 1988). All of these receptors share a common modular structure with a centrally located DNA-binding domain composed of two zinc fingers and a carboxy-terminal ligand-binding domain that is also involved in receptor dimerization and interactions with coactivators and corepressors. The receptors are nuclear proteins capable of binding to cognate DNA elements in the absence of their ligands. Binding of the ligand to the receptor alters the receptor conformation and subsequently enables the activation or repression of specific genes. Similar to the pattern for retinoid and vitamin D receptors, DNA-binding sites for thyroid hormone receptors include monomeric, palindromic, inverted repeat, and direct repeat response elements derived from a common AGGT(C/A)A motif. Multiple functional forms exist for thyroid hormone receptors, including monomers, homodimers, heterodimers between thyroid hormone isoforms, and heterodimers with retinoid and vitamin D receptors. In bone (Williams *et al.,* 1994; Williams *et al.,* 1995), as in other tissues (Glass, 1994; Brent *et al.,* 1991), DNA binding and transcriptional activation are enhanced when the thyroid hormone receptor isoforms are present as heterodimers with retinoid or vitamin D receptors. In osteoblast cell lines, interactions among the retinoid, vitamin D, and thyroid hormone ligands appeared to mediate specific responses (Williams *et al.,* 1994, 1995). Studies on the effects of treatment combinations on the expression of osteoblast phenotypic genes in the cell lines revealed complex responses that indicated the importance of dose, treatment duration, and degree of confluence in dictating the magnitude of the response (Williams *et al.,* 1995). In primary rat osteoblastic cells, alteration of the ligand combinations did not influence the responses (Bland *et al.,* 1997).

Most commonly, the unliganded thyroid hormone receptor represses gene transcription. Interaction of the unliganded receptor with a corepressor complex, including nuclear receptor corepressor (NCoR), silencing mediator for retinoic acid receptor, the repressor Sin3, and histone deacetylases (Torchia *et al.,*1998; Yen *et al.,* 2006), results in the condensation of chromatin structure and repression of transcription through decreased access of transcription factors (Koenig, 1998; Wu and Koenig, 2000). Interaction of the receptor with the active thyroid hormone triiodothyronine (T3) results in a conformational change that leads to dissociation from the repressor complex and interaction with activation complexes. One activation complex involves the steroid receptor coactivator (SRC) family, which can also interact with CREB-binding protein (CBP) and the p300 protein, which in turn interacts with p300/CBP associated factor (PCAF) (McKenna and O'Malley,

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2002; Torchia *et al.,* 1998). These factors have histone acetyltransferase activity. A second activation complex is the vitamin D receptor interacting protein/thyroid hormone receptor (TR) associated protein (DRIP/TRAP) complex (Sharma and Fondell, 2002; Burakov *et al.,* 2002). Recruitment of this complex can result in recruitment and stabilization of RNA polymerase II (Ito and Roeder, 2001; Rachez and Freedman, 2001). Other chromatin remodeling proteins having ATPase activity have also been proposed as components leading to activation (DiRenzo *et al.,* 2000). Differential recruitment of coactivators and histone acetylation have been demonstrated for different genes in GH3 pituitary cells (Liu *et al.,* 2006).

Thyroid hormone receptors are encoded by two genes: one found on chromosome 17 encoding TRα and one on chromosome 3 encoding TRβ. Alternative splicing of TRα transcripts results in the generation of several carboxy-terminal products (Izumo and Mahdavi, 1988; Chassande *et al.,* 1997). The TRα1 isoform is a commonly expressed active isoform of the receptor. TRα2, which is homologous to the v-erb A oncogene, is a nonbinding isoform resulting from alternative splicing of the TRα primary transcript. TRα2 fails to heterodimerize with retinoic acid receptors (RXR) (Reginato *et al.,* 1996) and may act as a dominant-negative repressor (Koenig *et al.,* 1989; Sap *et al.,* 1986). The TRβ1 and the amino-terminal splice variant, TRβ2, are both active. Tissue expression of TRβ2 is limited, and this isoform is expressed most significantly in the hypothalamus and pituitary (Lazar, 1993). TR isoforms have been found in skeletal tissues. mRNAs for TRα1, TRα2, and TRβ are found in MG63, ROS 17/2.8, and UMR-106 cell lines (Williams *et al.,* 1994; Allain *et al.,* 1996). TRβ2 mRNA has been found in osteoblasts (Abu, 2000). mRNA for TRα1 was 12 times higher than TRβ1 mRNA in tibia and femur of 7-week-old male mice (O'Shea *et al.* 2003). TRα1, TRα2, TRβ1, and TRβ2 mRNAs were expressed in chondrocytes at all stages of differentiation. TRα1, TRα2, and TRβ1 mRNAs were highly expressed in osteoblasts at bone-remodeling sites; and mRNA for all of the isoforms was present and highly expressed in multinucleated osteoclastic cells from an osteoclastoma (Abu *et al.,* 1997). TRα1, TRα2, and TRβ1 mRNA have also been detected in rat femurs and vertebrae (Milne *et al.,* 1999). Immunohistochemical staining with antibodies recognizing a TRα epitope or specific TRα2 and TRβ revealed the presence of receptor protein in osteoblast cell lines and in osteoclasts in tissue smears from a human osteoclastoma (Allain *et al.,* 1996). In contrast to mRNA expression, TRα1 protein expression was not seen in the osteoclastoma cells and was limited to osteoblasts at sites of remodeling and undifferentiated chondrocytes (Abu *et al.,* 2000).

To determine the role(s) of specific TR isoforms, TR-deficient mouse strains have been generated by homologous recombination. TR$\alpha1^{-/-}$ mice did not show bone defects (Wikstrom *et al.,* 1998), whereas mice in which both TRα isoforms were deleted showed growth retardation and impaired development of epiphyseal bone, with disorganization of chondrocyte columns, decreased hypertrophic chondrocytes, and low ossification (Fraichard *et al.,* 1997). The animals died shortly after weaning. TRβ knockout mice failed to show bone defects (Gauthier *et al.,* 1999; Gothe *et al.,* 1999), suggesting that the TRβ isoform is not essential for bone development in the mice. The TR$\alpha1^{-/-}$ TR$\beta^{-/-}$ double knockout produced viable mice, the majority of which survived at least through 12 months, although there was increased mortality compared to wild-type mice (Gothe *et al.,* 1999). TR$\alpha1^{-/-}$ TR$\beta^{-/-}$ double knockout mice exhibited retarded growth and significantly reduced levels of growth hormone (GH) and insulin-like growth factor I (IGF-I). Bone length was decreased significantly in limbs and vertebrae, with the effect being most marked in the femur. Growth plates were disorganized and epiphyseal ossification was delayed. Dual x-ray absorptiometry showed decreased bone area and bone mineral content but no significant effect on bone mineral density. Middiaphyseal peripheral quantitative computed tomography (pQCT) scans of the femurs revealed decreased cortical density, cortical area, bone mineral content, and periosteal circumference. Cross-sectional moment of inertia and moment of resistance were decreased significantly. It was noted that the phenotype was less severe than that resulting from thyroidectomy and this was postulated to be a reflection of the fact that in the case of thyroidectomy, T3 would not be available to alleviate the transcriptional repression effected by the TRs (Gothe *et al.,* 1999).

Skeletal alterations associated with mutations in the TRβ1 gene have been described in patients with resistance to thyroid hormones (RTH). In most reported cases, the defect shows an autosomal dominant pattern of inheritance. The mutations are clustered and largely located within domains in the carboxy-terminal region. They are mainly nucleotide substitutions that result in single amino acid changes (Refetoff, 1993). The mutant alleles may act by a dominant-negative mechanism to inhibit the ability of the normal allele to elicit normal receptor function (Chatterjee *et al.,* 1991; Sakurai *et al.,* 1990). The dominant-negative action appears to be at the level of DNA binding (Kopp *et al.,* 1996). The mutant phenotypes are heterogeneous, but some patients have shown evidence of retarded bone age and stippled epiphyses, similar to characteristics of hypothyroidism, with resulting short stature. In other patients there is accelerated bone age, accelerated chondrocyte maturation, and early epiphyseal closure, again resulting in short stature (Behr *et al.,* 1997). The target sites at which resistance occurs (pituitary or peripheral) may determine the phenotype. A novel syndrome resulting in advanced bone age was associated with a mutation in the MCT8 thyroid hormone transporter gene (Herzovich *et al.,* 2007). A single nucleotide change (Q261X) in exon 3 on the X chromosome resulted in low serum T4 and free T4, and elevated serum T3 and free T3, and slightly elevated TSH. The child had severe neurological abnormalities and normal growth, which had been previously noted in other patients with mutations in this gene (Dumitrescu *et al.*, 2004).

Replicating a human mutation resulting in severe resistance to thyroid hormone has resulted in mouse models that have provided considerable new information on the roles of TRα and TRβ in bone and other tissues (Kaneshige *et al.*, 2000; Kaneshige *et al.*, 2001; O'Shea *et al.*, 2003; O'Shea *et al.*, 2005; O'Shea *et al.*, 2006). The mutation (mutant PV) is a frameshift mutation that has complete loss of T3 binding and transactivation activity. Knockout of TRβ in the mouse model eliminated expression of DNA and ligand binding domains of TRβ1 and TRβ2. The knockout showed that TRβ was the predominant regulator of hypothalamic and pituitary responses to thyroid hormone, as these animals had marked increases in T3, T4, and TSH. There was advanced endochondral and intramembranous ossification in utero, premature closure of the growth plates and shortened body length, along with increased mineralization and craniosynostosis. The narrower growth plate appeared to be a consequence of faster transition through the proliferative zone. The TRβ knockout animals had decreased trabecular bone mass, more resorption and a greater number of TRAP-positive cells. Knockout of TRα in the mouse model abolished the generation of TRα1, TRα2, and the splice variants TR$\Delta\alpha$1 and TR$\Delta\alpha$2. The TRα knockout failed to markedly affect the hypothalamic pituitary axis. Thyroid hormones were normal and TSH was only slightly decreased in these animals. This knockout resulted in animals with a wider growth plate, transiently decreased bone calcification and transiently decreased bone growth, decreased resorption and and decreased TRAP-positive cells. The results suggested that TRα is functionally predominant in bone and that the hyperthyroid osteoporotic phenotype in the TRβ PV mutation was mediated predominantly by TRα.

The paradigm for the effects of thyroid hormone on bone was challenged by studies that proposed a role for TSH as an independent regulator of bone formation and resorption (Abe *et al.*, 2003; Hase *et al.*, 2006). A mouse model was generated in which the TSH receptor was knocked out through homologous recombination in embryonic stem cells. The mice exhibited a phenotype of decreased body weight, decreased femur length and decreased weight. Bone histology showed osteoporosis together with focal sclerosis, and histomorphometry revealed increased TRAP-labeled surface and decreased trabecular bone area. The results were consistent with the possibility that the bone loss in hyperthyroid state was due to the suppressed TSH. TNFα, JNK, and NFκB were implicated in the osteoclast response, and LRP-5 and Flk-1, but not Runx-2 or osterix, in the osteoblast response. Treatment of hematopoetic stem cells or RAW-C3 bone cells with rhTSH (at the rather high concentration of 10 U/ml) decreased osteoclast markers and stimulated apoptosis of osteoclast precursor cells and osteoclasts. In other studies, TSH increased alkaline phosphatase and osteocalcin and stimulated mineralization in osteoblastic cells (Sampath *et al.*, 2007). Several methods were used to demonstrate TSH receptors in bone cells. In the knockout model, exon 1 was replaced with a GFP cassette, allowing GFP fluorescence to be used as an indicator of the presence of TSH receptors in bone cells. Receptors were also demonstrated by specific ^{125}I-TSH binding (Abe *et al.*, 2003). In other studies, TSH stimulated cAMP production in SaOS-2 human osteosarcoma cells and expression of TSHR was demonstrated in SaOS-2 cells and NHOst normal human osteoblast cells by RT-PCR (Morimura *et al.*, 2005). In these cells, TSH (0.1–10 U/ml) stimulated type 2 iodothyronine deiodinase, which converts T4 to T3 (Morimura *et al.*, 2005). T4, rT3, and T3 all inhibited the deiodinase activity.

Other studies have challenged the conclusions about the role of TSH on the skeleton. In primary cultures of human osteoblast-like cells, the evidence for expression and function of TSH was weak, with no effects on calcium signaling and only small effects on cAMP (Tsai *et al.*, 2004). TSHR mRNA could not be demonstrated by RNase protection assay, although it could be detected by RT-PCR and nucleotide sequencing (Tsai *et al.*, 2004). Selective knockout of TRα or TRβ, described earlier, produced results that were inconsistent with the hypothesis of the role of TSH. The TRβ knockout animals, which had marked increases in T3, T4, and TSH owing to the essential role of the TRβ in mediating feedback inhibition at the hypothalamus and pituitary, had decreased trabecular bone and evidence of increased osteoclast activity (Bassett *et al.*, 2007). Thus, these results were inconsistent with the hypothesis that TSH was a suppressor of bone loss. Bassett *et al.* (2007) noted that in the study by Abe *et al.* (2003) the TSHR knockout mice received hormone replacement with thyroid extract after weaning, and therefore hypothyroidism during early development could have resulted in a period of catch-up growth and accelerated bone development when thyroid hormone was replaced. These apparently incompatible findings will undoubtedly be the subject of future investigations. An important question will be the local thyroid hormone concentrations within bone cells and their actions.

Differential expression of TRα and β isoforms could also allow for the development of thyroid hormone analogs that have tissue specificity owing to their preferential interaction with one receptor isoform. The TRβ-selective agonist GC-1 had greater effects on lipid metabolism and less on cardiac activity (Trost *et al.*, 2000). In a direct comparison of the effects of GC-1 and T3 on bone in female adult rats, T3 significantly reduced bone mineral density in lumbar vertebrae, femur, and tibia, and reduced trabecular volume, thickness, and number. GC-1 did not have these skeletal effects, although it did reduce serum TSH and cholesterol (Freitas *et al.*, 2003). Another agonist, tiratricol (3,5,3′-triiodothyroacetic acid), showed enhanced effects on hepatic lipids and skeletal metabolism (Sherman *et al.*, 1997). Interestingly, when used topically it was found to reverse glucocorticoid-induced skin atrophy (Yazdanparast *et al.*, 2006).

Competitive Binding Studies

Two studies of T3 binding to nuclei from ROS 17/2.8 cells gave the following parameters: K_d 5 n*M*, $B_{\max}$ 0.13 ng/mg DNA, with incubation for 60 min at 37° C (Rizzoli *et al.*, 1986) and K_d 150 p*M*, $B_{\max}$ 24 fmol/100 μg DNA, with incubation for 2.5 hours at 37° C (Sato *et al.*, 1987). In UMR-106 cells, two nuclear binding sites were identified: one with K_d 260 p*M*, $B_{\max}$ 7.7 pg/mg DNA, and one of lower affinity, K_d 1.8 n*M* (LeBron *et al.*, 1989). In MC3T3-E1 cells, K_d for T3 binding was 120 p*M* (Kasono *et al.*, 1988). T3 receptors were also found in cell lines (ROS 25/1, ROS 17/2.8–3) that did not show an alkaline phosphatase response to T3, suggesting that there is a postreceptor defect in these cell lines (Sato *et al.*, 1987). There was good agreement between the relative affinity of different ligands [T3 = 1, thyroxin (T4) = 0.1, 3,3′-diiodothyronine = 0.013, reverse T3 (rT3) = 0.002, monoiodotyrosine = 0, diiodotyrosine = 0] and their ability to increase alkaline phosphatase in ROS 17/2.8 cells (Sato *et al.*, 1987). In another study, T4 had a 20-fold lower and rT3 a 400-fold lower affinity compared with T3 in ROS 17/2.8 cells (Rizzoli *et al.*, 1986). In binding studies with a nuclear fraction from neonatal mouse calvaria, carried out for 60 minutes at 22° C, K_d for T3 was 3 n*M* and the $B_{\max}$ 1.9 pmol/mg DNA (Krieger *et al.*, 1988). The cardiotonic agent milrinone, which has structural homology to T4 (Mylotte *et al.*, 1985), did not compete for binding to the calvarial receptors. Both time and temperature dependence were observed in the binding studies, with binding being more rapid at 37° C than at 22° C (Krieger *et al.*, 1988; Sato *et al.*, 1987). Kinetic analysis of normal nuclear receptors gave K_a of $9 \times 10^8 M^{-1}$ min^{-1} and K_d of 0.016 min^{-1} with a $t_{1/2}$ of approximately 36 minutes (Krieger *et al.*, 1988). For binding studies in the cell lines, the method of Samuels *et al.* (1979) was used to remove thyroid hormones from serum used in the growth medium. The procedure involves treatment of the serum with AG1-X8 resin and removes more than 90% of the T4 and 95% of the T3.

Relevance of *in Vitro* Concentrations and *in Vivo* Dosages of Thyroid Hormone to Physiological and Pathophysiological Concentrations

It has been reported that the normal range for free T3 in serum is similar in rats and humans, and is 3.3–8.2 p*M* (Jurney *et al.*, 1983). Wide concentration ranges of thyroid hormones have been used in experimental studies, especially *in vitro,* and often markedly different dosages are required to obtain the same response in a different cell line, model system, or laboratory. The differentiation state and the production of modulating factors are potential variables that can affect the response in a given system. In addition, the presence of thyroid hormone in the added sera or the presence of binding sites in stripped sera can dramatically influence the free hormone available to the cells or tissue. Several studies have estimated the amount of free hormone available under the experimental conditions used (Sato *et al.*, 1987; Allain *et al.*, 1992). In one study, an equilibrium dialysis method was used to determine free T4 and T3 after treating fetal calf serum with AG1-X8 resin (Sato *et al.*, 1987). T4 and T3 concentrations in the fetal calf serum prior to extraction were 11.1 μg/dl and 157 ng/dl, respectively. It was determined that addition of 10 n*M* T4 to the stripped serum provided 80 p*M* free T4 and that addition of 1 n*M* T3 provided 40 p*M* free T3. In the other study, in which 10% neonatal calf serum was used, the free T3 was measured by radioimmunoassay (Allain *et al.*, 1992). It was determined that the addition of 10 p*M* T3 yielded a free T3 concentration of 2.1 p*M*, that 0.1 n*M* yielded 4 p*M*, that 1 n*M* yielded 2.1 p*M* and that 10 n*M* yielded >39 p*M* (Allain *et al.*, 1992). Although the type and percentage of serum would influence the final values, these measurements and calculations are of value in comparing studies and in relating *in vitro* concentrations to the concentrations of thyroid hormones in normal serum.

Membrane Actions and Signal Transduction Pathways

Thyroid hormones interact with several signal transduction pathways in bone cells. These results suggest that extranuclear actions could initiate some of the thyroid hormone effects on bone. Rapid (within 30 seconds) increases in inositol mono-, bis-, and trisphosphates are elicited by treatment of fetal rat limb bones with 100 n*M* and 1 μ*M* T3 (Lakatos and Stern, 1991). The inactive analogs diiodothyronine and rT3 did not increase inositol phosphates. This effect of T3 was inhibited by indomethacin and could represent an initiation pathway for the prostaglandin-dependent effects of thyroid hormones on bone resorption, discussed later. Thyroid hormones at high doses inhibit cyclic AMP phosphodiesterase (Marcus, 1975). T3 at 0.1 and 1 n*M* increased ornithine decarboxylase and potentiated the responses of this enzyme to parathyroid hormone (PTH) (Schmid *et al.*, 1986). Specific cellular functions associated with membrane receptors have not been identified, although it has been proposed that nongenomic actions of thyroid hormones serve homeostatic functions for membrane transport and may modulate genomic actions of the hormones (Davis and Davis, 1996).

GENE PRODUCTS

Osteoblast Phenotypic Markers

Thyroid hormone promotes the proliferation and differentiation of osteoblastic cells (Ohishi *et al.*, 1994; Ishida *et al.*, 1995). This is reflected in the increased expression of a number of markers.

Alkaline Phosphatase

T4 (10 n*M*) and 1 n*M* T3 increased alkaline phosphatase in ROS 17/2.8 cells (Sato *et al.*, 1987). The effect was seen within four days of culture. Responses were more robust in subconfluent cells and were inhibited by 1 μg/ml cycloheximide or 0.1 μg/ml actinomycin D. T3 modulated the stimulatory effect of hydrocortisone on alkaline phosphatase. At low hydrocortisone concentrations (1 n*M*, 10 n*M*), 1 μM T4 resulted in an additive effect, whereas at higher hydrocortisone concentrations (0.1 μM, 1 μM), coincubation with T4 decreased the stimulatory effect (Sato *et al.*, 1987). T3 also increased alkaline phosphatase in MC3T3-E1 cells (Kasono *et al.*, 1988; Klaushofer *et al.*, 1995). In one study, significant responses were elicited with 0.1 n*M* T3 and 10 n*M* T4; the effect of T4 was further increased by concentrations up to 1 μM and was less at 10 μM; the effect of T3, however, was maximal at 1 n*M*, and no increase was observed at 100 n*M* (Kasono *et al.*, 1988). T3 failed to affect alkaline phosphatase in UMR-106 cells (LeBron *et al.*, 1989; Huang *et al.*, 2000), possibly owing to the high basal expression of the enzyme in this cell line. T3 had biphasic effects on alkaline phosphatase in normal rat osteoblastic cells (Ernst and Froesch, 1987), stimulating at concentrations of 0.01 and 0.1 n*M* and inhibiting at a concentration of 10 n*M*. In neonatal rat calvarial cells, mRNA for alkaline phosphatase was decreased by 1 or 4 days exposure to 1 n*M* T3 (Schmid *et al.*, 1992). In cells derived from human trabecular bone explants and cultured in medium containing charcoal-stripped serum, alkaline phosphatase in the cell layer was increased by T3 at concentrations up to 200 n*M* (Kassem *et al.*, 1993). Alkaline phosphatase was also increased by thyroid hormone in isolated tibiae (Stracke *et al.*, 1986) and in primary human (Kassem *et al.*, 1993) and rodent (Egrise *et al.*, 1990) osteoblasts. Thus, most but not all osteoblastic cells respond to thyroid hormones with an increase in alkaline phosphatase. Effects are seen at concentrations in the physiologic range; however, the dose dependence of the effect is quite variable and may be dependent on cell type and culture conditions.

Osteocalcin

T3 increased osteocalcin in a dose-dependent manner in ROS 17/2.8 cells (Rizzoli *et al.*, 1986; Sato *et al.*, 1987). In medium containing 2% T3-depleted serum, a significant effect was seen at 1 n*M*, with a half-maximal effect at 2.5 n*M*; the osteocalcin concentration in the control medium was 9 ng/10^6 cells and was increased to 12.3 ng/10^6 cells by 1 n*M* T3 (Rizzoli *et al.*, 1986). A striking difference in the response of osteocalcin mRNA to T3 was observed between cells derived from femoral and vertebral bone marrow, cultured under conditions leading to osteogenic differentiation (Milne *et al.*, 1998). In cultures from femoral marrow, T3 supplementation (10 or 100 n*M*) prevented the time-dependent decrease in osteocalcin mRNA observed in untreated cells. In cultures from the vertebral marrow, osteocalcin mRNA expression was maintained over time in untreated cells, and T3 failed to augment the response. T3 failed to increase osteocalcin mRNA in the bone marrow-derived stromal cell line ST2 (Varga *et al.*, 2004). T3 stimulation of osteocalcin expression and its inhibition by 1,25(OH)$_2$D$_3$ in murine (MC3T3-E1) osteoblasts were shown to potentially be mediated through a sequence in the mouse OG2 promoter that was able to bind TRα1 and contained a rat VDRE-like sequence (Varga *et al.*, 2003).

Collagen

In cultured rat osteoblastic cells, decreases in collagen and noncollagenous protein synthesis were noted with 0.01 and 0.1 n*M* T3, but not with higher concentrations (Ernst and Froesch, 1987). Thyroid hormones did not inhibit collagen synthesis in rat calvaria (Canalis, 1980). In neonatal mouse calvaria precultured with indomethacin to inhibit prostaglandin synthesis, both collagen and noncollagenous protein synthesis were stimulated by T3 and by triiodothyroacetic acid at concentrations in the 0.01–10 n*M* range (Kawaguchi *et al.*, 1994a). Cells from human trabecular bone explants showed decreased type I procollagen carboxyterminal propeptide production when treated with T3 (Kassem *et al.*, 1993). The synthesis of collagen thus appears to be regulated by T3 in a complex manner and may be influenced by T3 stimulation of other cellular products, such as prostaglandins. Collagen type I gene expression was regulated differentially by T3 in marrow cultures from femoral and vertebral bones, with a more marked stimulatory effect in the femoral bones (Milne *et al.*, 1998).

Other Phenotypic Responses

A series of studies has characterized other changes elicited by T3 in MC3T3-E1 osteoblastic cells (Luegmayr *et al.*, 1996; Fratzel-Zelman *et al.*, 1997; Varga *et al.*, 1997, 1999; Luegmayer *et al.*, 1998, 2000; Fratzel-Zelman *et al.*, 2003). In addition to alkaline phosphatase, expression of c-fos, c-jun, and an osteocalcin-related protein were increased in T3-treated cells. Morphological changes were also observed. T3-treated cells ceased proliferation and became flattened, enlarged, and polygonal. The amount of F-actin increased and the patterns of actin expression were altered. Pancadherin/β catenin immunoprecipitation was increased by T3, which could reflect the organization of adherens junctions. Apoptosis was accelerated. Matrix metalloproteinase-13 was also markedly increased (Fratzl-Zelman *et al.*, 2003). T3 also increased the expression of osteoprotegerin in MC3T3-E1 cells, an effect that was antagonized by 1,25 $(OH)_2D_3$ (Varga *et al*., 2004). In ROS 17/2.8 cells, treatment with T3 increased expression of receptors for PTH: conversely, PTH increased binding of T3 (Gu *et al.*, 2001).

Insulin-like Growth Factors, IGF-Binding Proteins, FGF Receptor

IGF-I has significant anabolic effects on bone, increasing cell replication and both collagen and noncollagen protein synthesis (Canalis, 1980; Hock *et al.*, 1988; McCarthy *et al.*, 1989; Centrella *et al.*, 1990; Pirskanen *et al.*, 1993). IGF-I is increased in fetal rat bones treated with thyroid hormone (Schmid *et al.*, 1992; Lakatos *et al.*, 1993; Varga *et al.*, 1994; Klaushofer *et al.*, 1995). At 1 n*M*, T3 stimulates IGF-I production in neonatal rat calvarial osteoblasts (Schmid *et al.*, 1992). There is a dose-dependent, biphasic effect of T3 and T4 on IGF-I production in UMR-106 cells and fetal rat bone organ cultures (Lakatos *et al.*, 1993). IGF-I mRNA is increased by T3 treatment in MC3T3-E1 cells (Varga *et al.*, 1994; Klaushofer *et al.*, 1995). T3 increased IGF-I expression more markedly in cells from vertebral marrow than in cells from femoral marrow (Milne *et al.*, 1998). Interference with IGF-I action by decreasing expression or function of the IGF-I receptor by the use of antisense oligonucleotides, antibodies, and antagonist peptide decreased the anabolic effects of T3 on MC3T3-E1 cells and primary mouse calvarial osteoblasts, including effects on alkaline phosphatase, osteocalcin, and collagen synthesis (Huang *et al.*, 2000). The effects of thyroid hormones on IGFs may be modulated by changes in IGF-binding proteins (IGFBPs). The physiological role of the binding proteins is not fully understood; however, they can influence the cellular uptake and turnover of IGF-I. The binding proteins may represent a mechanism for retention of IGFs in the bone matrix (Bautista *et al.*, 1991). IGFBPs can also modulate IGF action in osteoblastic cells. IGFBP-2 and IGFBP-4 can inhibit IGF-I actions (Mohan *et al.*, 1989; Feyen *et al.*, 1991). Both enhancing and inhibitory (Schmid *et al.*, 1995) effects are produced by IGFBP-3 (Ernst and Rodan, 1990; Schmid *et al.*, 1991). In rat osteoblasts, T3 stimulates the production of IGFBP-2 and IGFBP-3 (Schmid *et al.*, 1992). T3 increases IGFBP-4 expression in MC3T3-E1 cells (Glantschnig *et al.*, 1996), which could regulate the response to T3 and contribute to the decreased anabolic effects observed at higher concentrations. Alterations in thyroid status *in vivo* influence the expression of IGFBPs in a complex manner. In hyperthyroid rats, IGFBP-3 gene expression in liver is decreased; however, in hypothyroid (propylthiouracil-treated) animals, IGFBP-1 and IGFBP-2 gene expression are increased and IGFBP-3 mRNA is decreased (Rodriguez-Arnao *et al.*, 1993). In hyperthyroid patients, serum IGFBP-3 and IGFBP-4, but not IGFBP-5, are increased (Lakatos *et al.*, 2000).

Fibroblast growth factor may also play an important role in thyroid hormone effects on bone. Treatment of osteoblasts with T3-stimulated expression of fibroblast growth factor receptor-1 (FGFR1) mRNA and protein (Stevens *et al.*, 2003). The effect was inhibited by actinomycin or cycloheximide, implicating an intermediate protein. TRα knockout mice had decreased expression of FGFR1 (Stevens *et al*., 2003).

CELL AND TISSUE PHENOTYPIC RESPONSES

Osteoblast Proliferation

T3 can increase proliferation of rodent and human osteoblastic cells (Ernst and Froesch, 1987; Kassem *et al.*, 1993). In the rodent cell cultures, 0.01 and 1 nM were stimulatory, and 10 nM was inhibitory in longer term cultures (Ernst and Froesch, 1987). Cell number was decreased after eight days of incubation with T4 in MC3T3-E1 cells; inhibition was observed with 10 n*M* T3 and was maximal at 1 μM (Kasono *et al.*, 1988). In other investigations, T3 did not significantly affect growth of ROS 25/1, UMR-106, or ROS 17/2.8 cells (Sato *et al.*, 1987; LeBron *et al.*, 1989; Williams *et al.*, 1994). The diversity of the responses obtained suggests that in addition to thyroid hormone dose, the cell type, passage number, degree of confluence, and the presence or production of other factors can determine the particular outcome that is observed. In explanted neonatal mouse calvaria, T3 stimulated thymidine incorporation in a dose-dependent manner and was significant at 10 pM (Kawaguchi *et al.*, 1994a). A preculture period was required to demonstrate the effect, as high levels of prostaglandin production from untreated tissues appeared to mask treatment effects.

Nodule Formation

Bone nodule formation has been used as a parameter of bone cell differentiation, presumably representing the capability of the cell to generate a mineralized matrix. T3 or T4, at concentrations of 1 nM–0.1 μM, decreased nodule formation by 21-day fetal rat calvarial cells cultured in medium containing 15% heat-inactivated fetal bovine serum, and lower concentrations, starting at 1 pM, had no effect (Ishida *et al.*, 1995). When serum was depleted of T3 with AG-1X-10 resin (Samuels *et al.*, 1979), basal bone nodule formation was increased (Ishida *et al.*, 1995). Dexamethasone (10 nM), enhanced bone nodule formation markedly. This was promoted by low concentrations of T3 (1 and 10 pM) and inhibited by higher concentrations (10 and 100 nM). Although the results suggest that high concentrations of thyroid hormones can inhibit mineralization, the authors point out that the procedure to strip serum of thyroid hormone could remove other inhibitory factors as well.

Osteoclast Activation

Two studies indicate that the resorptive effects of thyroid hormones on bone are mediated indirectly through the stimulation of osteoblasts or other cell types present in bone. T3 failed to activate isolated osteoclasts; however, when mixed bone cells were added to the cultures, a significant response was observed with 1 μM T3, although not with lower concentrations (Allain *et al.,* 1992). UMR-106 cells failed to activate the osteoclasts in the presence of T3, suggesting that a different cell type or a different osteoclast stage might be responsible for the activation observed with the mixed bone cells. In another study, either UMR-106-01 cells or rat calvarial cells were able to activate the osteoclasts (Britto *et al.,* 1994). Responses were detected at lower T3 concentrations in the latter study, perhaps owing to the use of stripped serum.

Resorption

T3 stimulates resorption in bone organ cultures. Fetal rat limb bones (Mundy *et al.,* 1979; Hoffmann *et al.,* 1986; Lakatos and Stern, 1992) and neonatal mouse calvaria (Krieger *et al.,* 1988; Klaushofer *et al.,* 1989; Kawaguchi *et al.,* 1994b) are the models that have been studied most extensively. In both the limb bones and calvaria, responses to T3 are slower to develop than the effects of PTH (Mundy *et al.,* 1979; Klaushofer *et al.,* 1989; Kawaguchi *et al.,* 1994b) and the dose-response curves are generally shallow (Mundy *et al.,* 1979; Hoffmann *et al.,* 1986; Krieger *et al.,* 1988). Higher doses of thyroid hormones *in vitro* can have inhibitory effects on resorption (Orbai and Gazariu, 1982). One of the most striking differences from the effects of PTH is that the maximal responses to thyroid hormones are lower (Mundy *et al.,* 1979; Lakatos and Stern, 1992; Kawaguchi *et al.,* 1994b), sometimes only about 50% of those elicited with maximal concentrations of PTH. The slower responses and lower efficacy of thyroid hormones compared with PTH may be the basis for the observation that thyroxin does not exhibit "escape" from the inhibitory effects of calcitonin (Krieger *et al.,* 1987; Klaushofer *et al.,* 1989). Alternatively, this may reflect a different mechanism for the direct effect of thyroid hormones compared with PTH. Further evidence for such a difference between the mechanism of T3 and PTH responses is the contrast in their interaction with TGFβ (Lakatos and Stern, 1992). TGFβ enhanced the early responses to PTH and inhibited the later effects, whereas the interaction with T3 displayed a somewhat reverse time course. A range of threshold concentrations was observed for both T3 and T4 in the different studies, with no clear basis in terms of the composition of the medium. 3,5,3′-Triiodothyroacetic acid, an analog that binds to nuclear receptors, especially β forms, with higher affinity than T3, was a more potent stimulator of resorption than T3 (Kawaguchi *et al.,* 1994b). In cultured fetal bones, T3 increases collagen degradation (Halme *et al.,* 1972). T3 increased mRNA for the metalloproteinases collagenase-3 and gelatinase B in cultures of osteoblastic cells, effects that were not inhibited by indomethacin (Pereira *et al.,* 1999).

Several mechanisms may mediate thyroid hormone-stimulated resorption. In neonatal mouse calvaria, resorption was inhibited by indomethacin, implicating a prostaglandin-dependent pathway (Krieger *et al.,* 1988; Klaushofer *et al.,* 1989; Kawaguchi *et al.,* 1994b). Other studies have shown prostaglandin-independent effects on the calvaria (Conaway *et al.,* 1998). In fetal rat limb bones, the T3 effect is not affected by indomethacin. However, in limb bone cultures, T3 potentiates the bone-resorbing effect of IL-1 (Tarjan and Stern, 1995), and the effect of IL-1 and the IL-1/T3 combination is sensitive to indomethacin. T3 also potentiates the IL-1-mediated production of IL-6 in this model (Tarjan and Stern, 1995), as well as in MC3T3-E1 osteoblastic cells (Tokuda *et al.,* 1998) and in bone marrow stromal cells (Kim *et al.,* 1999). In contrast, in MC3T3-E1 cells, T3 reduced the IL-6 production elicited by prostaglandin, cholera toxin, and forskolin, possibly reflecting crosstalk through effects on a cAMP pathway (Tokuda *et al.,* 1998). In mouse bone marrow cultures, T3 promoted calcitriol-induced osteoclast formation through an IL-6-dependent pathway (Schiller *et al.,* 1998). T3 also increased IL-6 production in MG-63 cells and human bone marrow stromal cells (Siddiqi *et al.,* 1999). These findings suggest that the indirect stimulation of osteoclast differentiation by IL-6 may be a component of the resorptive effect of thyroid hormone. The IL-1 receptor antagonist protein did not prevent the resorptive effect of thyroid hormones in limb bone cultures (Kawaguchi *et al.,* 1994b). Thyroid hormone effects on resorption were blocked by aphidicolin or cortisol (Kawaguchi *et al.,* 1994b) and by hydroxyurea (Conaway *et al.,* 1998), indicating the involvement of cell replication. Immunosuppressive cyclosporins blocked the thyroid hormone effects in limb bone cultures (Lakatos and Stern, 1992), and interferon-β (Klaushofer *et al.,* 1989) and the antibody to TGFβ (Klaushofer *et al.,* 1995) blocked the thyroid hormone effects in calvaria, consistent with the participation of other local factors in the resorptive response to thyroid hormone.

Remodeling

Most *in vitro* studies have focused on either anabolic or catabolic effects of thyroid hormone, under conditions designed to optimize the study of the particular response. However, because there are dose-dependent biphasic effects on formation parameters and delayed (Klaushofer *et al.,* 1989) and submaximal (Mundy *et al.,* 1979; Krieger *et al.,* 1988; Lakatos and Stern, 1992) effects on resorption, it may be that neither effect can be studied to the exclusion of the other, and the net effects on bone remodeling may be accessible to *in vitro* investigation. A model system designed to study growth, mineralization, and resorption in radii and ulnae of 16-day fetal mice (Soskolne *et al.,* 1990) revealed interesting differences between effects of T3 and PTH. Effects of T3 were studied over a 0.1 nM–10 μM dose range. T3 concentrations in the 10 nM–0.3 μM range resulted in increases in diaphyseal length, in calcium, phosphate, and hydroxyproline content, and in decreases in ^{45}Ca release. At higher concentrations (1 and 10 μM), T3 stimulated ^{45}Ca release. In contrast, when PTH was studied over a 1 pM–0.1 μM range, only resorptive effects were observed, these being at concentrations of 1 nM and higher.

Chondrocyte Responses

Thyroid hormones block clonal expansion of the proliferative cell layer of the epiphyseal growth plate and promote chondrocyte maturation (Nilsson *et al.,* 1994). In earlier studies on isolated cells, T3 was found to inhibit chondrocyte proliferation (Burch *et al.,* 1987). T3 suppressed the synthesis of DNA, protein, and type II collagen when added to rapidly proliferating chicken growth plate chondrocytes cultured in serum-free media (Ishikawa *et al.,* 1998). When T4 was added to a chemically defined medium containing insulin and growth hormone, there were dose-dependent increases in type X collagen and in alkaline phosphatase in chondrocytes (Ballock and Reddi, 1994). T3 was approximately 50 times more potent than T4 in promoting expression of the hypertrophic markers in prehypertrophic chondrocytes in cells cultured with insulin/transferrin/selenium (Alini *et al.,* 1996). There was a biphasic dose dependency of the effects of T3 and T4 to stimulate the synthesis of type II collagen and chondroitin sulfate-rich proteoglycans in cultured rabbit articular chondrocytes (Glade *et al.,* 1994). In an *in vitro* model of cartilage formation from a chondrocyte pellet, the developing cartilage assumed the structural architecture of the normal epiphysis if thyroid hormones were present, whereas the structure was random in their absence (Ballock and Reddi, 1994). In serum-free long-term micromass cultures of chick embryonic limb mesenchyme in which the sequence of chondrogenesis, chondrocyte maturation, hypertrophy mineralization, and apoptosis can be reproduced T3 inhibited proliferation and stimulated alkaline phosphatase and hypertrophy and promoted apoptosis (Mello and Tuan, 2006). TGFβ1 or serum disrupted the appearance of columnar organization of chondrocytes in control or T3-treated cultures. Findings from cocultures of vascular endothelial cells and chondrocytes suggest that vascular endothelial cells may produce factors that act synergistically with thyroid hormone to derepress the late differentiation of resting chrondrocytes and permit them to become hypertrophic and express type X collagen and alkaline phosphatase (Bittner *et al.,* 1998), leading to mineralization. The antiproliferative and differentiation effects of T3 on primary chondrocytes from mouse rib were shown to be mediated by the TRβ receptor isoform, but not the TRα isoform (Rabier *et al.,* 2006).

IN VIVO RESPONSES OF THE SKELETON TO THYROID HORMONES AND TSH: ANIMAL STUDIES

When thyroid hormones are administered to young rats, bone growth is enhanced (Glasscock and Nicoll, 1981). This response is not seen in older rats, suggesting that the stage of cellular differentiation or the environment in terms of other hormones and local factors can influence the manifestation of thyroid hormone responses. T3 treatment of neonatal rats elicited a narrowing of the sagittal suture and increased mineral apposition rates at the osseous edges of the sutures (Akita *et al.,* 1994). Histomorphometric analysis was consistent with the conclusion that T3 is critical for bone remodeling (Allain *et al.,* 1995). *In vivo* effects of TSH were investigated in ovariectomized Sprague-Dawley rats (Sampath *et al.,* 2007). Rat TSH prevented the bone loss and resulted in improvements in bone mineral density and histomorphological measurements. For many of the parameters, maximal effects were seen at the lowest dose administered (0.1 ug). Responses to TSH were seen in the absence of increases in T3 or T4, and with long-term (up to 6-week) treatment, thyroid hormone concentrations were decreased in some groups receiving the higher TSH doses.

Hypothyroidism

Animal models of hypothyroidism include the use of the antithyroid agents propylthiouracil or methimazole to block the synthesis of thyroid hormones. Treatment of young rats with methimazole for seven weeks resulted in a marked increase in trabecular bone volume of the subchondral spongiosa of the mandibular condyles and a decrease in cartilage cellularity (Lewinson *et al.,* 1994). IGF-I was present in the condyles of control rats, but lacking in hypothyroid rats. Replacement of T4 during the last two weeks of treatment restored the parameters to normal (Lewinson *et al.,* 1994). Histomorphometric studies in iliac crest biopsies of young rats made hypothyroid by a 12-week treatment with propylthiouracil showed that both osteoid surfaces and eroded surfaces were reduced and cancellous bone volume was increased (Allain *et al.,* 1995). In a study in which 21-day rats were made hypothyroid by administration of methimazole, T4 given daily at doses of 2 to 64 μg/kg/day for 21 days elicited biphasic effects on epiphyseal growth plate width and logitudinal growth rate (Ren *et al.,* 1990). The dose-response curve paralleled that of serum IGF-I concentrations, which were postulated to contribute to the growth responses (Ren *et al.,* 1990).

An interesting animal model for hypothyroidism utilizes transgenic mice (line TG66-19) in which the bovine thyroglobulin promoter drives the expression of the herpes simplex type I virus thymidine kinase gene in thyrocytes. This enzyme converts ganciclovir to ganciclovir-5′-phosphate, which inhibits DNA replication, resulting in loss of thyrocytes, loss of follicles, and undetectable T3 and T4; levels of PTH and CT are unaffected (Wallace *et al.,* 1991, 1995). In this transgenic mouse model, administration of 15 or 50 μg of ganciclovir to mouse dams during days 14–18 of gestation resulted in growth delay in pups carrying the transgene (Wallace *et al.,* 1995). The authors point out that the reason their effects were more dramatic than those obtained with the *hyt/hyt* mouse, a strain that has an inactivating mutation in the TSH receptor, is that in the latter model, circulating T4 is still 10–20% of normal (Adams *et al.,* 1989). Effects of mutations in thyroid hormone receptors in mouse models were discussed earlier.

Hyperthyroidism

A range of T4 regimens has been used to elicit hyperthyroidism in animal models. The duration of treatment is generally at least three weeks and dosages range from 200 μg to 1 g/day. Lower concentrations have been used in animals previously made hypothyroid with antithyroid drugs (Lewinson *et al.,* 1994). When the animals were rendered hyperthyroid by treatment with T4 (200 μg/day for 12 weeks), the mineral apposition rate and the mineral formation rate were increased markedly, with a smaller increase in eroded surfaces (Allain *et al.,* 1995). A greater sensitivity of cortical bone (femur) than trabecular bone (spine) to thyroid hormone-induced bone loss has been noted in animal models of hyperthyroidism (Ongphiphadhanakul *et al.,* 1993; Suwanwalaikorn *et al.,* 1996, 1997; Gouveia *et al.,* 1997; Zeni *et al.,* 2000). Tooth movement was greater in T3-treated rats undergoing orthodontic procedures than in control untreated animals, probably reflecting greater root resorption (Shirazi *et al.,* 1999). Ten-day-old rats treated with 100 μg/kg/day for up to 60 days displayed altered parameters of cranial width, narrowing of the suture gap of the sagittal suture, and intense immunohistochemical staining for IGF-I along the suture margins, consistent with the possibility that local IGF-I is involved in the effect of thyroid hormone to cause premature suture closure (Akita *et al.,* 1996).

Ovariectomized rats treated with a low dose of T4 (30 μg/kg/day for 12 weeks) showed increased bone turnover and decreased bone density compared with controls; however, in the presence of 17β-estradiol, their bone mass and mineral apposition rate were greater than those of controls (Yamaura *et al.,* 1994). T4, (250 μg/kg/day for five weeks) increased serum osteocalcin and urinary pyridinolines and produced a greater loss of bone mineral compared with either ovariectomy alone or T4 alone (Zeni *et al.,* 2000). In contrast to the effects of these high doses of T4, administration of a more physiological concentration (10 μg/kg/day) to ovariectomized rats resulted in a generalized increase in bone mineral density at both lumbar and vertebral sites (Gouveia *et al.,* 1997). Estradiol prevented T3-stimulated decreases in bone mineral density in ovariectomized thyroidectomized rats, but had no effect in animals that were not treated with T3 (DiPippo *et al.,* 1995). These results raise the possibility of crosstalk at the level of binding of estradiol and T3 receptors to DNA target sites.

PATHOPHYSIOLOGICAL EFFECTS OF ALTERED THYROID HORMONE STATUS IN HUMANS

Hypothyroidism

Bone turnover is decreased in hypothyroidism (Mosekilde and Melson, 1978). In juvenile hypothyroidism, there is delayed skeletal maturation and epiphysial dysgenesis. In a study of children with congenital hypothyroidism treated with T4, the bone age at 1.5 years was correlated positively with the dose of T4 administered during the first year and with the concentrations of serum T4 (Heyerdahl *et al.,* 1994). As discussed previously, multiple skeletal abnormalities have been described in syndromes of RTH, including short stature, delayed skeletal maturation, and stippled epiphyses (Refetoff *et al.,* 1993). Serum IGF-I is lower in hypothyroid patients (Lakatos *et al.,* 2000). Bone resorption is decreased in patients with hypothyroidism, as indicated by reduced urinary pyridinium cross-links (Nakamura *et al.,* 1996).

Hyperthyroidism

Since the initial description of bone loss in thyrotoxicosis by Von Recklinghausen (1891) more than a century ago, substantial additional evidence has shown that excessive thyroid hormone production can lead to bone loss. In patients with hyperthyroidism, markers of bone turnover are increased. Pyridinoline and hydroxypyridinoline cross-link excretion are elevated (Harvey *et al.,* 1991; Garnero *et al.,* 1994; Nagasaka *et al.,* 1997; Engler *et al.,* 1999), as are urinary N-terminal telopeptide of type I collagen (NTX) (Mora *et al.,* 1999; Pantazi *et al.,* 2000) and serum carboxyterminal-1-telopeptide (ICTP) (Loviselli *et al.,* 1997; Miyakawa *et al.,* 1996; Nagasaka *et al.,* 1997). Evidence of activation of osteoblasts in hyperthyroidism is the elevation of alkaline phosphatase (Mosekilde and Christensen, 1977; Cooper *et al.,* 1979; Martinez *et al.,* 1986; Nagasaka *et al.,* 1997; Pantazi *et al.,* 2000), osteocalcin (Martinez *et al.,* 1986; Lee *et al.,* 1990; Mosekilde *et al.,* 1990; Nagasaka *et al.,* 1997; Loviselli *et al.,* 1997; Pantazi *et al.,* 2000), and carboxyterminal propeptide of type I procollagen (PICP) (Nagasaka *et al.,* 1997). Osteocalcin showed a better correlation than alkaline phosphatase with thyroid hormone concentrations (Martinez *et al.,* 1986; Garnero *et al.,* 1994). Greater increases in the resorption markers than the formation markers suggest an imbalance between resorption and formation, leading to bone loss (Garnero *et al.,* 1994; Miyakawa *et al.,* 1996). Histomorphometric analyses show increased osteoclast numbers and resorbing surfaces, with loss of trabecular bone volume (Meunier *et al.,* 1972; Mosekilde and Melsen, 1978). Histomorphometric data yield a kinetic model demonstrating accelerated bone remodeling, with a disproportionately greater increase in resorption and a net loss of bone with each cycle of remodeling (Eriksen, 1986). Decreased bone mineral content in hyperthyroidism is well documented (Fraser *et al.,* 1971; Krolner *et al.,* 1983; Toh *et al.,* 1985), and fracture risk is increased in hyperthyroidism (Fraser *et al.,* 1971; Cummings *et al.,* 1995; Wejda *et al.,* 1995; Vestergaard *et al.,* 2000a). Mild hyperthyroidism may increase bone loss in postmenopausal women (Lakatos *et al.,* 1986). In children, however, thyrotoxicosis can lead to acceleration of growth and skeletal development (Schlesinger and Fisher, 1951; Saggese *et al.,* 1990). A study of Japanese male patients with Graves' disease showed significant prevalence of cortical bone loss, which was more marked in older patients (Majima *et al*., 2006a). There was a negative correlation between bone mineral density and TSH receptor autoantibodies In view of the proposed role of TSH on bone, studies of the bone effects of the TSH receptor autoantibodies could be interesting.

Stimulation of the production of local factors by thyroid hormone, which was observed *in vitro* and animal studies, is also seen in humans. Thyroid hormone increased circulating IL-6 (Lakatos *et al.,* 1997; Siddiqi *et al.,* 1999). A lack of correlation between IL-6 and remodeling markers was noted in a study of hyperthyroid patients (Akalin *et al*., 2002). In patients with Graves' disease, but not in patients with nontoxic nodular goiter, there was a correlation between TSH receptor autoantibodies and serum IL-6 (Bossowski and Urban, 2001). However, another study did not find a correlation (Salvi, 2000). Thyroid hormone increases IGF-I (Brixen *et al.,* 1995; Kassem *et al.,* 1998; Foldes *et al.,* 1997; Lakatos *et al.,* 2000). One can speculate that the greater rate of production of IGF-I in children could explain the findings of studies in which large doses of thyroid hormone were not deleterious to bone in children (Kooh *et al.,* 1996; Verrotti *et al.,* 1998; Dickerman *et al.,* 1997; Leger *et al.,* 1997; van Vleit *et al.,* 1999; Turner *et al.,* 1999). The anabolic effect of the increased IGF-I could compensate for or override the bone breakdown. Studies have shown effects of thyroid hormones and TSH on OPG. The mechanism and role of this response are not yet clear. Consistent with the molecular studies (Varga *et al*., 2004) showing that T3 increases OPG in osteoblastic cells, elevated OPG has been reported in patients with Graves' disease (Amato *et al*., 2004; Mochizuki *et al*., 2006). In the Amato study, the elevated OPG correlated with free T3 but not free T4. In the Mochizuki study, antithyroid treatment decreased the elevated OPG and this change correlated with free T4; the correlation of OPG with free T3 was close to statistical significance, and there was no correlation with TR antibodies. The possibility that the increase in OPG has a counterregulatory function to protect against excessive turnover stimulated by thyroid hormone was proposed (Amato *et al*., 2004). TSH was found to inhibit RANKL and upregulate OPG production in the thyroid gland (Hofbauer *et al*., 2002).

Several reviews (Greenspan and Greenspan, 1999; Lakatos, 2003) provide useful overviews of the bone effects of overt and subclinical hyperthyroidism and exogenous thyroid hormone treatment at suppressive and lower doses. The studies cited indicate that the hyperthyroid condition can result in bone loss and fracture risk and that subclinical hyperthyroidism could be an additional risk factor for postmenopausal women.

T4 Therapy and Bone Loss

A particularly critical issue regarding the effects of thyroid hormone on the skeleton is the question of what amounts of exogenously administered thyroid hormones increase the risk of bone loss, especially among individuals already at risk for osteoporotic fractures from other causes. Thyroid hormones are given as replacement therapy for hypothyroidism after thyroidectomy, as well as in other states where patients may have inadequate thyroid hormone secretion and goiter, such as autoimmune thyroiditis. Thyroid hormones are also used as suppressive therapy for toxic nodular goiter or for thyroid cancer. There may be patients who used excess thyroid hormones in the past for weight loss or as a tonic.

There have been, and continue to be diverse clinical findings on the skeletal effects of treatment with suppressant doses of thyroid hormones. Decreased bone density, accelerated bone turnover, and increased risk of fracture in patients treated with T4 have been documented extensively (Fallon *et al.,* 1983; Coindre *et al.,* 1986; Ross *et al.,* 1987; Paul *et al.,* 1988; Stall *et al.,* 1990; Taelman *et al.,* 1990; Adlin *et al.,* 1991; Diamond *et al.,* 1991; Greenspan *et al.,* 1991; Lehmke *et al.,* 1992; Frevert *et al.,* 1994; Garton *et al.,* 1994; Grant *et al.,* 1995; McDermott *et al.,* 1995; Campbell *et al.,* 1996; Affinito *et al.,* 1996; Jodar *et al.,* 1998; Hadji *et al.,* 2000). Several studies and

a meta-analysis (Marcocci *et al.,* 1994; Uzzan *et al.,* 1996; Greenspan and Greenspan, 1999; Campbell *et al.,* 1996; Affinito *et al.,* 1996) concluded that the dose of T4 and duration of treatment are major determinants of the occurrence of bone loss. Other factors that appear to amplify the risk include a previous history of hyperthyroidism (Grant *et al.,* 1995), age (Duncan *et al.,* 1994), and postmenopausal status (Greenspan *et al.,* 1991; Stepan and Limanova, 1992; Franklyn *et al.,* 1994; Garton *et al.,* 1994; Affinito *et al.,* 1996; Jodar *et al.,* 1998). It has been suggested that a low dietary calcium intake can contribute to the risk of T4-induced bone loss (Kung *et al.,* 1983). Estrogen and HRT protected against the bone loss associated with T4 treatment (Schneider *et al.,* 1994; Franklyn *et al.,* 1995). Consistent with the importance of dose and duration, TSH has been a useful predictive marker for bone loss (Wartofsky, 1991). A meta-analysis of 13 publications in which TSH was suppressed by thyroid hormone treatment projected that a premenopausal woman at an average age of 39.6 years, treated with L-T4, (164 μg/day for 8.5 years), would have an excess annual bone loss of 0.31% and 2.67% less bone mass than a control (Faber and Galloe, 1994).

In contrast with these findings, a number of studies report that T4 treatment failed to produce bone loss (Toh and Brown, 1990; Ribot *et al.,* 1990; Franklyn and Sheppard, 1992; Grant *et al.,* 1993; Fujiyama *et al.,* 1995; Hawkins *et al.,* 1994; Schneider *et al.,* 1995; DeRosa *et al.,* 1995; Saggase *et al.,* 1990, 1997; Marcocci *et al.,* 1997; Gurlek and Gedik, 1999; Rachedi *et al.,* 1999; Nuzzo *et al.,* 1998; Knudsen *et al.,* 1998; Hanna *et al.* 1998; Langdahl *et al.,* 1996a). One explanation for this apparent disparity, in the case of patients receiving T4 replacement therapy, is that their cortical bone density was initially higher owing to their hypothyroidism and that T4 replacement resulted in normalization (Ross, 2000). A longitudinal study would indicate bone loss, whereas a cross-sectional study would not reveal a significant difference from the control group. Another possible basis for some of the diversity of findings is that the accelerated bone turnover and increased fracture risk with T4 treatment can be a transient phenomenon. In one report, correlations between serum-free T4 and serum procollagen III peptide, which had been noted after six months of T4 treatment, were not found in patients treated chronically (Nystrom *et al.,* 1989). There may be an initial increase in cortical width and bone porosity that results in increased fracture risk until a new steady-state condition is established (Coindre *et al.,* 1986). Another study found a temporary increase in fracture risk in previously hypothyroid patients, which was most prevalent in patients over 50 years of age and limited to forearm fractures (Vestergaard *et al.,* 2000b). Studies published since the previous version of this chapter indicate that the issue of bone risk of suppressive therapy is still unclear. Some studies show no significant deleterious effect of treatment (e.g., Mikosch *et al.,* 2001, Reverter *et al.,* 2005). Close monitoring of TSH may allow suppressive treatment without risk to bone. A three-year study in women with benign nodular goiter showed that doses of LT4 that reduced TSH to below the lower limit of normal but maintained it above above 0.005 μU/ml and maintained normal free T3 and free T4 had no effect on BMD or serum markers of bone turnover in pre-post-menopausal women (Appetecchia, 2005). A recent review of 21 studies of TSH-suppressive thyroid hormone therapy on bone metabolism in patients with well-differentiated thyroid carcinoma concluded that postmenopausal women with DTC receiving TSH-suppressive therapy were at high risk for bone loss whereas premenopausal women and men did not share this risk (Heemstra *et al.*, 2006). OPG was elevated in patients receiving suppressive therapy for differentiated thyroid cancer (Mikosch *et al.*, 2006). A study in patients being monitored for thyroid carcinoma, all of whom had subclinical thyrotoxicosis as a consequence of T4 treted, tested the effects of short-term stimulation with recombinant human TSH on skeletal remodeling (Mazziotti *et al.*, 2005). Two intramuscular doses of 0.9 mg TSH were administered. Measurements made two days and one week after the treatment showed decreases in serum c-telopeptides of type-1 collagen (CrossLaps). in patients who were postmenopausal. Bone alkaline phosphatase was increased, and there was no significant change in OPG. The conclusion was that TSH inhibited bone resorption, but that it did not regulate OPG in bone.

Reversibility/Treatment/Prevention of Thyroid Hormone-Stimulated Bone Loss

Recovery of bone loss in hyperthyroid patients following antithyroid treatment has been inconsistent (Fraser *et al.,* 1971; Toh *et al.,* 1985; Saggese *et al.,* 1990; Diamond *et al.,* 1994; Mudde *et al.,* 1994, Oikawa *et al.,* 1999, Kumeda *et al.,* 2000, Barsal *et al.,* 2004), but may be achieved more readily in younger individuals (Fraser *et al.,* 1971; Saggese *et al.,* 1990). Studies have documented protective effects of methimazole (Langdahl *et al.,* 1996b; Nagasaka *et al.,* 1997; Mora *et al.,* 1999). Surgery and radioactive iodine also prevented bone loss in hyperthyroid patients (Langdahl *et al.,* 1996c; Arata *et al.,* 1997), but were less protective than methimazole (Vestergaard *et al.,* 2000a). The protective effects of estrogen during treatment with T4 were noted earlier, and androgen may also be beneficial (Lakatos *et al.,* 1989). Calcium and calcitonin were also found to offer some protective benefit (Kung and Yeung, 1996).

Bisphosphonates may protect against thyroid hormone-induced bone loss. Both animal (Ongphiphadhanakul *et al.,* 1993; Rosen *et al.,* 1993a; Yamamoto *et al.,* 1993; Kung and Ng, 1994) and human (Rosen *et al.,* 1993b; Lupoli *et al.,* 1996) studies have demonstrated that bisphosphonates are effective in preventing thyroid hormone-stimulated bone loss. Etidronate (0.5 mg/100 g administered twice weekly) prevented decreased bone mineral density and increased mRNA for alkaline phosphatase, tartrate-resistant acid phosphatase, and histone H4 in femurs of rats treated with L-T4 for 20 days (Ongphiphadhanakul *et al.*, 1993). The combination of L-T4 and etidronate resulted in lower expression of mRNA for type I collagen, osteocalcin, and osteopontin, which was lower than that of controls, although neither L-T4 nor etidronate alone affected these parameters. Alendronate (1.75 mg/kg orally twice weekly) prevented increased bone turnover resulting from the administration of excess T4 for three weeks (Lupoli *et al.*, 1996). The preventive effect was assessed by histomorphometry and measurement of osteocalcin. Bone volume was above control in all alendronate-treated groups in the study. Pretreatment of rats with pamidronate (5 μmol/kg/day subcutaneously for one week prior to T3) prevented increases in alkaline phosphatase and osteocalcin at one week and losses of bone mineral density at three weeks in the femur and spine (Rosen *et al.*, 1993b). Pamidronate pretreatment (30 mg IV, daily for two days) prevented increases in urinary calcium/creatinine ratio, urinary hydroxyproline, and urinary pyridinoline cross-links in normal male subjects treated with T3 (Rosen *et al.*, 1993a). The addition of risedronate, 2.5 mg/day to a methimazole regimen for 6 or 12 months improved bone mineral density at the lumbar spine and distal radius and reduced turnover markers in a study in male patients with newly diagnosed Graves' disease (Majima *et al.*, 2006b).

OVERVIEW, SPECULATIONS, AND FUTURE DIRECTIONS

Thyroid hormones act at receptor, cellular, and organismal levels to modulate or interact with many of the other factors and pathways that determine the status of the skeleton. There are interactions with both nuclear receptors and membrane-binding sites in bone cells and effects on many of the phenotypic responses of bone cells. The relative importance of the different nuclear receptor isoforms for bone responses and the mechanisms of this are now clearer. The responses mediated by the membrane-binding sites are still not defined. It is clear that thyroid hormones have importance in normal skeletal physiology, because deficiency or excess of thyroid hormone can alter skeletal development and maintenance. There is a dose dependence to the effects, with anabolic effects declining and catabolic effects becoming more prominent with the higher concentrations of thyroid hormone present in hyperthyroidism and with the higher doses that are used for suppressive therapy. Determination of TSH to guide thyroid hormone dosage can decrease the occurrence of bone loss resulting from the therapeutic use of thyroid hormone. However, factors other than dosage can influence the response to thyroid hormone. The anabolic effects of thyroid hormone on bone are more apparent in younger animals and children, consistent with the possibility that growth factors can have significant mediating or modulating effects. Thyroid hormones increase IGF-I in osteoblasts and experimental animals; elevated circulating thyroid hormones are associated with increases in IGF-I and IGFBPs in patients and other growth factors, including FGF, are also likely to be involved. Other physiological factors modulate the skeletal effects of thyroid hormones, e.g., estrogens can decrease the deleterious effects of excess thyroid hormone. The pharmacological inhibition of bone resorption with bisphosphonates can also diminish thyroid hormone-stimulated bone loss. Thyroid hormones stimulate osteoblast proliferation, promote the differentiation of this cell type, and stimulate differentiated functions, as shown by increases in alkaline phosphatase activity, osteocalcin expression, and stimulation of collagen synthesis. The variation in the responses when different model systems are used indicates that there are additional modulating factors that are not yet understood. The osteoblast is the target cell for thyroid hormone activation of mature osteoclasts. The promotion of resorption by thyroid hormones may be mediated through the activation of cytokine pathways that lead to osteoclast differentiation. The role of the thyroid hormone-stimulated increase in osteoprotegerin is an interesting and open question. TSH as an effector of responses in bone will undoubtedly be the topic of further basic and clinical investigations. Even as more information is gained regarding the actions of thyroid hormone, there are continuing and new unanswered questions.

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Chapter 44

Clinical and Basic Aspects of Glucocorticoid Action in Bone

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INTRODUCTION

Glucocorticoid-induced osteoporosis (GIOP) was first reported by Cushing when he described osteoporosis in patients with high levels of cortisol owing to an adrenocorticotrophin-producing tumor of the pituitary gland (Cushing, 1932). The problem became clinically significant in 1949 when pharmacological doses of glucocorticoids were introduced for therapeutic use because of their potent anti-inflammatory and immunosuppressive effects. It became clear that treatment with glucocorticoids causes a loss of bone mass and pathological fractures. Since then, efforts have been made to elucidate the cause of steroid-induced bone loss.

Cortisol, the glucocorticoid secreted by the adrenal gland, is essential in physiological doses for the differentiation and function of osteoblasts and osteoclasts, and it modulates the effects of other hormones and mediators of cell function, even though supraphysiological doses inhibit bone formation. These direct effects on bone, combined with effects on other systems that indirectly regulate bone metabolism, cause rapid bone loss in patients treated with glucocorticoids. The mechanisms involved and the resulting clinical picture are the subjects of this chapter.

Pharmacology of Glucocorticoids

Synthetic derivatives of cortisol with less mineralocorticoid effect have been developed. The compounds most frequently prescribed are prednisone, prednisolone, methylprednisolone, betamethasone, dexamethasone, and triamcinolone. Prednisone is metabolized to prednisolone. The 4,5-double bond and the 3-ketone structures are both necessary for typical adrenocorticoid activity. Introduction of 1,2-double bond, as in prednisone or prednisolone, enhances the ratio of carbohydrate-regulating potency to sodium-retaining potency. 6α-Methylation of the B ring (6α-methylprednisolone) increases anti-inflammatory potency while reducing electrolyte-retaining properties. 9α-Fluorination enhances all biological activities, whereas 16-methylation eliminates the sodium-retaining effect, but only slightly alters other effects on metabolism or inflammation. Substitution in the 17α-ester position produces a group of extremely potent steroids, beclomethasone dipropionate and budesonide, which are effective when applied topically to skin or administered by inhalation (Gilman *et al.*, 1990). The absorption of inhaled steroids is virtually equivalent to that of oral administration and absorption from skin is significant if applied over a large surface or under plastic film. Despite this, inhaled or topical steroids reduce side effects because the drugs are targeted to the site of the disease and lower doses can be used. Deflazacort, an oxazoline derivative of prednisone, has been developed with the hope of reducing the catabolic effects of glucocorticoids while maintaining anti-inflammatory effects (Gennari *et al.*, 1984), but the results have been disappointing. The recent development of synthetic glucocorticoids that dissociate transactivation and AP-1 transrepression and yet exhibit anti-inflammatory activity holds promise for the development of glucocorticoid-based drugs that separate beneficial from deleterious effects.

Glucocorticoids are widely used in the treatment of asthma, collagen-vascular disease, inflammatory bowel disease, and granulomatous and skin diseases. The skeletal response to glucocorticoids is not disease specific, and accelerated bone loss has been described in patients with each of these diseases when they are treated with steroids (de Deuxchaisnes *et al.*, 1984; Reid *et al.*, 1986a; Rizzato *et al.*, 1988).

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Characteristics of Bone Loss and Fracture Risk

Bone loss, measured by dual-energy x-ray absorptiometry in patients receiving glucocorticoids for more than a year, has been reported to average 0.6% to 6% per year (Laan *et al.*, 1993; Lukert *et al.*, 1992; Sambrook *et al.*, 1990). Trabecular bone and the cortical rim of the vertebral body are more susceptible to the effects of glucocorticoids than the cortical bone of the extremities (Laan *et al.*, 1993; Seeman *et al.*, 1982). Consequently, compression fractures of the spine are frequently the first sign of glucocorticoid-induced bone loss, and the proximal femur becomes more fragile. Although bone loss appears to be most rapid during the first 6 to 12 months of treatment, loss remains above average for the duration of treatment (Gennari, 1985; Lukert *et al.*, 1992). The risk for hip fracture is doubled and the risk for vertebral fracture is increased by 5-fold by oral doses of prednisone exceeding 7.5 mg/day (Van Staa *et al.*, 2000). The risk for fracture increases within the first 3 months after the initiation of glucocorticoid therapy and decreases within 3 months after discontinuation. Approximately 30% to 50% of patients taking glucocorticoids long term and 50% of patients with Cushing's disease (excessive endogenous production of steroids) have at least one atraumatic fracture (Adinoff *et al.*, 1983; Ross *et al.*, 1982). In patients who have been exposed to the equivalent of prednisone 10 mg/day for longer than 90 days, the risk of hip and spine fractures is increased by 7- and 17-fold, respectively, even in patients taking glucocorticoids intermittently (Steinbuch *et al.*, 2004). Vertebral compression fractures are commonly asymptomatic. In a cross-sectional study, 37% of postmenopausal women taking glucocorticoids for more than 6 months showed morphometric changes in vertebral bodies without symptoms (Angeli *et al.*, 2006). The fracture threshold for vertebral fractures appears to be higher for patients taking steroids than for those with involutional osteoporosis (Luengo *et al.*, 1990; Van Staa *et al.*, 2000); that is, fractures occur at a higher bone density in steroid-treated patients. Quantitative computed tomography (QCT) studies of the hip have shown lower integral, cortical, and trabecular bone mineral density (BMD) and cortical volume in glucocorticoid-treated postmenopausal women than in postmenopausal controls. These findings may partially explain why individuals treated with glucocorticoids have increased risk for hip fracture than age-matched controls (Lian *et al.*, 2005).

Glucocorticoid-induced bone loss is partially reversible after cessation of prednisone administration or removal of the cause of excessive endogenous production of cortisol (Laan *et al.*, 1993; Manning *et al.*, 1992; Rizzato *et al.*, 1993; Van Staa *et al.*, 2000). Bone loss is also partially reversible during treatment with estrogen/progesterone therapy, bisphosphonates, calcitonin, parathyroid hormone, or sodium fluoride, whereas prednisone is continued (Lane *et al.*, 2000; Luengo *et al.*, 1990; Lukert *et al.*, 1992; Meunier *et al.*, 1987; Reid *et al.*, 2000; Saag *et al.*, 1998; Struys *et al.*, 1995).

Histomorphometric studies on bone from glucocorticoid-treated patients show that glucocorticoids cause apoptosis of osteoblasts and osteocytes, and depress osteoblastic function, whereas, at the same time, the frequency of activation of bone remodeling units is increased. Thus, there is an increase in the number of sites at which bone is being resorbed, and the ability of osteoblasts to replace bone at each site is decreased. This results in reduced wall thickness of cancellous bone packets, and eventually, to perforation and removal of trabecular plates (Bressot *et al.*, 1979; Dempster, 1989; Meunier *et al.*, 1982; Plotkin *et al.*, 1999; Weinstein *et al.*, 1998).

Bio-markers of Bone Remodeling

Serum levels of osteocalcin, the most abundant noncollagen bone matrix protein and a biochemical marker of bone formation, are suppressed in patients receiving either oral or inhaled glucocorticoids (Lukert *et al.*, 1986; Puolijoki *et al.*, 1992). Surprisingly, urinary hydroxyproline and pyridinium cross-links, markers of bone resorption, are not increased by glucocorticoids (Cosman *et al.*, 1994; Lukert *et al.*, 1995). Conversely, serum tartrate-resistant acid phosphatase was elevated during short-term steroid therapy. It was felt that the high doses used in this study could have been toxic to osteoclasts causing cell death and liberation of cytoplasmic tartrate-resistant acid phosphatase (TRAP)into serum in the absence of increased bone resorption (Cosman *et al.*, 1994). The finding of a 96% increase in osteoclast perimeter observed in vertebrae taken from mice receiving prednisolone for 7 days makes it more likely that osteoclastic bone resorption is indeed increased early in the course of glucocorticoid administration (Weinstein *et al.*, 1998).

Risk Factors for Glucocorticoid-Induced Bone Loss

The usual risk factors for involutional osteoporosis (age, race, sex, weight, and parity) do not apply to the same extent to glucocorticoid-induced bone loss (Dykman *et al.*, 1985). Everyone taking high doses (greater than 10 mg/day of prednisone) loses significant amounts of bone (Garton *et al.*, 1993). Postmenopausal women receiving equivalent doses of steroids are more at risk for fractures than premenopausal women or men, presumably because they also have age- and menopause-related bone loss. It is unlikely that there is a threshold dose of glucocorticoid below which bone loss does not occur. A recent retrospective cohort study showed that the risk for fracture is increased even for doses below 7.5 mg/day and increases further with increasing daily and cumulative doses (Van Staa *et al.*, 2000). Even high doses of some inhaled steroids (Ip *et al.*, 1994), but not others (Medici *et al.*, 2000), cause bone loss.

The reasons for individual variation in response to glucocorticoids are numerous. Polymorphisms in the glucocorticoid receptor are associated with differences in BMD and body composition (Russcher *et al.*, 2005; van Rossum *et al.*, 2003, 2004). Responses to glucocorticoids may be related to peripheral enzymes that interconvert active and inactive glucocorticoids. Two 11β-hydroxysteroid dehydrogenases regulate the interconversion between cortisone and the active cortisol, and prednisone to prednisolone, thus playing a role in the modulation of glucocorticoid activity (Tomlinson *et al.*, 2004). 11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) is a glucocorticoid activator, converting cortisone to cortisol. 11β-Hydroxysteroid dehydrogenase type 2 (11β-HSD2) is a glucocorticoid inhibitor, converting cortisol to cortisone, and is present in mineralocorticoid target tissues. 11β-HSD1 is expressed in bone, as well as other glucocorticoid target tissues, and its ability to convert glucocorticoids to their most active form is enhanced by glucocorticoids (Cooper *et al.*, 2001; Tomlinson *et al.*, 2004). An inverse relationship has been observed between 11β-HSD1 activity and the differentiation of osteoblasts (Cooper *et al.*, 2002). 11β-HSD1 activity increases with age and this may partially explain the greater sensitivity of the elderly to glucocorticoid-induced bone loss (Cooper *et al.*, 2002). The underlying disease obviously affects the susceptibility to the adverse effects of glucocorticoids. Rheumatoid arthritis, chronic obstructive pulmonary disease, and inflammatory bowel disease have all been shown to be associated with bone loss without glucocorticoid treatment (Lekamwasam *et al.*, 2005; Romas, 2005; Sin *et al.*, 2003). Nutritional factors, such as inadequate protein, calcium, and/or vitamin D consumption, and nonspecific frailty and muscle weakness influence the response to glucocorticoids, in particular, the risk for fractures.

INDIRECT MECHANISMS FOR THE PATHOGENESIS OF GLUCOCORTICOID-INDUCED BONE LOSS

Glucocorticoids affect nearly every system in the body. We will first discuss the effects of glucocorticoids on systems that indirectly modulate bone metabolism to set the stage for a discussion of the direct effects of glucocorticoids on bone.

Effects on Pituitary Function

Growth Hormone

The secretion of growth hormone (GH) is partially controlled by glucocorticoids. Prednisone inhibits pituitary secretion of growth hormone in response to GH-releasing hormone in healthy men, most likely by increasing hypothalamic somatostatin tone (Giustina *et al.*, 1995; Kaufmann *et al.*, 1988). The growth hormone response to growth hormone-releasing hormone is reduced in patients receiving long-term inhaled steroids, illustrating that the control of growth hormone secretion is disrupted even by very small doses of glucocorticoids (Malerba *et al.*, 2005). Nevertheless, serum concentrations of growth hormone and insulin-like growth factor-1 (IGF-1) are normal in patients receiving glucocorticoids (Gourmelen *et al.*, 1982; Kaufmann *et al.*, 1988; Morris *et al.*, 1968). Despite normal levels, IGF-1 activity measured by bioassay is decreased in patients with glucocorticoid excess, perhaps because of an IGF-1 inhibitor that has been found in the serum of children receiving glucocorticoids (Unteman *et al.*, 1985). This inhibitory factor may be one of the IGF-binding proteins. Curiously, despite these findings, administration of growth hormone appears to reverse some of the adverse effects of chronic glucocorticoid treatment on bone (Giustina *et al.*, 1995).

A clearer understanding of the role of IGF-binding proteins on IGF activity has emerged and shed light on the mechanisms through which glucocorticoids may exert their effect. As discussed later, glucocorticoids may affect IGF-binding proteins (IGFBPs), which inhibit or enhance IGF activity. Glucocorticoids increase circulating levels of IGFBP-1, which may limit the activity of IGF-1; this effect has been associated with glucocorticoid-induced fetal growth retardation (Prince *et al.*, 1992). The importance of serum levels of growth factors or their binding proteins is unknown because growth factors are produced locally by bone cells. Growth hormone and parathyroid hormone (PTH) are trophic hormones (Ernst *et al.*, 1988; McCarthy *et al.*, 1989) for growth factors produced in bone, and the increase in bone density observed with the administration of PTH may be caused by stimulation of the production of growth factors in bone (Lane *et al.*, 2000).

Hypothalamic-Pituitary-Gonadal Axis

Glucocorticoids blunt pituitary secretion of luteinizing hormone (Sakakura *et al.*, 1975). A subset of gonadotropin-releasing-hormone (GnRH)-containing neurons in the rat hypothalamus possesses glucocorticoid receptors that bind dexamethasone *in vitro* with high affinity. Glucocorticoids repress transcription in a hypothalamic cell line, and glucocorticoid receptors present within GnRH neurons could be at least partly responsible for negative regulation of the hypothalamic-pituitary-gonadal axis. Suppression of gonadotropin levels by chronic elevations in glucocorticoids may be accounted for in part by suppression of GnRH mRNA levels, whereas the block of the gonadotropin surge by short-term glucocorticoids appears to involve other mechanisms including decreased follicle-stimulating hormone β (FSHβ) mRNA levels (Gore *et al.*, 2006).

Glucocorticoids also have direct effects on the gonads inhibiting FSH-induced estrogen production by ovarian granulosa cells and testosterone production by the testes (Hsueh *et al.*, 1978). The adrenal secretion of androgens is also decreased owing to suppression of ACTH secretion. Inhaled beclomethasone in doses of 1 mg/day or greater lower mean serum levels of DHEA by 35% in postmenopausal women (Smith *et al.*, 1994). As a result of these combined effects, serum concentrations of estradiol, estrone, dehydroepiandrosterone sulfate (DHEA), androstenedione, and progesterone are decreased in women; and DHEA and testosterone are decreased in men receiving glucocorticoids (MacAdams *et al.*, 1986). In mice even small doses dexamethasone impair ovarian production of androgens, estrogens, and progestins (Van Merris *et al.*, 2007). A single dose of dexamethasone was shown to disrupt gonadal function and fertility in rats for at least 30 days (Illera *et al.*, 2005).

It is very likely that deficiencies in these anabolic hormones accelerate bone loss. There is a direct correlation between bone mineral density and plasma estradiol levels in glucocorticoid-treated women (Montecucco *et al.*, 1992); furthermore, women receiving estrogen/progesterone replacement therapy and men given medroxyprogesterone acetate while taking glucocorticoids were protected against bone loss (Grecu *et al.*, 1990; Lukert *et al.*, 1992).

Calcium and Phosphorus Transport, Parathyroid Function, and Vitamin D Metabolism

Gastrointestinal Absorption of Calcium

Patients taking pharmacological doses of glucocorticoids have impaired gastrointestinal absorption of calcium, hypercalciuria and phosphaturia, and higher levels of serum PTH and 1,25(OH)$_2$D when compared with patients not taking steroids (Adams *et al.*, 1981; Bikle *et al.*, 1993; Favus *et al.*, 1973; Shrivastava *et al.*, 2000). Even very small oral doses of beclomethasone, similar to doses that may be swallowed by patients using the drug in inhaled form, decrease calcium absorption for the intestine (Smith *et al.*, 1993).

Calcium is absorbed from the intestine by passive (paracellular) and active (transcellular) transport. Active calcium absorption is primarily localized in the duodenum and tightly regulated. Transcellular calcium transport can be described in three sequential cellular steps, including transfer of luminal calcium into the enterocyte by the epithelial Ca^{2+} channel TRPV6, translocation of cytosolic Ca^{2+} toward the basolateral membrane by calbindin-D$_{9K}$, and active extrusion into the circulation by the plasma membrane ATPase 1b (PMCA1b) (Huybers *et al.*, 2007; van Abel *et al.*, 2003).

The diminished active Ca^{2+} absorption induced by glucocorticoids is associated with diminished TRPV6 and calbindin-D$_{9K}$ expression in the first part of the duodenum in the presence of normal levels of calcitriol suggesting a calcitriol-independent effect of glucocorticoids on calcium absorption. *In vitro* studies of calcium absorption have also shown evidence of a non-vitamin D-dependent inhibition of calcium transport (Adams *et al.*, 1980; Charney *et al.*, 1975). These effects on transport may not be caused solely by changes in calcium receptors or other proteins or enzymes involved in transport (a genomic effect), but also by direct effects on membrane permeability, post-transcriptional events, basolateral membrane transport, changes in paracellular transport, or other adverse effects. Possible mechanisms include depletion of mitochondrial adenosine triphosphate (Krawitt, 1972) or paracellular back flux caused by to stimulation of the sodium-potassium-ATPase pump by glucocorticoids (Adams *et al.*, 1980; Charney *et al.*, 1975).

Parathyroid Hormone and Vitamin D Metabolites

High PTH levels have traditionally been attributed to prolonged negative calcium balance, and the rise in 1,25(OH)$_2$D has been attributed to the stimulatory effect of PTH. However, recent acute longitudinal studies have shown that serum 1,25(OH)$_2$D levels increase and serum phosphorus levels decrease within 2 hours after intravenous administration of methylprednisolone, before PTH had risen significantly. These findings suggest that glucocorticoids alter transport across a number of biological membranes and may produce nongenomic effects. PTH levels then increased progressively during the first 2 weeks of high-dose treatment. All of these parameters returned to baseline at 3 weeks when methylprednisolone was given orally but still at a high dose (Cosman *et al.*, 1994).

Although the acute studies showed that the changes in PTH and 1,25(OH)$_2$D were transient, it is important to note that others have found PTH and 1,25(OH)$_2$D levels higher than disease-matched controls, although within the normal range, and higher levels of urinary cAMP and reduced tubular reabsorption of phosphate in patients taking glucocorticoids for more than a year and in those with Cushing's disease (Bikle *et al.*, 1993; Findling *et al.*, 1982; Lukert *et al.*, 1976). This suggests that glucocorticoids have an acute effect on transport, which inhibits gastrointestinal absorption of calcium, decreases renal tubular reabsorption of calcium and phosphorus, and may decrease intracellular calcium and phosphorus. This in turn promotes synthesis of 1,25(OH)$_2$D. Long-term glucocorticoid administration causes negative calcium balance, which perpetuates secondary hyperparathyroidism with its accompanying hypophosphatemia and elevated serum 1,25(OH)$_2$D levels. The high levels of cAMP and decreased tubular reabsorption of phosphate indicate that the increase in PTH (even though in the normal range) is of physiological significance because both of these changes are known effects of PTH on the kidney. Glucocorticoids decrease the tonic release of PTH and increase pulsatile bursts of the hormone. In healthy subjects, PTH is secreted by low-amplitude, high-frequency pulses superimposed on tonic secretion (Bonadonna *et al.*, 2005). The increase in pulsatile bursts may have important end-organ effects. The sensitivity of osteoblasts to PTH is increased by glucocorticoids. Glucocorticoids probably act on or near the stimulatory guanine nucleotide-binding regulatory protein complex. The potentiation of PTH-induced increases in cAMP response appears to be caused by increases in cAMP activity and inhibition of phosphodiesterase (Chen *et al.*, 1978). Whether or not renal tubules are more sensitive to PTH effects on cAMP response in the presence of glucocorticoids remains unclear.

Phosphate Transport

In addition to the effects of glucocorticoid-induced elevation of PTH levels on phosphate transport, glucocorticoids have direct effects, on renal tubular reabsorption of phosphate acting through the Na^+-H^+ exchange activity in the proximal tubule thus decreasing Na^+ gradient-dependent phosphate uptake (Frieberg *et al.*, 1982).

Osteonecrosis

Osteonecrosis [avascular necrosis (AVN) or aseptic necrosis] is a well-recognized complication of glucocorticoid excess. Glucocorticoid-induced osteonecrosis was first recognized in 1957. Previous administration of glucocorticoids can be implicated in 16% to 34% of patients presenting with “idiopathic” osteonecrosis (Fisher *et al.*, 1971). The femoral head is most frequently affected, followed by the head of the humerus and distal femur, but osteonecrosis may occur in other long bones and the bones of the feet. A similar lesion characterized by a transverse radiolucent cleft running under an endplate is seen in the vertebra and resembles subchondral fracture seen in long bones. The risk for osteonecrosis increases with both the dose of glucocorticoids and the duration of treatment (Zizic *et al.*, 1985). However, osteonecrosis may develop in patients who receive steroids in very high doses for a short period of time (Taylor, 1984), moderate doses over a long period of time (Metselaar *et al.*, 1985), or by intra-articular or epidural injection.

The mechanisms responsible for glucocorticoid-induced osteonecrosis remain obscure. Etiological considerations invoke several theories. One is a mechanical theory that attributes ischemic collapse of the epiphysis to osteoporosis and the accumulation of unhealed trabecular microcracks resulting in fatigue fractures. Others include a vascular theory proposing that ischemia is caused by microscopic fat emboli and a theory that increased intraosseous pressure owing to fat accumulation as part of Cushing’s syndrome leads to mechanical impingement on the sinusoidal vascular bed and decreased blood flow (Mankin, 1992). The number of apoptotic osteoblasts and osteocytes is increased in femoral necks removed from patients developing avascular necrosis while taking steroids, although this phenomenon was not observed in patients with AVN due to other causes (Weinstein *et al.*, 1998). The induction of early cell death may play a pivotal role in the etiology of steroid-induced AVN.

Clinically, pain is the usual presenting symptom and may be mild or vague in chronic forms of the disease, but it is usually acute and severe. Osteonecrosis may remain silent as long as it is not associated with epiphyseal collapse that appears to initiate symptoms (Maldague *et al.*, 1984). Early osteonecrosis of the hip may be managed by prolonged avoidance of weight bearing, but prosthetic replacement of the joint is frequently necessary. Surgical decompression may be attempted but the results are not encouraging (Mankin, 1992).

Summary of Cumulative Effects of Glucocorticoid-Induced Metabolic Changes on Bone

Overall effects of glucocorticoids are catabolic (Fig. 1). Inhibition of pituitary secretion of growth hormone and alterations in IGF-binding proteins leads to a fall in the biological activity of growth factors with loss of their anabolic effect on bone and other tissues. Gonadotrophin secretion is inhibited and, along with direct inhibitory effects of glucocorticoids on gonadal secretion of estrogen/testosterone, leads to a fall in circulating gonadal hormone concentrations. Deficiency in gonadal hormones causes an increase in bone resorption.

Membrane transport systems are altered by glucocorticoids resulting in inhibition of gastrointestinal absorption of calcium and decreased renal tubular absorption of calcium and phosphorus. Lowered intracellular phosphorus causes an acute rise in $1,25(OH)_2D$ synthesis. PTH secretion is increased despite elevated serum levels of calcium and $1,25(OH)_2D$. Chronically, increased PTH secretion and the resultant elevation in $1,25(OH)_2D$ production are perpetuated by negative calcium balance. The elevated levels of PTH and $1,25(OH)_2D$, along with deficiency of gonadal hormones, increase the number of sites undergoing bone resorption. The direct inhibition of osteoblastic bone formation at each bone remodeling site further augments the rate of bone loss.

Glucocorticoid-induced bone loss can be prevented by bisphosphonates, hormone replacement, PTH, and perhaps calcitonin (Cohen *et al.*, 1999; Lane *et al.*, 1998a; Luengo *et al.*, 1990; Lukert *et al.*, 1992; Reid *et al.*, 1996; Saag *et al.*, 1998). Fracture risk is reduced by bisphosphonates but fracture data are not available for the other modalities.

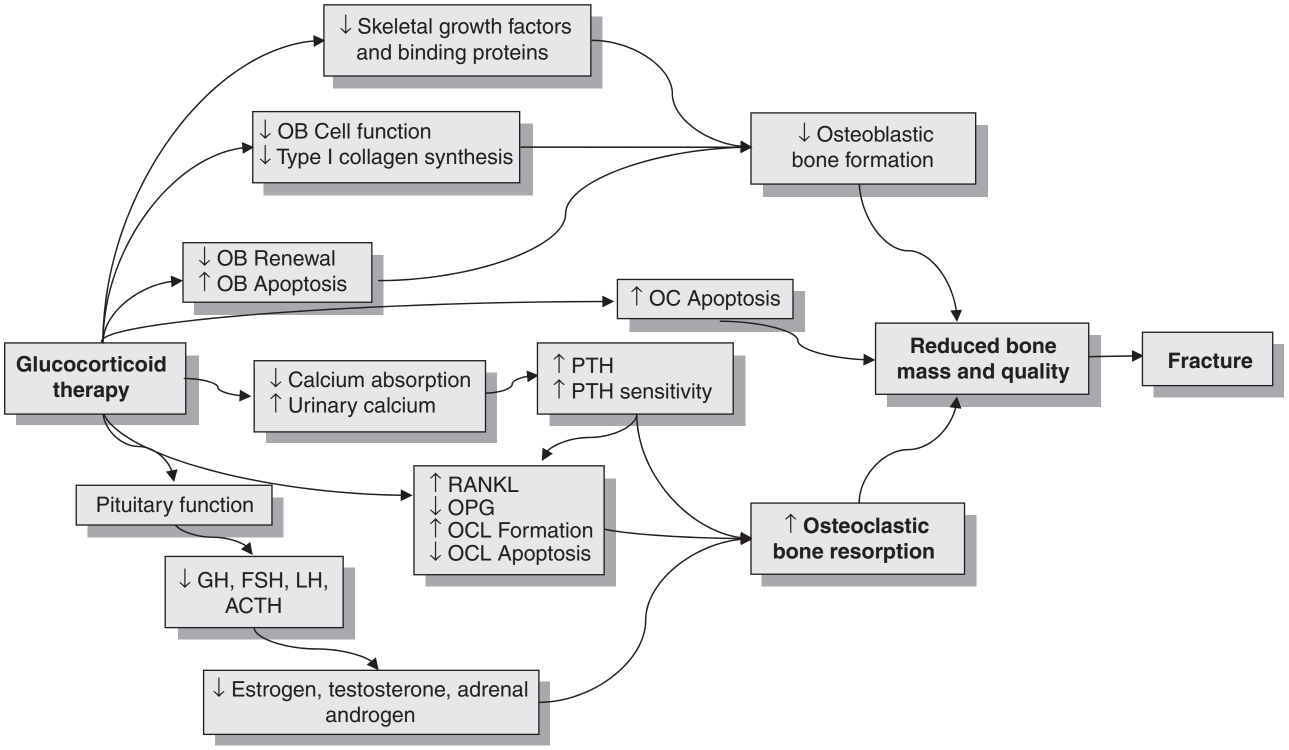
↓ Skeletal growth factors and binding proteins
↓ OB Cell function
↓ Type I collagen synthesis
↓ Osteoblastic bone formation
↓ OB Renewal
↑ OB Apoptosis
↑ OC Apoptosis
Glucocorticoid therapy
↓ Calcium absorption
↑ Urinary calcium
↑ PTH
↑ PTH sensitivity
Reduced bone mass and quality
Fracture
Pituitary function
↑ RANKL
↓ OPG
↑ OCL Formation
↓ OCL Apoptosis
↑ Osteoclastic bone resorption
↓ GH, FSH, LH, ACTH
↓ Estrogen, testosterone, adrenal androgen

FIGURE 1 Mechanisms of glucocorticoid-induced bone loss. PTH, parathyroid hormone; OB, osteoblast; OC, osteocyte; OCL, osteoclast; GH, growth hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; ACTH, adrenocotropic hormone; OPG, osteoprotegrin; RANKL, receptor activator of NF-κB ligand.

In addition to inhibiting bone resorption, bisphosphonates and calcitonin appear to decrease the number of apoptotic osteocytes and osteoblasts observed in bone biopsies from patients treated with prednisolone (Plotkin *et al.*, 1999). Estrogen has a similar effect in estrogen-deficient states but it is not known whether estrogen prevents apoptosis in the presence of glucocorticoids. Protection from glucocorticoid-induced apoptosis may play a major role in prevention of early bone loss in patients taking glucocorticoids. The major effects of glucocorticoids on bone are the direct effects on osteoblasts, which are discussed below.

DIRECT ACTIONS OF GLUCOCORTICOIDS ON BONE

Bone Formation and Osteoblast Differentiation

Glucocorticoids have many effects on osteoblasts that result in alterations in bone remodeling (Alesci *et al.*, 2005; Canalis *et al.*, 2007; Cooper *et al.*, 1999; Ishida *et al.*, 1998; Manolagas *et al.*, 1999; Mazziotti *et al.*, 2006). A hallmark of glucocorticoid-induced osteoporosis in humans is decreased mean wall thickness of trabecular bone, reflecting a reduction in the amount of new bone replaced in each remodeling cycle (Dempster *et al.*, 1983). Cells of the osteoblast lineage contain glucocorticoid receptors (Abu *et al.*, 2000; Chen *et al.*, 1977; Haussler *et al.*, 1980; Manolagas *et al.*, 1978), and high concentrations of glucocorticoids decrease protein, RNA, and DNA synthesis in primary bone cell cultures (Chen *et al.*, 1977; Choe *et al.*, 1978; Peck *et al.*, 1967; Wong, 1979). These studies are consistent with the well-described catabolic effects of high levels of glucocorticoids on human bone. Likewise, glucocorticoid treatment also decreases bone formation in dogs, rats, and mice (Altman *et al.*, 1992; Ortoft *et al.*, 1995; Quarles 1992; Turner *et al.*, 1995; Weinstein *et al.*, 1998). In some rat studies, glucocorticoid treatment decreases bone formation but does not induce osteopenia. This is because of an inhibition of turnover, because both bone resorption and formation are reduced, and bone mass does not decrease (Li *et al.*, 1996; Shen *et al.*, 1997). In mice, however, there is an increase in osteoclast surface shortly after glucocorticoid treatment, which is followed by a decrease in the rate of bone formation and a reduction in bone mass (Weinstein *et al.*, 1998). Thus, it has been suggested that mice, compared with rats, may be more like humans in the response of bone to pharmacological doses of glucocorticoids (Manolagas *et al.*, 1999).

By contrast to the striking inhibitory effect of pharmacological doses of glucocorticoids on bone formation *in vivo*, glucocorticoids cause both catabolic and anabolic effects on bone formation and osteoblast differentiation *in vitro*. Although the relevance of the *in vitro* anabolic effect is not completely clear, it may reflect the role of glucocorticoids in the maintenance of the osteoblast phenotype during bone remodeling. Analysis of data from *in vitro* studies is complicated by a plethora of experimental variables, including the concentration of hormone, the molecular form of the glucocorticoid used, the timing of hormone addition, the presence of serum, and the species, cellular heterogeneity, and developmental stage of the model system. There are examples of species differences in the response of osteoblasts to glucocorticoids. Mouse calvaria osteoblast cultures show a biphasic glucocorticoid dose response with respect to mineralized nodule formation in culture, whereas rat cells show a stimulatory effect over the same dose range (Bellows *et al.*, 1998). Moreover, the enhancement of nodule formation by glucocorticoids is less robust in mouse cultures (Bellows *et al.*, 1998). Other differences between the response of rat and mouse cells to glucocorticoids include the proliferative effects (Chen *et al.*, 1983a). In mouse bone marrow cultures, dexamethasone increases alkaline phosphatase activity despite its marked inhibition of cell proliferation (Chen, 2004). Thus, in mouse osteoblast cultures, the inhibition of proliferation may mask positive effects of glucocorticoids on differentiation. However, despite the differences in the models used to study the effects of glucocorticoids on bone cells, some generalizations can be made. *In vitro*, physiological concentrations of glucocorticoids enhance the differentiation of early osteoprogenitors and stimulate the formation of bone in developmental models of bone formation. By contrast, pharmacological concentrations of glucocorticoids inhibit cell proliferation, impair the function of more mature osteoblasts, and increase osteoblast and osteocyte apoptosis. These effects ultimately lead to a decrease in bone mass.

Organ cultures reflect both the anabolic and catabolic effects of glucocorticoids on bone formation. Organ explants of folded periostea from embryonic chick calvaria form new bone during culture (Tenenbaum *et al.*, 1985). When dexamethasone is added at the onset of culture, there is enhanced osteoid formation, alkaline phosphatase activity, and a transient increase in the replication of cells adjacent to the newly formed bone surface. However, when dexamethasone is added late in the culture period after bone has formed, there is a decrease in alkaline phosphatase activity (Tenenbaum *et al.*, 1985). Thus, it appears that glucocorticoids initially cause the proliferation and differentiation of a distinct population of osteoprogenitor cells that participate in bone formation, but then limit further cell proliferation in the cultures (McCulloch *et al.*, 1986).

Glucocorticoids both stimulate and inhibit type I collagen synthesis in serum-free organ cultures of fetal rat calvaria depending on the dose of hormone and duration of hormone treatment (Canalis, 1983; Dietrich *et al.*, 1978; Kream *et al.*, 1990b). In fetal rat calvaria, physiological concentrations of cortisol (30–100 nM) stimulate collagen synthesis after 24 hours, whereas pharmacological concentrations (1000 nM) are inhibitory at 48 to 96 hours (Dietrich *et al.*, 1978). Likewise, there is a rapid stimulatory effect of cortisol on collagen synthesis in newborn rat calvaria (Hahn, 1984). In fetal rat calvaria, the early stimulation of collagen synthesis is blocked by the addition of IGFBP-2, which binds and inactivates secreted IGFs (Kream *et al.*, 1997). These data suggest that the initial stimulation of collagen synthesis by glucocorticoids depends on the activity of endogenous IGF-1 and may be owing to increased osteoblastic differentiation.

Many studies show that glucocorticoids can enhance osteogenic differentiation in long-term primary calvaria cell cultures that form mineralized bone nodules in the presence of serum, ascorbic acid, and β-glycerol phosphate. These cultures are defined by the stages of cell proliferation, extracellular matrix maturation, and matrix mineralization, each characterized by the expression of cell growth and tissue-specific genes (Gerstenfeld *et al.*, 1987; Owen *et al.*, 1990; Stein *et al.*, 1990). Glucocorticoids increase the formation of bone nodules and the expression of genes associated with the osteoblast phenotype in primary rat osteoblastic cell cultures (Bellows *et al.*, 1987, 1989, 1990; Shalhoub *et al.*, 1992). The effect of glucocorticoids is biphasic: low concentrations of dexamethasone and hydrocortisone increase nodule formation, whereas pharmacological concentrations are less effective or not stimulatory (Bellows *et al.*, 1987). In this model, the anabolic effect of glucocorticoids has been attributed to the enhanced proliferation and differentiation of glucocorticoid-dependent osteoprogenitor (Bellows *et al.*, 1989, 1990). High concentrations of glucocorticoids inhibit osteogenic differentiation in MC3T3-E1 (Lian *et al.*, 1997) and primary murine osteoblast cultures (Bellows *et al.*, 1998).

Bone marrow stromal cell cultures grown in the presence of serum, ascorbic acid, and β-glycerolphosphate also have been used extensively as a model system to study the stages of osteoblast differentiation. Osteoprogenitor cells within the bone marrow stromal network, when activated to differentiate, provide a renewable source of osteoblasts for the endosteal and trabecular bone surfaces. Glucocorticoids enhance the expression osteoblastic phenotypic traits such as alkaline phosphatase activity, osteocalcin, type I collagen, osteopontin, and bone sialoprotein and the formation of mineralized bone nodules in cultures of chick, rat, and human bone marrow stromal cells (Aubin, 1999; Cheng *et al.*, 1994, 1996; Herbertson *et al.*, 1995; Kamalia *et al.*, 1992; Kasugai *et al.*, 1991; Malaval *et al.*, 1994; McCulloch *et al.*, 1991; Rickard *et al.*, 1994). Bone stromal cell cultures contain a glucocorticoid-dependent osteoprogenitor cell that gives rise to mineralized bone nodules (Aubin, 1999).

High concentrations of glucocorticoids inhibit osteoblast function and preosteoblast replication. Glucocorticoids inhibit collagen synthesis in organ cultures of fetal rat (Canalis, 1983; Dietrich *et al.*, 1978) and mouse calvaria (Woitge *et al.*, 2000). In subclones of osteoblastic ROS 17/2 osteosarcoma cells, glucocorticoids either stimulate or inhibit collagen synthesis depending on the state of maturation of the cells; in less mature osteoblasts, glucocorticoids stimulate collagen synthesis, whereas in more mature osteoblasts they inhibit collagen synthesis (Hodge *et al.*, 1988). In confluent cultures of primary osteoblastic cells, glucocorticoids generally inhibit collagen synthesis (Chen *et al.*, 1978; Kim *et al.*, 1989; Ng *et al.*, 1989). The inhibitory effect of glucocorticoids on collagen synthesis in fetal rat calvaria is accompanied by a decrease in periosteal cell content (Canalis, 1984; Chyun *et al.*, 1984; Dietrich *et al.*, 1978). Glucocorticoids also decrease the number of cells in the osteoblastic and periosteal layers of fetal rat parietal bone organ cultures (Gronowicz *et al.*, 1994). These effects in organ culture reflect, at least in part, the antiproliferative effect of glucocorticoids seen in osteoblastic cell cultures (Chen *et al.*, 1977; Hodge *et al.*, 1988; Hughes-Fulford *et al.*, 1992). Glucocorticoids decrease osteocyte formation in these cultures, which may reflect an inhibition of osteoblast renewal (Gohel *et al.*, 1995) and/or osteoblast apoptosis (Gohel *et al.*, 1999). High concentrations of glucocorticoids inhibit proliferation in primary human bone marrow stromal cell cultures (Silvestrini *et al.*, 2000; Walsh *et al.*, 2001). Glucocorticoids inhibit cell cycle progression in committed osteoblasts by activating glycogen synthase kinase-3β (GSK-3β), which in turn downregulates c-Myc (Smith *et al.*, 2000, 2002). Not all of the effects of glucocorticoids on osteoblast function can be attributed to an inhibition of cell replication. For example, the inhibitory effect of glucocorticoids on collagen synthesis, although blunted, still persists in the presence of DNA synthesis inhibitors, suggesting that glucocorticoids also inhibit the function of differentiated osteoblasts (Lukert *et al.*, 1991). In cultured bone marrow stromal cells, a low concentration of dexamethasone (10 nM) promotes the osteogenic differentiation, whereas a higher concentration (100 nM) was also osteogenic but decreased cell number. These data suggest that glucocorticoids enhance osteoblastic differentiation but that a decrease in proliferation of osteogenic precursors ultimately limits the extent of bone formation (Walsh *et al.*, 2001).

Glucocorticoids increase the apoptosis of osteoblasts (Gohel *et al.*, 1999; Silvestrini *et al.*, 2000; Weinstein *et al.*, 1998) and osteocytes (Plotkin *et al.*, 1999; Weinstein *et al.*, 1998). Chronic treatment of adult mice with prednisolone increases apoptosis of osteoblasts in vertebrae and osteocytes in metaphyseal cortical bone and decreases bone mass (Weinstein *et al.*, 1998), which is reduced by bisphosphonate treatment (Plotkin *et al.*, 1999). Acute treatment of neonatal mice with dexamethasone increases apoptosis of osteoblasts in calvaria (Gohel *et al.*, 1999) and this effect was reversed by cotreatment with 17β-estradiol. Apoptotic osteocytes and cancellous lining cells are seen in femoral heads from patients with glucocorticoid-induced osteonecrosis (Weinstein *et al.*, 2000). Glucocorticoids increase apoptosis in part by increasing caspase 3 activity (Liu *et al.*, 2004; Plotkin *et al.*, 1999). In primary fetal rat calvarial cell cultures, the increase in osteoblast apoptosis is associated with a decrease in the Bcl-2/Bax protein ratio (Gohel *et al.*, 1999). Bcl-2, an antiapoptotic integral membrane protein, has been targeted to mature osteoblasts of transgenic mice with a 2.3-kb fragment of the rat Col1a1 promoter (Col2.3-Bcl2). Transgenic mice are smaller than wild type and display an abrogation of the differences between male and female bone parameters and a blunting of age-related bone loss. *Ex vivo* primary calvarial osteoblast cultures show increased differentiation markers but decreased mineralization (Pantschenko *et al.*, 2005; Zhang *et al.*, 2007). In addition to increasing osteocyte apoptosis, glucocorticoids modify the osteocyte lacuna and affect bone quality. Mice treated with prednisolone show increased lacunae size, a halo of hypomineralized bone surrounding the lacunae, and a reduced mineral-to-matrix ratio. These changes are accompanied by decreased elastic modulus of bone adjacent to osteocytes and reduced whole bone strength (Lane *et al.*, 2006).

Chronic glucocorticoid administration decreases osteoblastogenesis (Manolagas, 1999, 2000). When mice are treated with pharmacological levels of glucocorticoids *in vivo*, the generation of fibroblast colony-forming units in *ex vivo* bone marrow cultures is decreased, suggesting that glucocorticoids deplete the bone marrow of osteogenic precursors (Simmons *et al.*, 1990). Likewise, glucocorticoid treatment of adult mice for one month suppresses osteogenic differentiation in *ex vivo* bone marrow cultures (Weinstein *et al.*, 1998).

Collectively, these data suggest that pharmacological doses of glucocorticoid induce osteoporosis owing to a decrease in bone formation that results from an impairment of osteoblast function, reduced osteoblastogenesis, and preosteoblast proliferation and increased osteoblast apoptosis (see Fig. 1). However, physiological concentrations of glucocorticoids may be important for maintaining osteoblast differentiation.

Bone Resorption and Osteoclast Differentiation

Glucocorticoids have both direct and indirect effects on osteoclasts that result in changes in bone resorption. Data from various studies may be conflicting owing to differences in experimental models and the dosage and timing of glucocorticoid addition. Glucocorticoids inhibit basal and agonist-stimulated resorption of fetal rat long bones (Raisz *et al.*, 1972) but increase resorption of fetal rat parietal bones (Gronowicz *et al.*, 1990) and mouse calvaria (Conaway *et al.*, 1996; Reid *et al.*, 1986b). Glucocorticoids decrease the activity and increase the apoptosis of rat osteoclasts (Dempster *et al.*, 1997; Tobias *et al.*, 1989). Dexamethasone increases osteoclastogenesis in mouse bone and spleen cell cocultures (Kaji *et al.*, 1997). Glucocorticoids increase macrophage colony-stimulating factor (M-CSF) in osteoblasts (Rubin *et al.*, 1998). They also increase the production of receptor activator of NF-κB (RANKL) and decrease the production of osteoprotegerin (OPG) in osteoblasts (Hofbauer *et al.*, 1999; Sivagurunathan *et al.*, 2005). An increase in the RANKL/OPG ratio is consistent with a stimulation of osteoclast formation and may explain the early stimulation of resorption in humans and mice. Glucocorticoids decrease osteoclast progenitor number but increase osteoclast lifespan (Weinstein *et al.*, 2002). The antiapoptotic effect is caused by glucocorticoid receptor-dependent glucocorticoid signaling in osteoclasts (Jia *et al.*, 2006; Kim *et al.*, 2006). At the same time, glucocorticoids disrupt actin ring formation resulting in dysfunctional osteoclasts *in vitro* and *in vivo* (Kim *et al.*, 2006). This study shows that glucocorticoids can decrease bone formation by signaling in osteoclasts (Kim *et al.*, 2006).

Permissive Effects of Glucocorticoids on Osteoblasts

Some of the physiological effects of glucocorticoids on bone may be in part owe to their ability to act as permissive hormones, thereby allowing other hormones to function optimally. Low doses of glucocorticoids enhance PTH-stimulated adenylate cyclase in rat, mouse, and human bone cells (Chen *et al.*, 1978; Rodan *et al.*, 1984; Wong 1980; Wong *et al.*, 1990) and PTH-mediated bioactivities (Wong, 1979). The enhancement of the PTH-dependent cAMP response may be caused by an increase in cAMP activity and a decrease in phosphodiesterase activity (Chen *et al.*, 1978). Glucocorticoids also increase the number of PTH receptors and levels of PTH/PTHrP receptor mRNA (Urena *et al.*, 1994; Yamamoto *et al.*, 1988).

The effect of glucocorticoids on 1,25-$(OH)_2$D receptors and biological activity, however, is not as clear. In rat bone cytosol and primary rat osteoblastic cells, glucocorticoids maintain or increase 1,25-$(OH)_2$D receptor number (Chen *et al.*, 1983b; Manolagas *et al.*, 1979) and enhance the biological actions of 1,25-$(OH)_2$D (Chen *et al.*, 1986). However, in one study using primary mouse osteoblastic cells, the effect of glucocorticoids on 1,25-$(OH)_2$D receptor number was dependent on the stage of growth of the cells; receptor number was decreased at early log phase growth and at confluence and increased at late log phase growth (Chen *et al.*, 1982). In another study, glucocorticoids were shown to increase 1,25-$(OH)_2$D biological activities in primary mouse osteoblastic cells (Wong, 1980). In human MG-63 cells, glucocorticoids decrease the expression of 1,25-$(OH)_2$D receptor mRNA (Godschalk *et al.*, 1992). Taken together, these findings indicate that glucocorticoids can increase or decrease 1,25-$(OH)_2$D receptor levels depending on the experimental model.

Glucocorticoids alter the IGF-1 pathway. They inhibit IGF-1 mRNA and protein expression by osteoblasts (as discussed later) but increase IGF-1 receptor number (Bennett *et al.*, 1984). Physiological concentrations of cortisol enhance the stimulatory effects of IGF-1 on collagen synthesis, producing a larger anabolic effect than with IGF-1 alone (Kream *et al.*, 1990a). The ability of glucocorticoids to augment IGF-1 activity may represent a compensatory response that helps maintain bone mass and growth during periods of diminished IGF-1 supply, such as starvation. A similar enhancing effect of glucocorticoids on IGF-1 action occurs in fibroblast cultures (Bird *et al.*, 1994; Conover *et al.*, 1986). Cortisol enhances the anabolic effects of exogenous prostaglandins on collagen and DNA synthesis in organ cultures of rat calvaria, which may be dependent partly on the IGF-1 pathway (Raisz *et al.*, 1993). Physiological concentrations of glucocorticoids may amplify the stimulatory effect of PGE_2 on the IGF-1 promoter through induction of C/EBP family transcription factors (McCarthy *et al.*, 2000b).

Target Cell Metabolism of Glucocorticoids in Bone Cells

Target cell metabolism has emerged as an important mechanism for regulating the sensitivity of cells to glucocorticoids (Eyre *et al.*, 2001). As discussed earlier, glucocorticoids undergo target cell metabolism by two 11β-hydroxysteroid dehydrogenases that catalyze the interconversion of cortisol and cortisone in humans and corticosterone and 11-dehydrocorticosterone in rodents (Chapman *et al.*, 2006a; Draper *et al.*, 2005; Krozowski 1999; Krozowski *et al.*, 1999; Stewart *et al.*, 1999; Tomlinson *et al.*, 2004). The 11β-HSD enzymes control the local concentration of glucocorticoids available to engage in receptor binding and subsequent signaling (Rabbitt *et al.*, 2002). The 11β-HSD system is distinct from both the glucocorticoid biosynthetic pathway in the adrenal and the degradative pathway in the liver, both of which involve different enzymatic cascades. The NAD-dependent enzyme 11β-HSD2 has dehydrogenase activity and catalyzes the unidirectional conversion of biologically active glucocorticoids to inactive metabolites, and the bidirectional interconversion of dexamethasone to 11-dehydrodexamethasone. 11β-HSD2 is highly expressed in kidney where it protects the mineralocorticoid receptor from activation by glucocorticoids and is abundant in placenta where it protects the fetus from maternal glucocorticoids. Mice with a targeted deletion of 11β-HSD2 develop hypertension because glucocorticoids, which fail to be metabolized in kidney cells, evoke mineralocorticoid effects via the mineralocorticoid receptor (Kotelevtsev *et al.*, 1999). The NADP-dependent 11β-HSD1 has oxoreductase activity and catalyzes the bidirectional conversion of inactive glucocorticoids to active metabolites. In cell-free systems, 11β-HSD1 is also capable of catalyzing the conversion of cortisol to cortisone (dehydrogenase activity). However, in intact cells, oxoreductase activity predominates most likely because of the topography of 11β-HSD1 in the endoplasmic reticulum, where the active site of the enzyme faces the lumen in close proximity to hexose-6-phosphate dehydrogenase, which generates NADPH for oxoreductase activity (Atanasov *et al.*, 2004; Lavery *et al.*, 2006; Seckl *et al.*, 2001). Deletion of *Hsd11b1* abolishes the conversion of 11-dehydrocorticosterone to corticosterone in liver, providing strong evidence that 11β-HSD1 is the only 11-reductase in mice (Holmes *et al.*, 2001).

Nearly all glucocorticoid target tissues express 11β-HSD1 as a means of generating a locally acting pool of active hormone. 11β-HSD1 expression in the liver provides a ready source of cortisol for the stimulation of gluconeogenesis (Kotelevtsev *et al.*, 1997). The reactivation of cortisone by 11β-HSD1 in macrophages aids in the resolution of inflammation (Chapman *et al.*, 2006a, 2006b; Gilmour *et al.*, 2006). A noteworthy function of 11β-HSD1 is its role in promoting adipocyte differentiation and the development of visceral obesity (De Sousa Peixoto *et al.*, 2008a; Morton *et al.*, 2008; Tomlinson *et al.*, 2002). Glucocorticoids stimulate adipocyte differentiation and 11β-HSD1 expression, which ensures an adequate local source of hormone for fat differentiation (Kim *et al.*, 2007). Transgenic mice with adipocyte-targeted 11β-HSD1 have visceral obesity and elevated levels of adipose corticosterone despite normal serum levels of corticosterone (Masuzaki *et al.*, 2001). By contrast, Hsd11b1 knockout mice are protected against obesity and the metabolic consequences of a high fat diet (De Sousa Peixoto *et al.*, 2008a).

Rat and mouse calvarial osteoblast cultures can convert inactive glucocorticoids to active metabolites, and the 11β-HSD inhibitor carbenoxelone partially blocks the effects of corticosterone on bone nodule formation in rat and mouse osteoblast cultures, indicating that osteoblasts contain 11β-HSD1 activity (Bellows *et al.*, 1998). Both 11β-HSD1 and 11β-HSD2 are expressed in osteoblasts of rat and human osteoblasts (Cooper *et al.*, 2000). Rat and human osteoblastic osteosarcoma cell lines express 11β-HSD2 (Bland *et al.*, 1999; Eyre *et al.*, 2001). Primary human osteoblasts and adult human bone explants express both 11β-HSD1 and 11β-HSD2 (Bland *et al.*, 1999). The glucocorticoid sensitivity of osteosarcoma cell lines with equivalent numbers of glucocorticoid receptors is directly correlated with the level of 11β-HSD2 expression (Eyre *et al.*, 2001). Moreover, ROS 17/2.8 and MC3T3-E1 cells transfected with 11β-HSD2 show reduced responsiveness to natural glucocorticoids but remain responsive to the synthetic glucocorticoid dexamethasone (Woitge *et al.*, 2001). In human osteosarcoma cell lines, 11β-HSD1 expression is induced by the inflammatory cytokines IL-1β and TNFa (Cooper *et al.*, 2001). In primary human osteoblasts, 11β-HSD1 expression increases with donor age and may play a role in age-related bone loss (Cooper *et al.*, 2002). 11β-HSD1 is highly expressed during the early stages of osteoblast cultures and expression declines as differentiation ensues (Eijken *et al.*, 2005, 2006). Thus, 11β-HSD1 may regulate the sensitivity of osteoblasts to glucocorticoids and play a role in the early stages of osteoblast differentiation. The *Hsd11b1* knockout model, however, shows normal bone physiology in young and old mice (Justesen *et al.*, 2004). A confounding feature of the model is a 2-fold increase in circulating corticosterone and adrenal hyperplasia owing to activation of the hypothalamic-pituitary-adrenal axis (Holmes *et al.*, 2001). Thus, it is possible that the high serum levels of glucocorticoid in *Hsd11b1* knockout mice may have reduced the requirement for locally produced glucocorticoids. Moreover, bone marrow adipocytes were absent in this model, raising the possibility that the number of pluripotent progenitor cells available for osteoblast recruitment was increased.

11β-HSD2 cDNA has been cloned upstream of osteoblast-targeted promoters to develop glucocorticoid loss-of-function models based on the role of 11β-HSD2 in kidney and placenta to inactivate glucocorticoids. These models would prevent glucocorticoid signaling by the glucocorticoid receptor, and possibly the mineralocorticoid receptor, in osteoblasts. A 2.3-kb promoter fragment of the rat Col1a1 gene has been used to target 11β-HSD2 expression to differentiating osteoblasts (Sher *et al.*, 2004, 2006). Col2.3-HSD2 mice have decreased vertebral trabecular bone mass, decreased femoral cortical width and area, and reduced osteoblast markers and mineralization in *ex vivo* calvarial cultures. The Col2.3-HSD2 transgene led to a shift in mesenchymal cell fate owing to a decrease in Wnt signaling, suggesting a feedback signal from mature osteoblasts to early progenitors (Zhou *et al.*, 2008). 11β-HSD2 has been used to target mature osteoblasts with the osteocalcin (OG2) promoter (O'Brien *et al.*, 2004). OG2-HSD2 transgenic mice do not have alterations in bone development and turnover, but are protected from dexamethasone-induced osteoblast apoptosis and diminution of vertebral strength (O'Brien *et al.*, 2004). Differences between the models are likely owing to distinct temporal and spatial patterns of transgenic 11β-HSD2 expression as well as the level of transgene expression.

GLUCOCORTICOID-REGULATED GENE EXPRESSION IN BONE

Molecular Mechanisms of Glucocorticoid Action

At the molecular level, glucocorticoids alter the expression of a wide variety of genes in osteoblastic cells, including those for structural proteins, growth factors, receptors, and enzymes. Glucocorticoids elicit biological responses in their target cells by binding to and activating the intracellular glucocorticoid receptor. The structure and function of the glucocorticoid receptor, its intracellular trafficking, and glucocorticoid receptor-dependent transcription are discussed in detail in many excellent reviews (Beato *et al.*, 1996; Defranco 2000; Duma *et al.*, 2006; McKay *et al.*, 1999; Webster *et al.*, 1999; Zhou *et al.*, 2005). The glucocorticoid receptor contains an amino-terminal domain that encodes a transactivation function, and a carboxyl-terminal domain that specifies ligand binding, dimerization, heat shock protein (hsp) binding, and transactivation functions. The most highly conserved region is the 66-amino-acid DNA-binding domain consisting of two zinc finger motifs with cysteine residues that are coordinated with zinc atoms (Freedman *et al.*, 1988). Unligated glucocorticoid receptors are found in the cytoplasm in association with a variety of molecular chaparone proteins including hsp90, an FK506-binding immunophilin protein, and p23 (Cheung *et al.*, 2000; Defranco, 2000). Upon hormone binding, a conformational change enables the receptor to translocate to the nucleus, dimerize, and bind to DNA. Transcriptional activation by a glucocorticoid receptor homodimer occurs when the DNA-binding domains interact with a glucocorticoid response element (GRE). The GRE consensus sequence is GGTACAnnnTGTTCT (Beato, 1989). Transcriptional activation involves protein–protein interactions between the receptor dimer and basal transcription factors and RNA polymerase II (Mitchell *et al.*, 1989). Cofactors are recruited to the GRE by activated glucocorticoid receptor including those with histone acetyltransferase and methyltransferase activity (Lonard *et al.*, 2005), which facilitate alterations in chromatin structure and the recruitment of additional components of the transcriptional complex (Lu *et al.*, 2006). Glucocorticoid-dependent inhibition of gene expression has become a molecular paradigm for understanding mechanisms of transcriptional repression by steroid hormone receptors (Webster *et al.*, 1999). Transcriptional repression by glucocorticoid receptors occurs by direct interaction with DNA through negative GREs (Sakai *et al.*, 1988), by blocking the access of positive transcription factors to DNA sequences (Akerblom *et al.*, 1988), and by protein–protein interaction with transcription factors (Chatterjee *et al.*, 1991). An example of the latter mechanism is glucocorticoid inhibition of collagenase expression, which is thought to occur by interaction of the glucocorticoid receptor with the AP-1 transcription factor complex (Jonat *et al.*, 1990; Schule *et al.*, 1990; Yang-Yen *et al.*, 1990).

Effects on Gene Expression

Primary Response Genes and Transcription Factors

Glucocorticoids cause a rapid and transient increase in the mRNA levels of c-fos (Birek *et al.*, 1991; Shalhoub *et al.*, 1992; Shur *et al.*, 2005; Subramaniam *et al.*, 1992) and c-myc (Subramaniam *et al.*, 1992) in human and rodent osteoblasts and chick periosteal cultures. The rat c-fos promoter contains a putative GRE that may mediate glucocorticoid-dependent induction (Wang *et al.*, 1994). Cell lines prepared from tumors of c-fos transgenic mice show changes in osteoblast phenotypic markers but have unaltered glucocorticoid responsiveness (Grigoriadis *et al.*, 1993). The induction of primary response genes may be a key event in the regulation of downstream genes such as those that encode growth factors and matrix proteins. Id (inhibitor of differentiation) is a member of the helix-loop-helix (HLH) family of transcription factors that binds to other HLH factors and suppresses differentiation (Benezra *et al.*, 1990). Id mRNA is detectable in early cultures of MC3T3-E1 cells and then decreases as the cells differentiate (Ogata *et al.*, 1993). Dexamethasone maintains the high levels of Id mRNA in confluent MC3T3-E1 cells (Ogata *et al.*, 1993).

Type I Collagen

Type I collagen is the most abundant protein in the bone matrix and its expression is regulated by a wide variety of hormones, growth factors, and cytokines (Raisz, 1988). Glucocorticoids decrease a1(I) collagen (Col1a1) mRNA levels in osteoblastic cells and calvarial organ cultures (Delany *et al.*, 1995a; Kim *et al.*, 1989; Kream *et al.*, 1990a; Lukert *et al.*, 1991) and in calvaria of neonatal mice given *in vivo* dexamethasone (Advani *et al.*, 1997). Glucocorticoid downregulation of Col1a1 mRNA occurs by an inhibition of Col1a1 transcription and a decrease in the stability of Col1a1 mRNA (Delany *et al.*, 1995a). Dexamethasone decreases the activity of transfected Col1a1 mRNA promoter–reporter constructs in stably transfected osteoblastic cells, indicating a transcriptional effect (Petersen *et al.*, 1991). The precise molecular mechanisms by which glucocorticoids inhibit Col1a1 transcription in osteoblastic cells have not been elucidated. However, studies performed in fibroblasts provide mechanistic clues. Glucocorticoids decrease type I collagen mRNA levels in fibroblasts by decreasing transcription of collagen genes and the stability of collagen mRNA (Cockayne *et al.*, 1986; Hamalainen *et al.*, 1985; Raghow *et al.*, 1986). Glucocorticoids also decrease Col1a1 mRNA stability by affecting protein binding to 3' UTRs (Maatta *et al.*, 1993). Glucocorticoids decrease the activity of transfected murine Col1a2 promoter–reporter constructs in fibroblasts through sites contained in regions from −2048 to −981 bp and −506 to −351 bp (Perez *et al.*, 1992). In stably transfected fetal skin fibroblasts, the inhibitory effect of dexamethasone on rat Col1a1 promoter activity is maintained when the promoter is deleted to −900 bp. This region contains a putative GRE half-site; however, a mutation of this site does not block glucocorticoid-dependent inhibition of Col1a1 promoter activity (Meisler *et al.*, 1995). In this study, it was suggested that glucocorticoids decrease

Col1a1 transcription in fibroblasts by acting at a TGFβ-responsive site (Meisler *et al.*, 1995).

Noncollagen Proteins of Bone

Glucocorticoids alter the expression of a variety of non-collagen proteins in bone. Glucocorticoid treatment of rats and mice decreases osteocalcin mRNA levels in bone (Advani *et al.*, 1997; Ikeda *et al.*, 1992). Glucocorticoids inhibit basal and agonist-induced osteocalcin production and mRNA levels in osteoblasts (Schepmoes *et al.*, 1991; Wong *et al.*, 1990) and repress 1,25-$(OH)_2D$-mediated osteocalcin transcription (Morrison *et al.*, 1989). It has been proposed that this occurs by binding of the glucocorticoid receptor the TATA box in the proximal promoter region of the osteocalcin gene (Meyer *et al.*, 1997; Stromstedt *et al.*, 1991). Glucocorticoids suppress osteocalcin expression through an Egr2/Krox20 DNA-binding motif just upstream of a Runx2 site in the osteocalcin promoter (Leclerc *et al.*, 2005). Glucocorticoids also decrease Krox20 mRNA levels in osteoblasts (Leclerc *et al.*, 2008).

Glucocorticoids increase alkaline phosphatase activity and mRNA levels in human osteoblastic cells (Subramaniam *et al.*, 1992), SaOS-2 osteosarcoma cells (Murray *et al.*, 1987), and ROS 17/2.8 cells (Majeska *et al.*, 1985). In ROS17/2.8 cells, the increase in mRNA occurred after a lag period of 12 hours and is blocked by cycloheximide, indicating the requirement for new protein synthesis (Green *et al.*, 1990). Actinomycin D blocked the stimulatory effect of glucocorticoids on alkaline phosphatase mRNA levels, indicating transcriptional regulation (Green *et al.*, 1990). Osteoblasts synthesize the bone/liver/kidney/placenta form of alkaline phosphatase; this gene contains two alternative promoters spaced 25 kb apart; baseline and glucocorticoid-stimulated alkaline phosphatase mRNA in calvaria and ROS 17/2.8 cells is transcribed from the upstream promoter (Zernick *et al.*, 1991).

Bone sialoprotein is a glycoprotein containing an arginine-glycine-aspartic acid (RGD) sequence that mediates attachment of cells to extracellular matrix proteins. Glucocorticoids increase bone sialoprotein mRNA levels in fetal rat calvarial, bone marrow, ROS 17/2.8, and UMR106-06 cells in part by a transcriptional mechanism (Ogata *et al.*, 1995). The bone sialoprotein promoter contains a GRE between −906 and −931 bp that may mediate this transcriptional effect of glucocorticoids (Ogata *et al.*, 1995). There have been few studies examining the direct effect of glucocorticoids on the expression of osteonectin, an abundant noncollagenous glycoprotein. In one study, dexamethasone increased osteonectin mRNA levels and the activity of an osteonectin promoter–reporter construct in preosteoblastic UMR 201 cells (Ng *et al.*, 1989).

Glucocorticoids decrease fibronectin (Gronowicz *et al.*, 1991) and β1 integrin (Doherty *et al.*, 1995) mRNA levels in fetal rat parietal bone organ cultures. The inhibitory effect on β1 integrin expression is accompanied by a disruption of osteoblast organization on the bone surface and a decrease in calcification of the bone (DiPersio *et al.*, 1991). In primary rat osteoblast cultures and ROS 17/2.8 cells, glucocorticoids decrease plasma membrane β1 integrin staining, adhesion of the cells to bone matrix proteins, and β1 integrin mRNA levels (Gronowicz *et al.*, 1995). Glucocorticoids decrease the expression of cells containing the α2 and α4 integrin subunits in bone marrow stromal cultures (Walsh *et al.*, 2001).

Glucocorticoids decrease interstitial collagenase mRNA levels in human skin fibroblasts by reducing the half-life of collagenase mRNA (Delany *et al.*, 1992). By contrast, glucocorticoids increase the expression of collagenase mRNA in rat osteoblastic cells (Delany *et al.*, 1995b; Shalhoub *et al.*, 1992) by a mechanism that involves increased collagenase mRNA stability (Delany *et al.*, 1995b). Cortisol antagonized the phorbol ester-mediated increase in activity of a transiently transfected rat collagenase promoter–reporter construct (Delany *et al.*, 1995b). Glucocorticoid induction of interstitial collagenase expression in osteoblasts may be related to biological activities such as growth factor activation or the activation of osteoclastic bone resorption (Delany *et al.*, 1995b).

Growth Factor Pathways

IGF-1 is an important anabolic growth factor for bone (Rosen, 2004). The inhibitory effects of glucocorticoids on bone formation may be caused in part by a decrease in the production of IGF-1 (McCarthy *et al.*, 1990). Glucocorticoids decrease IGF-1 mRNA expression in rat tibia, organ cultures of fetal rat calvaria, and primary osteoblastic cell cultures (Chen *et al.*, 1991; Luo *et al.*, 1989; McCarthy *et al.*, 1990). However, glucocorticoids do not regulate IGF-II mRNA levels in primary rat osteoblastic cells (McCarthy *et al.*, 1992) but they decrease IGF-II peptide production in fetal rat calvarial cultures (Canalis *et al.*, 1991). Inhibitory effects of glucocorticoids on bone formation persist when IGFBP-2 is added to cultures of fetal rat calvaria to inactivate IGFs (Kream *et al.*, 1997). Moreover, calvaria from mice with a complete ablation of the Igf1 gene maintain responsiveness to glucocorticoids (Woitge *et al.*, 2000). These studies suggest that inhibitory effects of glucocorticoids are partly independent of the IGF-1 pathway.

IGFBPs regulate the storage, transport, and bioactivities of IGFs (Clemmons *et al.*, 1993). Six IGFBPs, termed IGFBP-1 through -6, have been identified in a variety of tissues (Shimasaki *et al.*, 1991). The expression of IGFBPs in osteoblastic cells of different origins is cell line specific (Hassager *et al.*, 1992). IGFBPs generally inhibit IGF-1 action *in vitro* (Feyen *et al.*, 1991; Mohan *et al.*, 1989), except for IGFBP-5, which may act as an anabolic growth factor (Andress *et al.*, 1992; Miyakoshi *et al.*, 2001). Glucocorticoids decrease IGFBP-3, -4, and -5 production in the normal human osteoblastic cells (Okazaki *et al.*, 1994), and decrease IGFBP-3 production in transformed osteoblastic cell lines (Nakao *et al.*, 1994). Glucocorticoids decrease IGFBP-5 transcription in rat osteoblasts (Gabbitas *et al.*, 1996b), and decrease IGFBP-2 production in rat calvarial osteoblastic cells (Chen *et al.*, 1991) and immortalized rat osteoblastic PyMS cells (Schmid *et al.*, 1988). However, glucocorticoids increase the expression of IGFBP-6 in fetal rat calvarial cell cultures (Gabbitas *et al.*, 1996a). Because IGFBP-6 has higher affinity for IGF-2 than IGF-1, glucocorticoid stimulation of IGFBP-6 may limit the availability of IGF-2 as an anabolic agent (Gabbitas *et al.*, 1996a). Because glucocorticoids decrease IGF-1 production in bone, the inhibitory effect of glucocorticoids on IGFBP expression may provide a mechanism by which osteoblastic cells are more responsive to the residual pool of IGF-1. Alternatively, downregulation of IGFBP-5 production could represent the removal of an anabolic factor and result, in part, in the inhibitory effects of glucocorticoids. Glucocorticoids alter the expression of other growth factor systems in cultured fetal rat calvarial osteoblasts such as mac25 (IGFBP-related peptide), connective tissue growth factor, and hepatocyte growth factor and its receptor c-met (Blanquaert *et al.*, 2000; Pereira *et al.*, 1999, 2000). As of yet, the role of these factors in mediating glucocorticoid responses in bone is not known.

Some of the inhibitory effects of glucocorticoids may be because of the antagonism of the Wnt pathway, a positive regulator of osteoblast differentiation and proliferation and bone mass accrual (Manolagas *et al.*, 2007; Westendorf *et al.*, 2004). Glucocorticoids inhibit canonical Wnt/β-catenin signaling in osteoblasts (Ohnaka *et al.*, 2005), and enhance the expression of Dickkopf, an inhibitor of Wnt signaling that prevents Wnt ligand from binding to its receptor complex on the cell membrane (Ohnaka *et al.*, 2004, 2005). Glucocorticoids maintain the activity of GSK-3β, which phosphorylates β-catenin and targets it for degradation and inhibits LEF/TCF-dependent transcription (Smith *et al.*, 2005).

TGFβ is anabolic for bone formation and either stimulatory or inhibitory for bone resorption depending on the experimental model and the culture conditions (Centrella *et al.*, 1994). TGFβ binds to three cell surface receptors, termed TGFβRI, II, and III, which have been demonstrated in osteoblastic cells (Centrella *et al.*, 1991). The type I and II receptors are thought to mediate TGFβ signaling (Massague, 1992); the type III receptor, betaglycan, is a cell surface proteoglycan that is more abundant than the types I and II receptors but has lower affinity for TGFβ1 (Lopez-Casillas *et al.*, 1993). Glucocorticoids modify the expression of molecules in the TGFβ pathway (McCarthy *et al.*, 2000a). Glucocorticoids decrease the stimulatory effects of TGFβ1 on DNA synthesis and collagen synthesis in fetal rat osteoblastic cells and increase the binding of TGFβ1 to betaglycan in primary cultures of fetal rat osteoblastic cells (Centrella *et al.*, 1991). Dexamethasone increases betaglycan mRNA levels in immortalized MC3T3-E1 and RCT1 osteoblastic cells (Nakayama *et al.*, 1994). If the function of betaglycan is to decrease the amount of TGFβ available for signaling, these effects of glucocorticoids would reduce the anabolic effects of TGFβ1 on osteoblastic cells. In fetal rat osteoblasts, glucocorticoids suppress Cbfa1 (Runx2) expression, which is associated with a decrease in the expression and activity of the TGFβRI (Chang *et al.*, 1998).

Glucocorticoids can affect signaling by members of the bone morphogenetic protein (BMP) family of proteins. The molecular pathway by which glucocorticoids enhance osteogenic differentiation *in vitro* may involve BMP pathway signaling. BMP-2 and glucocorticoids exert a synergistic enhancement of the osteogenesis in rat bone marrow stromal cells (Rickard *et al.*, 1994) and fetal rat calvarial cells (Boden *et al.*, 1996). In the calvarial model, BMP-4 and BMP-6 are synergistic with glucocorticoids in promoting osteogenesis (Boden *et al.*, 1996). Moreover, glucocorticoid-dependent differentiation of fetal rat calvarial cells is blocked by antisense oligonucleotides to BMP-6 (Boden *et al.*, 1997). However, in a gene-profiling experiment of MC3T3-E1 cells, dexamethasone added at the time of commitment (near confluency) was shown to antagonize the BMP pathway. Dexamethasone increased the expression of the BMP antagonists *Dan* and *Follistatin* and decreased the expression of *Tieg* (TGF-β-inducible early growth response) (Leclerc *et al.*, 2004), which is stimulated by BMPs (Hefferan *et al.*, 2000).

Plasminogen activator is a serine protease that activates plasminogen to the serine protease plasmin. The plasminogen activator–plasmin system may have a role in bone resorption by activating latent collagenase or TGFβ (Hamilton *et al.*, 1985). Glucocorticoids decrease plasminogen activator activity in normal rodent osteoblasts and UMR 106-01 cells (Hamilton *et al.*, 1985); this is primarily because of an increase in plasminogen activator inhibitor-1 mRNA and protein level (Fukumoto *et al.*, 1992). Glucocorticoid inhibition of plasminogen activator activity, therefore, might limit the activation of locally produced TGFβ, leading to a decrease in bone formation (Fukumoto *et al.*, 1992). However, glucocorticoids enhance the activation of latent TGFβ1 in normal human osteoblastic cells without an alteration of TGFβ1 mRNA levels (Oursler *et al.*, 1993). Such an effect of glucocorticoids might be expected to increase the availability of TGFβ as an anabolic bone growth factor; alternatively, enhanced TGFβ activation might lead to increased bone resorption. Taken together, the effect of glucocorticoids on the TGFβ activity in bone may result from a combination of the actions described earlier.

Prostaglandins

Prostaglandins are produced by bone cells and can affect both bone formation and resorption. Prostaglandins directly inhibit the activity of isolated osteoclasts (Fuller *et al.*, 1989) but increase bone resorption by increasing the formation of new osteoclasts (Dietrich *et al.*, 1975). Prostaglandins have both stimulatory and inhibitory effects on bone formation in organ cultures of rodent calvaria depending on the dose and hormonal milieu that is used (Raisz *et al.*, 1990). Glucocorticoids decrease baseline and agonist-induced prostaglandin production in bone (Hughes-Fulford *et al.*, 1992; Klein-Nulend *et al.*, 1991; Marusic *et al.*, 1991). The mechanisms for this inhibition likely include both a decrease in arachidonic release from membranes and a decrease in the expression of the cyclooxygenases that convert arachidonic acid to prostaglandins. Osteoblasts express two cyclooxygenases, the constitutive prostaglandin synthase-1 (PGHS-1) and the inducible prostaglandin synthase-2 (PGHS-2) (Kawaguchi *et al.*, 1995; Pilbeam *et al.*, 1993). Endogenous glucocorticoids suppress PGHS-2 in mice, and this suppression is relieved when the animals are adrenalectomized (Masferrer *et al.*, 1992). The induction of PGHS-2 by interleukin-1 and PTH in mouse calvaria and by serum in MC3T3-E1 cells is antagonized by glucocorticoids (Kawaguchi *et al.*, 1994). In summary, glucocorticoid inhibition of prostaglandin production in bone occurs primarily by a decrease in agonist-induced PGHS-2 expression.

PREVENTION AND TREATMENT OF GLUCOCORTICOID-INDUCED BONE LOSS

Prevention Recommendations

The severity and rapid onset of the increased risk of fracture with glucocorticoid use, along with the effectiveness of prevention and treatment strategies, have lead several specialty organizations to develop aggressive prevention and treatment recommendations (Recommendations for the Prevention and Treatment of Glucocorticoid-Induced Osteoporosis: 2001 Update. American College of Rheumatology Ad Hoc Committee on Glucocorticoid-Induced Osteoporosis, 2001; Compston, 2004). The American College of Rheumatology recommends a combination of calcium (1500 mg daily), vitamin D (800 IU daily), lifestyle modification (smoking cessation and reduction in alcohol, if excessive), and weight-bearing physical exercise for all patients beginning therapy with glucocorticoids or receiving chronic glucocorticoid therapy. For patients just beginning therapy with glucocorticoids (prednisone or equivalent ≥5 mg daily) with plans for a treatment duration of at least 3 months, bisphosphonate (alendronate or risedronate) therapy is recommended. Calcitonin is recommended as a second-line agent if the patient has intolerance or a contraindication to a bisphosphonate. For patients who are receiving chronic glucocorticoid therapy, replacement of gonadal sex hormones if deficient or otherwise clinically indicated is recommended. For patients receiving chronic glucocorticoid therapy, bisphosphonate therapy is recommended if the BMD is below normal (*T* score below −1). Again, calcitonin is recommended if there is a contraindication or intolerance to a bisphosphonate. The Royal College of physicians recommends bisphosphonates for primary prevention if the patient is older than 65 years of age or has a history of fragility fracture. They recommend bisphosphonate therapy based on a BMD *T* score less than −1.5 or with a reduction in BMD of more than 4% after one year.

Calcium and Vitamin D

Evidence supports the effectiveness of calcium and vitamin D in preventing the bone loss associated with glucocorticoid therapy. Calcium alone does not appear to prevent the bone loss observed in glucocorticoid-treated patients compared with no therapy (Amin *et al.*, 1999). In the randomized placebo-controlled trials investigating the effectiveness of bisphosphonate therapy, both treatment and placebo groups received calcium and vitamin D therapy. Bone density in the lumbar spine was maintained in the group receiving calcium and vitamin D without bisphosphonate (Cohen *et al.*, 1999; Saag *et al.*, 1998). Meta-analysis of retrospective clinical trails with calcium and vitamin D demonstrate improved bone density in the spine and radius for glucocorticoid-treated patients receiving calcium and vitamin D (Amin *et al.*, 1999; Homik *et al.*, 2000b). The active vitamin D metabolites, alphacalcidiol and calcitriol, have also been shown effective in the prevention of bone loss in patients starting glucocorticoid therapy. These agents may have an additional direct effect to increase bone mass or reduce bone resorption (Reginster *et al.*, 1999; Sambrook *et al.*, 1993). However, there was significant toxicity in the patients treated with calcitriol and calcium with nearly 25% of patients developing hypercalcemia. A meta-analysis estimating the effects of vitamin D and calcitriol on bone loss did not demonstrate an improved effect of calcitriol over vitamin D_3 (Amin *et al.*, 2002). Therefore, until further studies provide more information, it seems most appropriate to provide vitamin D_3 as opposed to the active metabolites. At present, there is a lack of prospective data to confirm a reduction in fracture risk with calcium and vitamin D in glucocorticoid-treated patients (Homik *et al.*, 2000b). Patients receiving calcium and vitamin D should have urinary calcium excretion determined over a 24-hour period. Patients who excrete more than 300 mg may benefit from a low-dose thiazide diuretic and dietary sodium restriction. Serum potassium should be monitored and replaced as needed. Hypokalemia may be avoided with the use of a combination potassium-sparing diuretic such as amiloride. Long-term effects of thiazide diuretics on bone loss in glucocorticoid-treated patients have not been done but, in the absence of glucocorticoid therapy, thiazide use is associated with higher bone density and reduction in fracture risk (Cauley *et al.*, 1993).

Bisphosphonates

Bisphosphonate therapy has become an important part of the prevention and treatment of GIOP because evidence has shown it results in improvement in bone density and a reduction in the risk of fracture (Adachi *et al.*, 2001; Cohen *et al.*, 1999; Reid *et al.*, 2000; Saag *et al.*, 1998; Wallach *et al.*, 2000). In a study of 477 men and women, alendronate, calcium, and vitamin D increased the bone density of the spine and hip compared with the group receiving only calcium and vitamin D; additionally, there was a reduction at 2 years in vertebral fractures (Saag *et al.*, 1998). The effect of alendronate was greater in postmenopausal women than in premenopausal women. In a prospective placebo-controlled trial with risedronate, there was a 70% overall reduction in the relative risk for vertebral fracture, 66% reduction in men, and 73% reduction in postmenopausal women. There were no fractures observed in premenopausal women (Wallach *et al.*, 2000). A recent meta-analysis of bisphosphonates for GIOP examined 13 controlled clinical trials reporting on 842 participants. All 13 studies reported data on lumbar spine BMD. On average, the lumbar spine BMD of the bisphosphonate-treated groups was 4% higher than the group not receiving bisphosphonates. Four of the trials reported data for the femoral neck BMD, which was 2.1% higher in the bisphosphonate-treated groups (Homik *et al.*, 2000a). In this meta-analysis, there was a 24% reduction in the odds of vertebral fracture, which did not reach statistical significance. Pamidronate has also been demonstrated to increase vertebral bone density in patients treated with glucocorticoids when given orally or intravenously. An intravenous route of 90 mg once yearly or 90 mg initially followed by 30 mg intravenously every 3 months both prevented bone loss (Boutsen *et al.*, 2001; Reid *et al.*, 1988). Etidronate has also been shown to improve bone density in glucocorticoid-treated patients when given cyclically with calcium and vitamin D (Amin *et al.*, 2002). There are no fracture reduction data available with the use of pamidronate or etidronate in glucocorticoid-treated patients. At present, alendronate and risedronate have Food and Drug Administration (FDA) approval for use in prevention and treatment of GIOP.

Hormone Replacement

Evidence suggests that postmenopausal women are at greatest risk for GIOP. In a trial of women receiving glucocorticoids for rheumatoid arthritis (RA), individuals were randomized to receive hormone replacement therapy (HRT) or placebo. A 2% increase in lumbar spine density was observed in the HRT group compared with controls (Hall *et al.*, 1994). In another study of 15 women receiving glucocorticoid therapy for asthma, HRT was associated with an increase in bone density of the lumbar spine at 1 year (Lukert *et al.*, 1992). There are no data showing the effect of HRT on hip BMD in glucocorticoid-treated patients and no trials at present demonstrating fracture reduction with HRT in glucocorticoid-treated patients. The recent report from the Women's Health Initiative demonstrated an increased risk of breast cancer and cardiovascular disease in postmenopausal women treated with long-term HRT (Rossouw *et al.*, 2002). The routine use of HRT in postmenopausal women for prevention of GIOP may not be indicated in light of these results. Raloxifene, a selective estrogen receptor modulator, has been shown to increase vertebral BMD in postmenopausal women and reduce vertebral fracture risk; however, there are few data on its use in GIOP (Ettinger *et al.*, 1999).

Glucocorticoid therapy is associated with a reduction in testosterone in men. A recent randomized placebo-controlled trial examined the effects of testosterone or nandrolone replacement in 51 glucocorticoid-treated men. The mean dose of glucocorticoids was 12.6 mg of prednisone daily. At 12 months, both testosterone and nandrolone increased muscle mass significantly over placebo by 3.5% and 5.8%, respectively. Muscle strength was increased as well. Testosterone increased the BMD of the lumbar spine significantly by 4.7%. There was no significant change seen with nandrolone and no significant change demonstrated with testosterone or nandrolone for BMD of the hip or total body. Testosterone but not nandrolone was associated with an improvement in quality-of-life assessment. As testosterone but not nandrolone may undergo peripheral aromatization, the lack of effect of nandrolone on BMD suggests that aromatization of testosterone may be important for the effect on bone density (Crawford *et al.*, 2003). These data would support the current recommendation to replace testosterone in testosterone-deficient men receiving glucocorticoid therapy (Recommendations for the Prevention and Treatment of Glucocorticoid-Induced Osteoporosis: 2001 Update. American College of Rheumatology Ad Hoc Committee on Glucocorticoid-Induced Osteoporosis, 2001; Compston, 2004). There are no data at present demonstrating a reduction in fracture with testosterone replacement in GIOP.

There are few studies examining HRT in premenopausal women receiving glucocorticoid therapy. Menstrual irregularities may occur in these patients owing to the effects of glucocorticoid therapy. In premenopausal women athletes with oligomenorrhea or amenorrhea, oral contraceptive therapy has been associated with a higher BMD than a comparison group who did not take oral contraceptive therapy (Drinkwater *et al.*, 1986). It seems reasonable, until further information is available in this area, to offer oral contraceptives or cyclic estrogen and progesterone if no other contraindications exist.

CALCITONIN

Calcitonin has been shown to reduce bone loss in the spine in patients receiving glucocorticoid therapy. A recent meta-analysis studied the effects of nine retrospective clinical trials investigating calcitonin for GIOP in 221 patients. The BMD of the spine was increased over placebo at all time points from 6 to 24 months. The weighted mean difference at 24 months was 4.5%. There was an increase in the radius BMD of 3.1% at 12 months but not at 24 months. There was no difference in BMD at the femoral neck and there have been no trials to date demonstrating a reduction in fracture risk with calcitonin treatment for GIOP (Cranney *et al.*, 2000). This evidence supports the use of calcitonin as a second-line drug if bisphosphonate therapy is contraindicated or not tolerated.

Parathyroid Hormone and Other Anabolic Agents

PTH is the most encouraging of the agents at present that simulates bone formation. A recent 18-month randomized controlled trial studied 428 men and women receiving prednisone equivalent to 5 mg daily for at least 3 or more months (Saag *et al.*, 2007). Subjects were randomized to teriparatide at 20 μg daily or alendronate at 10 mg daily. The bone density of the lumbar spine increased more in the teriparatide group than the alendronate group (7.2±0.7% versus 3.4±0.7%, $P < 0.001$). At 18 months, there was a greater improvement in the density of the total hip in the teriparatide group compared with the alendronate group (3.8±0.6% versus 2.4±0.6%, $P = 0.0005$). Though fracture was not the primary outcome there were fewer vertebral fractures in the teriparatide group compared with the alendronate group (0.6 versus 6.1%, $P = 0.004$). The nonvertebral fractures were greater in the teriparatide group, but this did not reach significance (5.6% versus 3.7%, $P = 0.36$). In this prospective trial of glucocorticoid-treated patients at high risk for fracture, there was greater bone density gain with teriparatide in both the hip and spine and significantly less fracture of the spine over the 18-month trial. An earlier randomized placebo-controlled trial over a 12-month time period studied 49 women receiving glucocorticoids (5–20 mg prednisone per day). All women received HRT. Subjects had a baseline T score at the femoral neck or spine of less than -2.5. Women receiving PTH and HRT demonstrated an increase in BMD of the spine of 11.9% over baseline at 12 months. Women receiving only HRT had a stable spine BMD of +1% over baseline. The BMD changes at the radius and hip were not significant (Lane *et al.*, 1998b). A follow-up study examined this group 12 months after discontinuing PTH. The gain in BMD in the spine by DXA was maintained at 13% over the baseline pretreatment BMD. At 24 months (12 months off PTH) there was a 4.7% increase in BMD at the femoral neck. BMD of the lumbar spine and femoral neck remained unchanged in the group receiving HRT only (Lane *et al.*, 2000). These results are encouraging and for patients at highest risk for fracture PTH therapy may be the best initial choice.

Sodium fluoride has been studied in GIOP along with calcium and vitamin D and in combination with cyclical etidronate. In these studies, fluoride was associated with an increase in BMD in the spine but not in the hip. In the group receiving etidronate plus fluoride the fractures were higher than the group receiving only etidronate (Lems *et al.*, 1997a, 1997b). In light of the lack of data on the reduction of fracture risk and data suggesting a possible increased risk of fracture with sodium fluoride, it is not currently recommended for the treatment of GIOP.

SUMMARY AND CONCLUSIONS

A variety of *in vitro* models have been developed to examine the direct effects of glucocorticoids on bone formation and resorption. These experiments show that glucocorticoids have diverse and complex direct effects on bone and can modify the expression of a wide variety of genes in osteoblastic cells. It is likely that the experimental outcomes in different models are affected by the concentration of glucocorticoid used, the timing of glucocorticoid addition, the presence of serum and growth factors, and the developmental stage of the model and species differences. However, several general principles can be drawn from these studies. Glucocorticoids can either stimulate or inhibit bone formation *in vitro* and these effects depend on the developmental stage of the model. Low concentrations of glucocorticoids are permissive for hormone action in bone (allowing other hormones to have optimal activity) and are associated with increased osteoblastic differentiation and bone formation. The daily secretion of physiological concentrations of cortisol may render osteoblasts and/or osteoprogenitor cells highly responsive to the effects of systemic and locally produced hormones. The ability of glucocorticoids to enhance the activity of some anabolic hormones may represent a compensatory response that helps maintain bone mass during periods of diminished growth factor supply. Pharmacological doses of glucocorticoids inhibit osteoprogenitor proliferation, osteoblast renewal, osteoblast function, and osteoblast and osteocyte apoptosis; glucocorticoids also increase osteoclast formation and life span. These multiple actions lead to a decrease in bone mass and quality (see Fig. 1). Understanding the molecular pathways that mediate these diverse effects of glucocorticoids will enable the development of effective therapeutic modalities for GIOP.

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Effects of Diabetes and Insulin on Bone Physiology

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INTRODUCTION

The term "diabetic bone disease" is used to describe the changes in bone growth, remodeling, and density as well as fracture risk imparted by the presence of type 1 diabetes (T1DM) or type 2 diabetes (T2DM). T1DM is caused by pancreatic β-cell destruction, usually leading to an absolute insulin deficiency; Northern Europeans are among the populations with the highest prevalence of T1DM. However, T2DM is currently by far the most prevalent form of diabetes in all continents. Its pathophysiology is heterogeneous, ranging from predominantly insulin resistance – i.e., at any of the classical insulin-sensitive tissues: liver, skeletal muscles, and adipose tissue – with relative insulin deficiency, to a predominantly insulin secretory defect with variable insulin resistance. Hence, insulin levels in T2DM vary widely, anywhere between hyper- and hypoinsulinemia. The majority of patients with T2DM are obese, and obesity itself aggravates insulin resistance. Body weight is, therefore, an important confounding factor when examining the pathophysiology of bone in T2DM individuals.

EFFECT OF DIABETES AND INSULIN ON ENDOCHONDRAL BONE GROWTH

T1DM and Skeletal Growth and Maturation in Children

At onset of T1DM, there is no difference in height compared with nondiabetic children. In fact, some but not all studies have documented that children are slightly taller at onset of diabetes compared with reference values (Holl *et al.*, 1998).

However, growth is affected from the clinical onset onward. In the preinsulin era, prepubertal growth virtually stopped, and stunted growth (Mauriac syndrome) was frequently observed in later decades of irregular insulin treatment. In addition, there was a delay in pubertal development and growth. Retarded growth and pubertal development remain common to this day among African children with T1DM (Elamin *et al.*, 2006). In Western countries, the majority of recent studies documented a mild reduction in growth from height [***] (*z*) charts. This deficit was more pronounced in children with a prepubertal compared to a pubertal onset of T1DM (Holl *et al.*, 1998). Poor glycemic control predicts growth retardation: indeed, glycohemoglobin levels (a reflection of glycemic control in the previous two to three months) correlated inversely with height velocity (Danne *et al.*, 1997; Holl *et al.*, 1998). In one study, final height was reduced by a median of 0.5 SD, or 2–3 cm (Danne *et al.*, 1997).

At diagnosis, bone maturation – determined by radiographs of hand and wrist – was not different compared with nondiabetic children (Holl *et al.*, 1994; Danne *et al.*, 1997). However, there was a small but significant retardation of bone maturation with increasing T1DM duration (i.e., a one-year difference between chronological and bone age after eleven years of diabetes) (Holl *et al.*, 1994).

Skeletal Growth in T1DM Animal Models

The most frequently used animal models are rats or mice with T1DM induced by β-cell-destroying drugs: alloxan or – in the large majority of studies – streptozotocin (SZ); and BB (Bio-Breeding) rats with spontaneous immune-mediated diabetes. Although T1DM can be drug-induced at any age, BB rats develop diabetes past the peak growth rate (~7 weeks). In both rodent models, insulin levels are very low or undetectable. The growth plate width as well as the endochondral bone growth – assessed by double fluorochrome labeling of the calcifying cartilage – at the proximal tibia were consistently lower in untreated, severely hyperglycemic SZ-induced or spontaneously diabetic BB

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[***] age-matched standard deviation(Z) charts

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TABLE I Histomorphometric Data from Proximal Tibial Metaphysis in Untreated and Insulin-Treated Male Spontaneously Diabetic BB Rats[a]

	Nondiabetic ($n = 14$)	Diabetic ($n = 11$)	Diabetic + insulin ($n = 15$)
Growth plate width (μm)	178 (8)	135 (8)***	230 (9)***,‡
Osteoblast surface (%)	1.5 (0.3)	0.04 (0.04)***	4.3 (0.8)**,‡
Osteoid surface (%)	1.5 (0.4)	0.04 (0.04)**	4.8(1.0)**,‡
Osteoclast surface (%)	0.4 (0.1)	0*	0.5 (0.2)†

[a] *From* Verhaeghe et al. (1992), *with permission of the* Journal of Endocrinology Ltd. *Measurements were performed about* 4 *weeks after onset of diabetes in diabetic rats and in nondiabetic littermates. Insulin-treated rats were treated with* 3 U/day *of insulin, infused* SC *with a miniosmotic pump for* 14 *days.* Data *are expressed as means* (SEM). Statistical *analysis:**, *versus nondiabetic rats* ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$);†, *versus diabetic rats* ($^{\dagger}P < 0.05$,$^{\ddagger}P < 0.001$).

rats, and was remedied by systemic insulin treatment (Bain *et al.,* 1997; Epstein *et al.,* 1994; Scheiwiller *et al.,* 1986; Verhaeghe *et al.,* 1992) (Table I). The femur was shorter after twenty-eight days of SZ-diabetes (Lucas, 1987).

The effect of insulin on bone growth might be direct and/or indirect, for example by restoring the depressed hepatic release of insulin-like growth factor-I (IGF-I). Although it was reported that systemic administration of IGF-I in T1DM rats partly normalized growth plate width (Scheiwiller *et al.,* 1986), we did not corroborate this finding (Verhaeghe *et al.,* 1992). The low $^{35}SO_4$ uptake by rib cartilage explants from T1DM rats was also unresponsive to exogenous IGF-I (Kelley *et al.,* 1993). These data suggest that diabetic cartilage is, at least in part, resistant to the actions of IGF-I.

Effects of Insulin on Cartilage in Nondiabetic Animal Models and *in Vitro*

The classic experiments of Salter and Best (1953) demonstrated that insulin administration increased growth plate width in hypophysectomized rats. This could be a local effect, because insulin injection into the proximal tibial growth plate (Heinze *et al.,* 1989) or insulin infusion into one hind limb (Alarid *et al.,* 1992) produced widening of the treated growth plates only. Such local effect might be mediated by *in situ* production of IGF-I, because the trophic effect of insulin was nullified by coinfusion of an IGF-I antiserum (Alarid *et al.,* 1992). Mice with absent expression of insulin receptors in bone showed normal bone length (Irwin *et al.,* 2006). However, they overexpressed the IGF-I receptor, which could represent a compensatory response.

In vitro studies have documented the presence of insulin receptors in a chondrosarcoma cell line (Foley *et al.,* 1982). Chondrocyte proliferation and $^{35}SO_4$ incorporation were stimulated by insulin in several tissue and cell culture systems (Foley *et al.,* 1982; Heinze *et al.,* 1989; Maor *et al.,* 1993). These effects were obtained at physiological levels of insulin, as low as 1n*M*, but equimolar IGF-I was more potent than insulin.

Collectively, the available data suggest that IGF-I is more important than insulin for chondrocyte proliferation and maturation, but further research is mandatory.

EFFECTS OF DIABETES AND INSULIN ON BONE IN HUMANS

Introductory Remarks

Two critical issues hamper the interpretation of published data on bone in diabetic individuals. First, there is the sample size and heterogeneity issue: series are often small – especially for T1DM, and most contained female and male subjects with varying age, pubertal stage, diabetes duration, long-term glycemic control, and current insulinization; a variable proportion exhibited diabetes complications (retinopathy, neuropathy, atherosclerotic disease) that may have affected physical activity and capabilities; and some of the individuals smoked and/or used other medications than insulin – e.g., statins and thiazolidinediones (TZDs), which may have influenced bone mineral density (BMD) and fracture risk, as we will discuss later. Second, there is the methodology issue, because bone and mineral measurements present specific problems in diabetic subjects. A few examples suffice to illustrate this. Diabetes affects body weight and bone size, which must be taken into account when interpreting areal bone mineral density (aBMD) results obtained by dual-energy x-ray absorptiometry (DXA), currently the reference densitometry technique. Serum total alkaline phosphatase is elevated in T1DM subjects (Bouillon *et al.,* 1995) owing to hypersecretion of the intestinal and hepatic but not the bone isoenzyme (Tibi *et al.,* 1988). Similarly, raised serum osteoprotegerin levels in T1DM subjects may well be derived from the vascular wall rather than bone (Rasmussen *et al.,* 2006). Finally, diabetes-induced changes in collagen metabolism in tissues other than bone may interfere with the measurement of the excretion of collagen breakdown products, particularly hydroxyproline or pyridinoline cross-links. Yet, in spite of these limitations, robust data have emerged over the last two decades that have much clarified the nature of diabetic bone disease.

T1DM and Bone Remodeling

Bone histomorphometry data are lacking, and the measurement of biochemical markers of bone formation/mineralization produced variable results. Yet in the largest study to date (229 T1DM individuals), Bouillon *et al.* (1995) documented that serum osteocalcin concentrations were 24–28% lower among diabetic children, adolescents, and adults compared to age- and gender-matched nondiabetic subjects (Fig. 1). Serum bone-specific alkaline phosphatase was decreased by 24% in diabetic adolescents, but there was no change among diabetic adults; serum PICP (procollagen carboxyterminal extension peptide) was unchanged in adolescents and adults. Of note, serum osteocalcin concentrations were well below the control range in children with new-onset T1DM but returned to the control range following two weeks of insulin treatment, and osteocalcin and glycohemoglobin levels were inversely correlated (Guarneri *et al.,* 1993).

No definitive data have been produced regarding biochemical markers of bone resorption in T1DM. In some studies, resorption markers were unchanged, or even slightly increased. Yet, total and free deoxypyridinoline excretion measured by high-performance liquid chromatography (HPLC) – the gold standard biochemical method to assess bone resorption – was reduced by ~20% in 84 adult T1DM individuals compared with 99 controls, and the decrease was proportional to their glycosuria (Cloos *et al.,* 1998).

T1DM and Bone Density

Thrailkill *et al.* (2005b) summarized the data obtained from numerous but generally small studies in T1DM children and adolescents, using a variety of methods [radiogrammetry, single and dual photon absorptiometry, DXA and quantitative computed tomography (QCT)]. Most though not all studies documented a mild reduction in bone area and BMD (~10% difference with controls, on average), but the average BMD *z* score in the diabetic group was generally at or above -1.0 SD. The differences were somewhat more pronounced when expressed relative to height or muscle mass; however, the reduction in volumetric BMD (vBMD, expressed in g/cm^3) appeared to be smaller than that in bone mineral content (BMC, in g) or aBMD (expressed in g/cm^2) (Moyer-Mileur *et al.,* 2004).

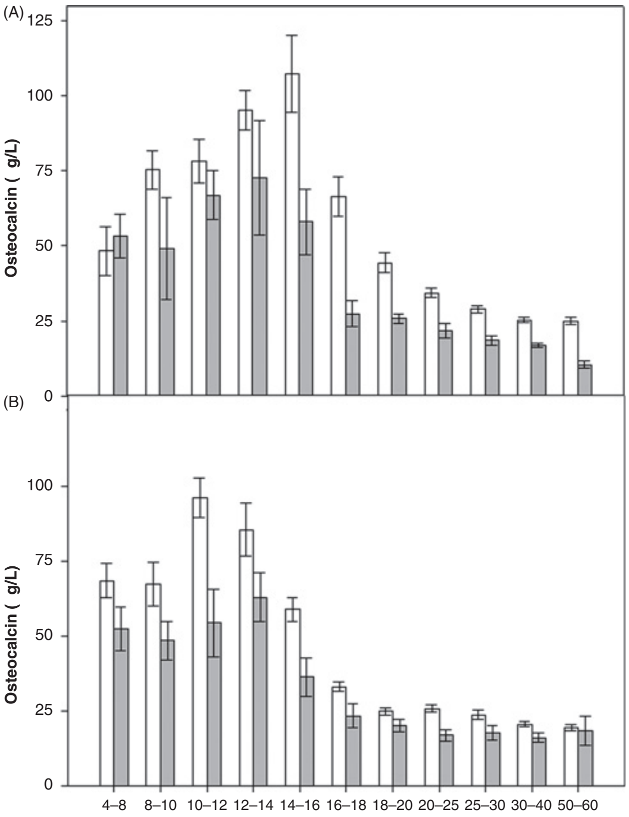

FIGURE 1 Serum osteocalcin concentrations in normal individuals (open bars) and individuals with type 1 diabetes (hatched bars): **(A)** male and **(B)** female. Data are presented as means ± SEM. Multiple regression analysis showed a positive correlation between age and serum osteocalcin levels below 16 and 12 years for boys and girls, respectively, and a negative correlation above those ages ($p < 0.001$). A significant ($p < 0.005$) decreasing effect of diabetes on serum osteocalcin was observed for both genders. From Bouillon *et al.* (1995), with permission. © The Endocrine Society.

Comparable data were obtained for aBMD measurements at the lumbar spine and hip in T1DM adult women before menopause (Strotmeyer *et al.,* 2006). Thus, the available data indicate that there is an early but mild BMD deficit in T1DM individuals that does not appear to deteriorate over time (Krakauer *et al.,* 1995; Moyer-Mileur *et al.,* 2004). Whether or not long-term glycemic control is a determinant of this BMD deficit remains a matter of controversy (Thrailkill *et al.,* 2005b). The aBMD deficit is more pronounced among T1DM adults with peripheral neuropathy (Rix *et al.*, 1999) or other microvascular complications (Strotmeyer *et al.*, 2006). The dynamics of bone loss during the peri- and postmenopausal years among T1DM women has not been investigated to date.

T1DM and Fractures (Table II)

The available epidemiologic studies concur that the risk of fractures is substantially increased in individuals with T1DM, more than could be explained by the mild BMD deficit – in effect, a 1-SD reduction in aBMD would translate into a twofold increased risk of hip fracture (Johnell *et al.*, 2005). A caveat is that many studies contained few incident fractures in the diabetic group (n = 1–18). The most robust evidence comes from a Swedish study, which identified >24,000 individuals hospitalized before age 31 for diabetes (presumably T1DM in the large majority of them). The incidence of hip fracture through 13 years was compared with that in the general population, and showed an eight- to tenfold elevated risk (Miao *et al.*, 2005). In another Swedish study, diabetes (presumably T1DM in the majority) was an independent risk factor for hip fracture among middle-aged men and women, and an independent risk factor for vertebral fracture among middle-aged women (Holmberg *et al.*, 2006).

T2DM and Bone Remodeling

Like T1DM, T2DM is accompanied by low bone formation and remodeling. In a small but interesting series of eight diabetic subjects (ages 37–67, diabetic for 2–36 years, 6 with T2DM), a transiliac bone biopsy was performed because of a low BMD; histomorphometry showed a significant decrease in osteoid thickness, mineralizing surface and mineral apposition rate (Krakauer *et al.*, 1995). Biochemical bone remodeling markers are also lower than in nondiabetics. This was demonstrated, for example, in middle-aged women (ages 42–52, from various ethnic backgrounds) examined in the Study of Women's Health Across the Nation (SWAN): diabetes (167/2,374 women, presumably T2DM in the majority of them) was associated with reduced serum osteocalcin concentrations and urinary N-telopeptide of type I collagen (uNTx) excretion (Sowers *et al.*, 2003). In Pima Indians, osteocalcin levels correlated negatively with postload glucose levels (Bouillon *et al.*, 1995). Similar findings were obtained in European women living in a nursing home (age 83 on average): serum osteocalcin and C-terminal telopeptide cross-links (β-CTxs) concentrations were 10–15% lower in 360 women with diabetes than in 588 women without diabetes (Dobnig *et al.*, 2006). Thus, reduced bone remodeling persists in elderly diabetic individuals. Improved glycemic control augmented serum osteocalcin concentrations and urinary pyridinoline excretion (Rosato *et al.*, 1998).

TABLE II Fracture Risk in Individuals with T1DM

First author (year)	Country (Study)	Sample size (Age at baseline)	Follow-up	Outcome#	N with diabetes (+ fractures)	Age-adjusted RR (95%CI)
Meyer *et al.* (1993)	Norway	24,513 ♂ (35–49 y) 23,979 ♀	10–13 y	Hip #	180 (3)* 118 (4)*	7.7 (2.4–24.5) 5.8 (2.15–15.7)
Forsén *et al.* (1999)	Norway	13,238 ♂ (50–74 y) 13,708 ♀	1986–95	Hip #	28 (1)[†] 23 (3)[†]	4.0 (0.6–28.2) 5.7 (1.8–17.9)
Nicodemus (2001)	Iowa, U.S.A.	30,424 ♀ (55–69 y)	1986–97	Hip #	47 (5)[†]	14.1(5.85–34.2)
Miao *et al.* (2005)	Sweden	12,551 ♂ (<30 y) 12,054 ♀	1975–98	Hip #	100% (70)[†] 100% (51)[†]	7.6 (5.9–9.6) 9.8 (7.3–12.9)
Janghorbani (2006)	U.S.A. (Nurses' Health)	101,635 ♀ (34–59 y)	1980–2002	Hip #	292 (18)[†]	7.1 (4.4 –11.4)
Ahmed *et al.* (2006)	Tromsø, Norway	12,691 ♂ (25–98 y) 14,094 ♀	1994–2001	Non-vertebral #	52 (5)[†] 29 (3)[†]	3.1 (1.3–7.4) 3.0 (0.98–9.4)
Holmberg *et al.* (2006)	Malmö, Sweden	22,444 ♂ (44–50 y) 10,902 ♀	19 (7–25) y 15 (7–22) y	Any low-energy #	218 (31)* 105 (27)*	2.5 (1.75–3.6) 1.95 (1.3–2.9)

#, *fracture*; N: *number*; RR: *relative risk*; *y*: *years*

*: *any type of diabetes* (*Scandinavian countries*); [†]: *specified as* T1DM

Hyperinsulinemia and Bone Density

Hyperinsulinemia is the compensatory answer to hepatic and muscle insulin resistance; when inadequate, impaired fasting glucose and/or postload hyperglycemia (impaired glucose tolerance) ensue. Several population studies have demonstrated a correlation between – on the one hand – fasting insulin, the HOMA insulin resistance index, and/or postchallenge glucose and insulin concentrations and – on the other hand – the aBMD at the hip and/or the spine (Barrett-Connor and Kritz-Silverstein, 1996; Dennison *et al.*, 2004; Stolk *et al*, 1996; Thomas *et al.*, 2001). Yet adjusting for body mass index (BMI) or fat mass attenuated or abolished the correlation. Thus, insulin is thought to mediate the link between fat mass and aBMD, in addition to other hormonal mediators such as leptin and estrogens.

Established T2DM and Bone Density

The seminal report by Meema and Meema (1967) that the cortical thickness at the radius was higher in aged (65–101 years) white women with T2DM than in nondiabetics was followed by numerous studies using DXA. The majority of these studies showed that aBMD is mildly increased in T2DM subjects, even after controlling for BMI (Thrailkill *et al.*, 2005b). For example, in the Study of Osteoporotic Fractures, T2DM ($n \approx 500$) was associated with a 4.8% increase in aBMD at the radius and a 3.4% increase in aBMD at the femoral neck in multivariate analyses (Bauer *et al.*, 1993; Orwoll *et al.*, 1996). In the Rotterdam Study, lumbar spine and the femoral neck aBMD were also 3–4% higher in T2DM individuals ($n = 578$) (van Daele *et al.*, 1995). Similarly, in the Health ABC Study, total hip aBMD was 4–5% higher in black and white men and women with T2DM ($n = 566$) after adjusting for their altered body composition (i.e., higher fat and lean mass) (Strotmeyer *et al.*, 2004). However, total hip aBMD was inversely related to T2DM duration (Strotmeyer *et al.*, 2004), and white women with T2DM showed increased aBMD loss compared with their nondiabetic counterparts (Schwartz *et al.*, 2005); in addition, the aBMD of insulin-treated T2DM individuals was not different from that of controls in some studies (van Daele *et al.*, 1995; Tuominen *et al.*, 1999). The data suggest that aBMD decreases with waning β-cell function and insulin secretion. Indeed, aBMD in T2DM subjects correlated with fasting insulin and urinary C-peptide levels (Wakasugi *et al.*, 1993).

The contrasting effects of T1DM and T2DM on aBMD were confirmed in a Finnish study (Tuominen *et al.*, 1999). They examined subjects (ages 52–72) who developed diabetes after 30 years of age (i.e., after achievement of peak bone mass) and were insulin-treated, but differed in their baseline insulin secretory response (C-peptide levels after glucagon). Subjects with deficient insulin secretion (classified as T1DM) showed a lower aBMD at the hip than subjects with normal insulin secretion (classified as T2DM) or controls. After adjusting for age, BMI, and other factors, the difference was attenuated but still demonstrable.

However, the effects of T2DM on vBMD were much less pronounced than those on aBMD, and generally not significant (Register *et al.*, 2006; Strotmeyer *et al.*, 2004), indicating that T2DM induces larger rather than denser bones.

T2DM and Fractures

Table III shows that the risk of fractures is elevated in individuals with T2DM, but much less so than in T1DM. The largest cohorts – the Study of Osteoporotic Fractures (Schwartz *et al.*, 2001) and in particular the Women's Health Initiative Observational Study (Bonds *et al.*, 2006) – both confirmed a mildly increased risk (between one- and twofold). The latter study documented an elevated age-adjusted risk of fracture at all skeletal sites except the forearm/wrist/hand.

Limited evidence indicates that diabetes (T1DM or T2DM) delays the healing of clinical fractures, taking about twice as long (Loder, 1988).

Clinical Risk Factors for Fractures in T1DM and T2DM (Table IV)

Regarding T1DM, the Swedish study (Miao *et al.*, 2005) reported that the age-adjusted risk of hip fracture increased with longer diabetes duration. Longer diabetes duration means a greater likelihood of micro- and macrovascular complications; indeed, the risk of fracture was particularly high among T1DM patients with eye, nephropathic, neurologic, or cardiovascular complications. When controlling for diabetes complications, the risk of hip fracture among Norwegian T1DM patients was no longer significantly elevated (Forsén *et al.*, 1999).

For T2DM, the elevated risk of fractures was observed in both white and black U.S. women (Bonds *et al.*, 2006). BMI is not a critical factor, because the risk was comparably elevated in nonobese and obese T2DM women, and controlling for BMI did not meaningfully alter the risk ratio (Janghorbani *et al.*, 2006; Nicodemus and Folsom, 2001; Schwartz *et al.*, 2001). In some studies, the risk appeared to be higher among insulin-treated versus non-insulin-treated T2DM individuals (Janghorbani *et al.*, 2006; Ottenbacher *et al.*, 2002). This may reflect the association between longer duration of T2DM and fracture: in individuals with >12 years of T2DM, the risk was increased more than twofold (Janghorbani *et al.*, 2006; Nicodemus and Folsom, 2001). Again, longer diabetes duration means more complications. In the Australian Blue Mountains Eye Study, which examined 216 subjects (ages 49–97) with T1DM or T2DM, cortical cataract and especially retinopathy increased the risk of fractures (Ivers *et al.*, 2001). Neuropathy with or without

TABLE III Fracture Risk in Individuals with T2DM

First author	Country	Sample size	Follow-up	Outcome	N with diabetes	Age-adjusted
(Year)	(Study)	(Age at baseline)			(+ fractures)	RR (95%CI)
Forsén (1999)	Norway	13,430 ♂ (50–74 y) 2963 ♂ (≥ 75 y) 13,959 ♀ (50–74 y) 4027 ♀ (≥75 y)	1986–95	Hip #	220 (4)* 118 (7)* 274 (20)* 196 (26)*	1.0 (0.4–2.6) 1.1 (0.6–2.5) 1.7 (1.1–2.7) 1.4 (0.9–2.0)
Schwartz (2001)	U.S.A. (SOF)	9,654 ♀ (≥65 y)	9.4±2.4 y	Nonvertebral #	551 (162)† 106 (36)‡	1.16(0.99–1.37) 1.58 (1.14–2.2)
Nicodemus (2001)	Iowa, U.S.A.	32,059 ♀ (55–69 y)	11 y	Hip #	1682 (38)	1.75 (1.25–2.43)
de Liefde (2005)	the Netherlands	2691 ♂ (≥55 y) 3964 ♀	6.8±2.3 y	Nonvertebral #	309 (25) 483 (79)	1.34 (0.87–2.06) 1.03 (0.81–1.31)
Strotmeyer (2005)	U.S.A. (Health ABC)	2802 ♂ + ♀ (70–79 y)	4.5±1.1 y	Any #	566 (29)	1.23(0.82–1.86)§
Ahmed (2006)	Tromsø, Norway	12,814 ♂ (25–98 y) 14,263 ♀	1994–2001	Nonvertebral #	175 (9) 198 (23)	1.19(0.61–2.31) 0.89(0.59–1.35)
Bonds (2006)	U.S.A. (WHI)	93,405 ♀ (PM)	7 y	Any # Vertebral # Hip/pelvis/femur #	5,285 (899) (99) (128)	1.29(1.20–1.38) 1.28 (1.04–1.56) 1.41(1.17–1.70)
Dobnig (2006)	Austria	1664 ♀ (>70 y)	2 y	Hip #	583 (81)	0.90(0.60–1.34)
Janghorbani (2006)	U.S.A. (Nurses'Health)	109,691 ♀ (34–59 y)	1980–2002	Hip fracture	6969 (83)† 1379 (36)‡	1.4 (1.1–1.8) 2.4(1.8–3.5)

#:*fracture*, N: *number*; RR: *relative risk*; *y*: *years*; PM: *postmenopausal*

*: T2DM > 5 *years*

†: T2DM *not treated by insulin*; ‡ T2DM *treated by insulin*

§: *age- and sex-adjusted*

foot ulcers was another important predisposing factor for fractures in general (Strotmeyer *et al.*, 2005), and foot fractures in particular (Cavanagh *et al.*, 1994). Diabetic individuals with eye, neuropathic, and vascular complications – and poor physical health in general – are more likely to fall. Indeed, insulin-treated T2DM individuals showed a fourfold increased risk of falls (Schwartz *et al.*, 2002), and repeated falls in diabetic individuals with a foot ulcer predisposed to fractures (Wallace *et al.*, 2002). It is likely, therefore, that falls mediate a significant part of the diabetes-associated risk of fractures. As a corrolary, effective fall-prevention strategies should be developed to prevent fractures in individuals with long-standing diabetes or diabetes complications.

POSSIBLE MECHANISMS OF FRACTURES IN T1DM AND T2DM (Table IV)

T1DM bones are smaller and show a mild deficit in BMD (∼10% or ∼1 SD). Smaller bones are predisposed to brittleness and damageability under loading conditions (Tommasini *et al.*, 2005). As mentioned earlier, an average BMD *z* score of -1.0 in T1DM individuals would translate in about a doubling of the fracture risk (1.73 relative risk of any osteoporotic fracture, and 2.11 relative risk of hip fracture) (Johnell *et al.*, 2005).

In addition, both T1DM and T2DM are associated with reduced bone formation and remodeling. Interestingly, there appears to be a J- or U-shaped relationship between bone remodeling activity and fracture risk (Heaney, 2003). It has been postulated that diabetes impairs the healing of fatigue microfractures (also called microdamage, or microcracks) in load-bearing bones because of suppressed bone formation ("frozen bones"); and that accumulated microdamage may predispose diabetic individuals to overt fractures (Krakauer *et al.*, 1995). This interesting hypothesis has yet to be formally tested, but until such time we may gain some insight from examining the evidence in a similar debate: i.e., whether prolonged treatment with potent bisphosphonates in osteoporosis ultimately produces a detrimental effect on bone strength by accumulated microdamage. In a

TABLE IV Possible Clinical Risk Factors and Possible Mechanisms of Increased Fracture Incidence in Diabetic Individuals

Possible Clinical Risk Factors		Possible Mechanisms
Diabetes duration	Related to Bone:	Smaller bones (T1DM)
Eye disease: Diabetic retinopathy, cataract		Reduced BMD (T1DM)
Peripheral neuropathy		Low bone formation
Transient ischemic attack/stroke		Accumulation of microdamage
Atherosclerotic disease		Altered collagenous matrix
Hypoglycemic seizures		(eg, accumulation of AGEs)
Nycturia	Neuromuscular:	Higer incidence of falls
Use of thiazolidinediones		
Organ transplantation		

detailed study in dogs treated with clinically relevant doses of bisphosphonates, microdamage did indeed occur dose-dependently, but was not associated with bone fragility (Allen *et al.*, 2006). The authors proposed that the deleterious effects of microdamage were offset by the increase in mineralized bone volume. However, such "compensation" would be absent in individuals with T1DM.

Effect of Concurrent Medication use on Bone

A Danish case-control study found that diabetic individuals who used sulphonylureas had a lower risk of hip fracture than diabetic individuals in general (Vestergaard *et al.*, 2005). In counterpart, TZDs or peroxisome proliferator-activated receptor (PPAR)-γ agonists (e.g., pioglitazone, rosiglitazone) worsen diabetic bone disease. Indeed, aBMD loss at the spine and the whole body was higher among women – but not men – with T2DM who used a TZD (Schwartz *et al.*, 2006). In line with these data, in the ADOPT trial – which compared metformin, glyburide, and rosiglitazone during four years in 4,360 T2DM patients – the number of fractures (any site) was higher among women – but not men – treated with the TZD compared with the other two drugs (Kahn *et al.*, 2006). In mice, TZD treatment enhanced bone marrow adiposity and caused trabecular bone loss by uncoupling resorption (activated) and formation (suppressed) (Li *et al.*, 2006), the latter perhaps as a result of osteoblast/osteocyte apoptosis (Sorocéanu *et al.*, 2004). Clearly, further research is needed on the bone effects of TZDs. Statins are another frequently used class of drugs in diabetic patients. A small study suggested that statins increase bone density at the hip in T2DM patients (Chung *et al.*, 2000). However, the beneficial effects of statins on BMD and fracture risk are not undisputed at this time (Nguyen *et al.*, 2007).

Anti-osteoporosis drugs appear to be as effective in diabetic as in nondiabetic individuals. In a post hoc analysis of the Fracture Intervention Trial, alendronate 5–10 mg for three years increased aBMD at the spine and hip and reduced bone remodeling indices comparably in diabetic and nondiabetic postmenopausal women (Keegan *et al.*, 2004). Regarding estrogens, clinical trial data (e.g., from the Women's Health Initiative trial) have not been reported yet, but in the observational Iowa Women's Health Study, diabetic women who were using estrogen did not have an elevated hip fracture risk (Nicodemus and Folsom, 2001).

Advanced Diabetic Renal Disease and Bone

Several histomorphometric studies showed that the indices of bone formation and resorption are set lower in diabetic than in nondiabetic patients with chronic renal failure. Thus, high-turnover osteodystrophy is distinctly uncommon (Pei *et al.*, 1993, and references therein). Diabetic patients with chronic renal failure also have less Tc-99m methylenediphosphonate uptake on bone scintigraphy, confirming reduced osteoblastic activity (So *et al.*, 1998). These findings are echoed by comparable data obtained in experimental models: histomorphometric bone formation indices were repressed in 5/6-nephrectomized rats with SZ-induced diabetes compared with nondiabetic rats, which was reversed by insulin treatment (Jara *et al.*, 1995).

Fractures are well known to be more common among kidney transplant recipients. In one U.S. series, they were four times more likely to be hospitalized for a fracture in the three years following renal transplantation; diabetes (T1DM or T2DM) was an additive risk factor, conferring a twofold increased relative risk (Abbott *et al.*, 2001). Similarly, in 86 renal transplant recipients followed for a median of ten years, the relative risk of fracture at any site was 4.8 (95%CI, 3.6–6.4). Again, diabetes was an additional independent risk factor for fractures, raising the relative risk to 13 (95%CI, 8.2–20), with a preponderance of fractures at the lower limbs (Vautour *et al.*, 2004). The possible interaction between diabetes and cumulative corticosteroid, cyclosporine, or tacrolimus dosages needs further study. However, cyclosporine treatment did not affect bone volume and bone remodeling parameters in SZ diabetic rats (Epstein *et al.*, 1994).

THE EFFECT OF DIABETES AND INSULIN ON BONE IN EXPERIMENTAL ANIMALS

Bone Remodeling in Animal Models of T1DM and T2DM

Several animal models have been used for T1DM [alloxan- or SZ-induced diabetic rats and mice, spontaneously diabetic BB rats and nonobese diabetic (NOD) mice] and T2DM (db/db mice, Goto-Kakizaki rats). Untreated severe T1DM (hyperglycemia >300 mg/dl) was associated with low bone formation as shown by biochemical markers, in particular osteocalcin concentrations. Plasma osteocalcin dropped exponentially after onset of T1DM in BB rats to ~25% of nondiabetic levels after five weeks (Verhaeghe *et al.,* 1997b). Interestingly, diabetic BB rats already had lower circulating osteocalcin than their nondiabetic littermates at onset of glycosuria (Verhaeghe *et al.,* 1997b), similarly to what was reported in children (Guarneri *et al.,* 1993). Osteocalcin levels responded poorly, if at all, to exogenous 1,25$(OH)_2D_3$ (Verhaeghe *et al.,* 1989, 1993). The half-life of plasma osteocalcin was similar in diabetic and nondiabetic BB rats, indicating that faster clearance does not explain reduced circulating osteocalcin (Verhaeghe *et al.,* 1989). Plasma osteocalcin gradually returned to the control range with increasing insulin dosage in BB rats (Verhaeghe *et al.,* 1997b), and was normalized by pancreas transplantation in SZ diabetic rats (Ishida *et al.,* 1992).

Histomorphometry consistently showed low bone formation on all bone surfaces (trabecular-endosteal, endocortical, and periosteal) in SZ-diabetic and BB rats. Static morphometry demonstrated a marked decline in osteoblast and osteoid surface or volume (Table I), and dynamic morphometry a decrease in both mineralizing surface and mineral apposition rate. These changes were (partly) reversed by insulin (Bain *et al.,* 1997; Epstein *et al.,* 1994; Glajchen *et al.,* 1988; Shires *et al.,* 1981; Verhaeghe *et al.,* 1992). Electron microscopy of the endocortical surface confirmed that active, cuboidal osteoblasts are virtually absent in SZ-diabetic rats and are replaced by inactive bone-lining cells with flattened nuclei and little or no endoplasmic reticulum (Sasaki *et al.,* 1991). Thus, most of the bone surface is in a quiescent state in severely hyperglycemic animals.

Osteoblast differentiation and maturation are disturbed in T1DM animals. After marrow ablation of the tibia (a validated model of intramembraneous bone formation), SZ-diabetic mice showed a quantitatively reduced but normal temporal expression pattern of genes involved in osteoblast differentiation (c-fos, Cbfa1/Runx-2, collagen type 1, osteocalcin) (Lu *et al.,* 2003). However, osteocalcin but not Runx-2 or alkaline phosphatase mRNA levels were reduced in tibias from SZ-diabetic mice (Botolin *et al.,* 2005), reinforcing the conclusion that loss of mature osteoblasts is a key finding in T1DM. Apoptosis of osteoblastic cells may be stimulated as well, as shown in calvaria from T2DM db/db mice following bacterial inocculation (a model of periodontal bone loss) (He *et al.,* 2004).

Regarding bone resorption, T1DM rats displayed diminished total and creatinine-corrected deoxypyridinoline excretion (Horcajada-Molteni *et al.,* 2001; Verhaeghe *et al.,* 2000). Most but not all histomorphometric data confirmed that the osteoclast surface/number is decreased moderately to severely in T1DM rats, and is reversed by insulin treatment (Glajchen *et al.,* 1988; Shires *et al.,* 1981; Verhaeghe *et al.,* 1992). Electron microscopy showed that most osteoclasts in T1DM rats lack a ruffled border-clear zone complex and that acid phosphatase activity is rarely detected (Kaneko *et al.,* 1990). Bacterial inocculation of the scalp in db/db mice also generated less osteoclasts (He *et al.,* 2004).

Interestingly, bone marrow adiposity was increased in SZ-diabetic mice and NOD mice, with upregulation of adipogenic genes such as PPAR-γ_2, adipocyte fatty acid binding protein (aP2), and resistin (Botolin *et al.,* 2005). More research is needed on the significance of this finding for bone physiology.

Bone Size and Density in Animal Models of T1DM and T2DM

Experimental T1DM had a negative effect on bone size (wet and dry weight, diaphyseal width) that was related to at least three factors: earlier age at which diabetes was induced or occurred spontaneously; longer diabetes duration; and more severe hyperglycemia (Dixit and Ekstrom, 1980; Einhorn *et al.,* 1988; Locatto *et al.,* 1993; Lucas, 1987). Thus, the impact on bone size was modest in T1DM models with onset of diabetes past the peak growth rate (Verhaeghe *et al.,* 1994, 2000). However, the calcium/phosphate concentrations of long bones (expressed per dry weight) was within the normal range in all T1DM models indicating that the mineralization of bone matrix remains adequate (Bain *et al.,* 1997; Dixit and Ekstrom, 1980; Shires *et al.,* 1981; Verhaeghe *et al.,* 1989, 1990). On histomorphometry, total bone area and cortical bone area at the tibial diaphysis were reduced by ~10% in T1DM rats, while the percentage of cortical/total bone area remained unchanged (Epstein *et al.,* 1994); μCT analysis confirmed that cortical thickness at the tibial midshaft is reduced in NOD mice (Thrailkill *et al.,* 2005a). In addition, femoral middiaphyseal BMC, as measured by DXA, was decreased in BB rats but the aBMD and bone mineral apparent density (BMAD, i.e., BMC/area^2) were comparable with values obtained in nondiabetic rats (Verhaeghe *et al.,* 1997a, 2000).

T1DM affects not only cortical bone, but also trabecular bone. The reduction in trabecular bone volume, measured by histomorphometry at the proximal tibia (Bain *et al.,* 1997; Glajchen *et al.,* 1988; Verhaeghe *et al.,* 1990), was related to the severity of hyperglycemia (Locatto *et al.,* 1993). This reduction was the result of thinner trabeculae, while the number and separation of trabeculae remained normal (Botolin *et al.,* 2005; Epstein *et al.,* 1994; Suzuki *et al.,* 2003; Thrailkill *et al.,* 2005a); thinner trabeculae are indeed compatible with osteoblast dysfunction. Although diabetic BB rats did not show a significant decrease in trabecular bone volume in many of our studies (Verhaeghe *et al.,* 1989, 1992, 1993, 1994, 2000), they showed the expected trabecular bone loss after ovariectomy (Verhaeghe *et al.,* 1994, 1997a), but not after immobilization (unilateral paralysis by sciatic neurectomy) (Verhaeghe *et al.,* 2000).

In contrast to T1DM, bone size was increased in T2DM models such as the Goto-Kakizaki rat, particularly at the diaphysis. Whereas cortical BMD, measured by QCT, was only mildly affected, trabecular BMD was markedly (33–53%) decreased in these rats (Ahmad *et al.,* 2003). T2DM may well have complex effects on bone with divergent responses generated by obesity and hyperglycemia. Disruption of leptin or leptin signaling in mice creates massive obesity with T2DM and increases bone mass via central nervous system pathways, some of which still need to be uncovered (Karsenty, 2006).

Bone Strength in Animal Models of T1DM

The breaking strength of the femoral or tibial midshaft was assessed in several T1DM models (SZ-diabetic and BB rats, NOD mice) by various methods (perpendicular pressure, torsion, three-point bending) (Dixit and Ekstrom, 1980; Einhorn *et al.,* 1988; Horcajada-Molteni *et al.,* 2001; Thrailkill *et al.,* 2005a; Verhaeghe *et al.,* 1990, 1994, 2000). Most though not all of these studies concluded that strength parameters were reduced after a critical period of diabetes (e.g., eight weeks in rats), even when correcting for their smaller bone size. Decreased strength was also documented at the femoral neck, which was partly restored by insulin treatment (Hou *et al.,* 1993); and at the femoral metaphysis (Suzuki *et al.,* 2003; Verhaeghe *et al,* 2000). However, there was no effect on vertebral body breaking strength (Verhaeghe *et al,* 2000).

One of the biomechanical parameters most affected was the energy absorption (reflecting bone brittleness versus toughness) (Verhaeghe *et al.,* 1994). Diabetic bone brittleness may be related to the reduced amount and altered composition of the collagenous matrix. In spontaneously WBN/Kob rats, a model of T1DM occurring at an advanced age (i.e., from 12 months), enzymatic collagen cross-links in femoral bone were reduced from the prediabetic stage (eight months), while non-enzymatic (glycosylated) cross-links increased from 12 months. Interestingly, the collagen cross-link parameters in prediabetic/diabetic WBN bones strongly correlated with biomechanical properties (Saito *et al.,* 2006).

Bone Repair in Animal Models of T1DM

Several models have been examined: a closed femur, tibia, or fibula fracture in SZ-diabetic or BB rats (Funk *et al.,* 2000; Gandhi *et al.,* 2006; Kawaguchi *et al.,* 1994; Kayal *et al.,* 2007); tibial distraction following a fibula fracture in NOD mice (Thrailkill *et al.,* 2005a); and surgical bone defects [a 1.0–2.1 mm hole in the parietal bone of SZ-diabetic mice (Santana *et al.,* 2003), or a 0.4–1.6 mm hole in the distal femur of BB rats (Follak *et al.,* 2004)]. In all models, defect repair was impaired at least temporarily in the diabetic animals, with less new bone formed – as assessed by radiographic, μCT, and histomorphometric analyses. Both endochondral and periosteal postfracture repair mechanisms were defective. Increased cartilage resorption may explain in part the impaired endochondral bone repair, because there was more osteoclast activity at the callus site in SZ-diabetic rats (Kayal *et al.,* 2007). Finally, callus strength was delayed by at least one week (Funk *et al.,* 2000). Again, the bone-healing impairment depended on the degree of glycemic control, and was reversed by systemic insulin treatment (Follak *et al.,* 2004). However, some local treatments also (partially) reversed the defective fracture repair, e.g., intramedullary delivery of insulin (Gandhi *et al.,* 2005), and topically applied autologous platelet-rich (and growth factor-rich) plasma (Gandhi *et al.,* 2006) or fibroblast growth factor (FGF) (Kawaguchi *et al.,* 1994).

CONCLUSION: INSIGHTS GAINED FROM T1DM ANIMAL MODELS

Experiments in T1DM models have confirmed that reduced bone formation is a key feature of severe hyperglycemia. They have taught us that osteoblast differentiation and maturation are downregulated, resulting in suppressed production of osteoid tissue and mineralized bone, and thus mostly "quiescent" bone surfaces. Osteoblast apoptosis may be increased, but this finding must be buttressed in future research. Although bone resorption is suppressed as well, there is likely some degree of uncoupling with formation being more repressed than resorption, as suggested by the reduced trabecular bone volume with thinner trabeculae. Hence, diabetic bone disease should be regarded as an osteoblast-induced low-turnover osteopenia. Decreased periosteal bone formation (modeling) results in thinner cortical shells and thinner bones. Biomechanical studies have demonstrated that bone strength is impaired, which could be related to the amount and composition of the collagenous bone matrix. Finally, T1DM substantially delays bone defect or fracture repair.

LOCAL EFFECTS OF GLUCOSE AND INSULIN ON BONE

The effects of supraphysiological glucose concentrations were examined *in vitro* in human MG-63 and mouse MC3T3-E1 osteoblast-like cells. Glucose effects on cell proliferation were contradictory, but mineralization parameters (osteocalcin expression, calcium uptake) were generally downregulated. Whether this is the result of osmotic stress or a specific effect of glucose is controversial (Balint *et al.*, 2001; Terada *et al.*, 1998; Zayzafoon *et al.*, 2000).

Abundant, high-affinity insulin receptors were documented in mature osteoblastic cell lines (UMR-106 and ROS-17/2.8) but not in preosteoblastic cells (UMR-201). In UMR-106, half-maximal displacement of ^{125}I-labeled insulin was attained at physiological insulin concentrations (between 0.5 and 1.0 n*M*), while proinsulin had 0.1% of insulin's binding potency; insulin binding was downregulated at supraphysiological insulin concentrations (10^{-7} *M*). Physiological concentrations of insulin (0.25–10 n*M*) stimulated the proliferation of osteoblastic cells, as well as their glucose uptake, expression of the glucose transporter GLUT1, active K^+ uptake, $PO^{2-}{}_4$ uptake, and collagen synthesis (Hickman and McElduff, 1989; Ituarte *et al.*, 1989; Thomas *et al.*, 1996).

In fetal rat calvaria organ cultures, insulin at 3 n*M* stimulated collagen synthesis and bone matrix apposition, whereas osteoblast replication was boosted at supraphysiological insulin concentrations only (10^{-7}–10^{-6}*M*). This discrepancy suggests that the stimulating effect of insulin on collagenous matrix production is not explained by its effect on osteoblast replication (Hock *et al.*, 1988).

Insulin binding *in vivo* was demonstrated by autoradiography in rat osteoblasts but not osteocytes (Martineau-Doizé *et al.*, 1986). Insulin receptor-α was also detected by immunohistochemistry in osteoblast precursors present in healing distraction gaps in mice (Thrailkill *et al.*, 2005a). Insulin, administered locally to the periosteum of one hemicalvaria of normal mice, markedly stimulated morphometric parameters of bone formation (osteoblast number/perimeter, osteoid area) (Cornish *et al.*, 1996). As mentioned previously, local insulin delivery also normalized fracture healing in T1DM rats (Gandhi *et al.*, 2005).

Cultured osteoclasts also contained abundant insulin receptors, and insulin dose dependently inhibited pit formation in a dentine slice assay (Thomas *et al.*, 1998). However, local injections of insulin did not increase osteoclast number/perimeter in mouse calvariae (Cornish *et al.*, 1996).

However, the significance of these findings was called into question by a study that examined mice with absent insulin receptor expression in bone. These mice showed normal bone remodeling and trabecular bone morphology, while cortical thickness was slightly increased. These surprising findings might be explained in part by the upregulation of IGF-I receptor expression in bone (Irwin *et al.*, 2006). Nonetheless, the remarkable effects on bone of circulating insulin deficiency appear to be largely if not completely indirect.

WHAT CAUSES DIABETIC BONE DISEASE?

The Diabetic Hormonal *Milieu*

The effects of diabetes on bone growth and remodeling *in vivo* can be replicated in vitro using diabetic serum. Indeed, sera from T1DM individuals with poor glycemic control inhibited osteoblastic collagen synthesis (Brenner *et al.*, 1992). Similarly, diabetic rat serum inhibited collagen production by articular cartilage, which was reversed by *in vivo* insulin treatment of the animals but not by the direct addition of insulin to diabetic serum (Spanheimer, 1992).

Lower Circulating IGF-I

Lower circulating IGF-I is likely a crucial factor. Downregulation of hepatic IGF-I release resulted in a 73% drop in serum IGF-I levels in children with new-onset T1DM (Bereket *et al.*, 1995); circulating IGF-I remained below control levels in T1DM adolescents and adults receiving insulin treatment (Bouillon *et al.*, 1995; Léger *et al.*, 2006). IGF-I had powerful effects on osteoblast proliferation and bone matrix formation *in vitro* (Hock *et al.*, 1988). Also, disruption of the IGF-I gene resulted in 25–40% smaller bones in prepubertal mice and prevented periosteal expansion and BMD gain during puberty; serum osteocalcin levels in IGF-I knockout mice were reduced by 24–54% (Mohan *et al.*, 2003). Depletion of circulating IGF-I (while skeletal expression of IGF-I remained normal) equally resulted in decreased growth and periosteal expansion, which was restored by exogenous IGF-I (Yakar *et al.*, 2002). Insulin and IGF-I initiate cellular responses by binding to and activating the insulin and IGF-I (tyrosine kinase) receptors, and phosphorylation of insulin receptor substrate (IRS)-1. As would be predicted, IRS-1 knockout mice showed smaller bones with thinner growth plates, lower trabecular and cortical bone volume, thin trabeculae, and low bone formation and resorption (Ogata *et al.*, 2000). Our studies in T1DM animals showed a strong correlation between plasma IGF-I and osteocalcin levels (Verhaeghe *et al.*, 1997b). Similarly, IGF-I concentrations were correlated with biochemical bone formation markers in T1DM individuals (Bouillon *et al.*, 1995). In addition, serum IGF-I was an independent predictor of total body BMC in children and adolescents with T1DM (Léger *et al.*, 2006).

Upregulation of the hepatic expression of IGF-binding protein-1 (IGFBP1) by insulin deficiency caused a sevenfold increment in circulating IGFBP1 in children with new-onset T1DM (Bereket *et al.*, 1995). Through the accrued formation of binary IGF-I:IGFBP1 complexes (Frystyk *et al.*, 2002), the decrement in circulating free (bioavailable) IGF-I in

T1DM is more pronounced than that of total IGF-I. It is not surprising, therefore, that IGFBP1 inhibited the osteoblastic effects of IGF-I (Campbell and Novak, 1991). Elevated IGFBP1 levels might also explain, in part, why bone growth and remodeling in T1DM rats were resistant to the anabolic effects of exogenous IGF-I (Verhaeghe *et al.,* 1992).

Hypercortisolism

Hypercortisolism may also play an important role. New-onset T1DM and poorly controlled T1DM and T2DM are accompanied by an activation of the hypothalamo-pituitary-adrenal axis with increased cortisol levels (e.g., Bereket *et al.,* 1995); similarly, elevated serum corticosterone (the major glucocorticoid in rodents) was found in some though not all studies in animal models of severe T1DM (Unterman *et al.,* 1993; Verhaeghe *et al.,* 1997b). We documented that low osteocalcin levels in diabetic BB rats were normalized four days after adrenalectomy, whereas they remained suppressed when diabetic adrenalectomized animals were substituted with corticosterone (Verhaeghe *et al.,* 1997b). The effect of glucocorticoid excess on bone is probably mediated in part by decreased IGF-I and increased IGFBP1 gene expression in both liver and osteoblasts (Unterman *et al.,* 1993, Verhaeghe *et al.,* 1997b). In addition, glucocorticoids have direct effects on bone. Glucocorticoid excess, like diabetes, results in a suppression of osteoblastogenesis and osteoclastogenesis, and a stimulation of osteoblast/osteocyte apoptosis and adipogenic activity (Weinstein *et al.,* 1998).

Calciotropic Hormones

There is a great deal of controversy regarding the effects of T1DM on circulating PTH and $1,25(OH)_2D_3$ (reviewed by Verhaeghe *et al.,* 1999). Nonetheless, there may be some degree of "functional hypoparathyroidism" among T1DM individuals, perhaps related to urinary Mg loss. Decreased PTH levels were also reported in elderly T2DM women (Dobnig *et al.,* 2006). Intermittent administration of $hPTH_{1-34}$ to T1DM rats improved trabecular bone formation, volume, and strength (Suzuki *et al.,* 2003); however, intermittent PTH is a powerful anabolic agent for trabecular bone in various osteopenic conditions not necessarily associated with hypoparathyroidism (e.g., postmenopausal or glucocorticoid-induced osteopenia). Although T1DM rats displayed a higher metabolic clearance rate of $1,25(OH)_2D_3$ and lower (i.e., total but not free) circulating $1,25(OH)_2D_3$, $1,25(OH)_2D_3$ infusion did not affect the osteoblast and osteoid surface on histomorphometry and barely raised serum osteocalcin (Verhaeghe *et al.,* 1993).

Low Amylin

Amylin is a peptide co-secreted with insulin by pancreatic β-cells in response to nutrient stimuli. Amylin treatment of SZ-diabetic rats resulted in some improvement of the diabetic syndrome, and prevented the low-formation osteopenia observed in untreated rats (Horcajada-Molteni *et al.,* 2001). The role of amylin must be clarified in future research.

Impaired Vascularization of Diabetic Bones

It has been postulated by some that diabetic bone disease is another manifestation of microangiopathy, but evidence supporting this hypothesis is lacking. To be sure, the reduced bone remodeling is present well before microangiopathy develops. Nevertheless, bone vascularization is of great importance for bone growth, modeling, and remodeling. One study in SZ-diabetic rats showed that the blood flow to the tibia – examined by the injection of microspheres – was reduced by 65% after 14 days of T1DM and remained at the same level until day 56 (Lucas 1987). More research on the vascularization of diabetic bones (including the growth plate), and the factors that regulate vascularization (e.g., VEGFs), is sorely needed.

Altered Collagenous Bone Matrix

Increased Collagen Breakdown

Stimulation of collagenolysis has been documented in non-bone tissues (e.g., skin and periodontium) of T1DM rats (Bain *et al.,* 1997, and references therein). Tetracyclines inhibit collagenase (matrix metalloproteinase) activity in nonbone tissues as well as in bone via a mechanism independent of their antimicrobial effect. SZ-diabetic rats treated with minocycline exhibited a normalization of growth plate width and trabecular bone volume and an improvement of periosteal bone formation (Bain *et al.,* 1997). In addition, osteoblasts and osteoclast regained their normal morphology after tetracycline treatment (Kaneko *et al.,* 1990; Sasaki *et al.,* 1991). Low IGF-I levels and glucocorticoid excess in diabetic serum might be involved, because collagenase expression in osteoblastic cells was repressed by IGF-I but stimulated by glucocorticoids.

Increased Collagen Glycosylation

Nonenzymatic protein glycosylation and oxidation results in the gradual accumulation of "advanced glycation end products" (AGEs) in serum and various tissues. Proteins with a long half-life such as collagen are particularly susceptible to glycosylation. AGE accumulation in bone occurs with normal aging but is much accelerated in diabetes (Katayama *et al.,* 1996). Accumulation of AGEs in aging bone was associated with increased brittleness (Wang *et al.,* 2002), and may also be a determinant of diabetic bone brittleness. Moreover, AGE accumulation in diabetic bones affected bone cell function: inhibition of osteoblastic cell differentiation and function (Katayama *et al.*, 1996), and stimulation of osteoblast apoptosis (Alikhani *et al.*, 2007). RAGE, one of the receptors for AGEs, was overexpressed in healing bone tissue of SZ-diabetic mice, and local delivery of a RAGE-ligand delayed bone healing in nondiabetic mice (Santana *et al.*, 2003). These data would indicate that accumulation of AGEs explains in part the impaired fracture repair in diabetes.

Reduced Enzymatic Collagen Cross-Linking

Spontaneously diabetic WBN/Kob rats showed reduced serum vitamin B6 (pyridoxal and pyridoxamine) and immature enzymatic collagen cross-links in femoral bone from the prediabetic stage onward; however, mature cross-links (pyridinoline and deoxypyridinoline) remained normal while non-enzymatic cross-links increased from the diabetic stage. As mentioned earlier, the altered collagenous matrix composition was correlated with reduced bone strength parameters (Saito *et al.*, 2006).

TABLE V Key Findings in Diabetic Bone Disease (T1DM)

• Metabolic and Hormonal milieu	↓Insulin → ↑Glucose → ↓IGF-I (free > total) → ↑Cortisol
• Bone Cell Dynamics	↓Chondrocyte Proliferation ↓Osteoblast Differentiation ↑Osteoblast Apoptosis?
• Bone Cell Quantity –Morphometry	↓N Mature Osteoblasts ↓N Multinucleated Osteoclasts
• Bone Cell Function –Morphometry	↓Mineralizing Perimeter (all Bone Surfaces) ↓Mineral Apposition Rate
-Biochemistry:	↓Serum Bone-Specific Alkaline Phosphatase ↓Serum Osteocalcin ↓Deoxypyridinoline Excretion
• Bone Density – Morphometry	↓Cortical Bone Area ↓Trabecular Bone Volume ↓Trabecular Thickness
–Densitometry:	↓Areal Bone Density
• Bone Size	↓Bone Length ↓Bone Thickness ↓Bone Weight
• Biochemical Bone Composition	↓Enzymatic collagen cross-links ↑Glycosylated Collagen (AGEs) =Calcium and Phosphate
• Bone Vascularization	↓Blood flow
• Bone Biomechanics	↓Breaking Strength ↓Energy Absorption

↑: *increased*; ↓: *decreased*; =: *similar*; N: *number*

GENERAL CONCLUSIONS (see also Table V)

- Insulin has been called "an anabolic agent for bone" (Thrailkill *et al.*, 2005b) because bone cells (chondrocytes, preosteoblasts and osteoblasts, and osteoclasts) contain insulin receptors, and because insulin stimulates growth and bone formation *in vitro* and *in vivo*. However, absence of insulin receptor expression in bone does not cause diabetic bone disease, indicating that the effect of insulin-deficiency on bone is largely if not completely indirect.
- Severe T1DM causes stunted prepubertal and pubertal growth (Mauriac syndrome). In T1DM animal models, endochondral bone growth is retarded, resulting in smaller bones after medium- and long-term diabetes.
- Diabetes is accompanied by low bone remodeling (both formation and resorption). This is very apparent in animal models of T1DM with severe hyperglycemia, but has been demonstrated in humans with T1DM and T2DM as well. Circulating osteocalcin is the parameter most affected, and is reduced from the diagnosis of diabetes. Impaired osteoblast differentiation and perhaps also accrued osteoblast apoptosis contribute to the defect in mature osteoblasts.
- Bone formation appears to be more affected than bone resorption, because there is a reduction in the amount of trabecular and cortical bone in animal models of T1DM; trabecular thickness but not the number of trabeculae is reduced, consistent with reduced osteoblast activity. Less cortical bone results in thinner bones. Bone size and bone density are also mildly reduced in T1DM children/adolescents and adults (∼10% or ∼1 SD reduction), but this reduction does not appear to be progressive at least until menopause. Future studies should assess under which circumstances (gender, age, diabetes duration, menopause status, etc.) T1DM may constitute an indication for bone densitometry screening in clinical practice.
- T1DM is accompanied by low free IGF-I in serum while cortisol levels are raised. The bone morphology in T1DM animals is reminiscent of that observed in other "catabolic" conditions, e.g., in IGF-I and IRS-1 knockout animals or animals treated with glucocorticoids. It is likely that low circulating IGF-I and hypercortisolism orchestrate in part the events that ultimately result in the diabetic bone phenotype.
- In contrast to T1DM, pre-T2DM (impaired glucose tolerance) and early T2DM are associated with a mild

(3–5%) increase in bone size and areal bone density, but changes in volumetric density appear to be smaller or nonexistent. Accelerated bone loss may occur in long-lasting T2DM, although this needs further research. There is also a need for amplified bone research in obesity and T2DM animal models.

- Both T1DM and T2DM are accompanied by a higher incidence of fractures, although the risk is far greater for T1DM individuals. The increased fracture incidence in T1DM is more pronounced than could be explained by the relatively small bone density deficit. Fracture risk increases with diabetes duration, and is higher among individuals with diabetes complications and limited physical capabilities; the higher incidence of falls in diabetic individuals is likely an important mediator.
- Bone strength is clearly impaired in T1DM animals. Diabetes-induced changes in the collagenous matrix are related to the biomechanical properties. These include: reduced enzymatic collagen cross-links but increased glycosylated collagen (AGEs). Accumulation of AGEs, in turn, appears to affect osteoblastic function.
- New bone formation following a fracture is impaired in T1DM animals and diabetic individuals. Whether diabetes results in microdamage accumulation, and whether this contributes to decreased bone strength, remains unknown.

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Chapter 46

Androgens Receptor Expression and Steroid Action in Bone

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INTRODUCTION

Much of the research describing the general action of gonadal steroids in bone has focused on the specific effects of estrogen (see Chapter 14) because of the obvious impact of the menopause on skeletal health. Nevertheless, it is clear that androgens, in both men and women, also have important beneficial effects on skeletal development and on the maintenance of bone mass. It has been demonstrated that androgens (a) influence growth plate maturation and closure, helping to determine longitudinal bone growth during development, (b) mediate regulation of cortical bone mass in a fashion distinct from estrogen, leading to a sexually dimorphic skeleton, (c) modulate peak bone mass acquisition, and (d) influence trabecular (cancellous) bone and inhibit bone loss (Wiren, 2005). In castrated animals, replacement with nonaromatizable androgens (e.g., 5α-dihydrotestosterone, DHT) yields beneficial effects that are clearly distinct from those observed with estrogen replacement (Turner *et al.,* 1989; Turner *et al.,* 1990b). In intact females, blockade of the androgen receptor (AR) with the specific AR antagonist hydroxyflutamide results in osteopenia (Goulding and Gold, 1993). Furthermore, treatment with nonaromatizable androgen alone in females results in improvements in bone mineral density (Coxam *et al.,* 1996). Finally, combination therapy with estrogen and androgen in postmenopausal women is more beneficial than either steroid alone (Castelo-Branco *et al.,* 2000; Miller *et al.,* 2000; Raisz *et al.,* 1996), indicating nonparallel pathways of action. Taken together, these reports illustrate the divergent actions of androgens and estrogens on the skeleton. Thus, in both men and women it is probable that androgens and estrogens each have important yet distinct functions during bone development, and in the subsequent maintenance of skeletal homeostasis in the adult. With an increased awareness of the importance of the effects of androgen on skeletal homeostasis (Vanderschueren *et al.,* 2004), and the potential to make use of this information for the treatment of bone disorders, much nevertheless remains to be learned.

THE ROLE OF ANDROGEN METABOLISM

Metabolism of Androgens in Bone: Aromatase, 17β-hydroxysteroid Dehydrogenase (17β-HSD), and 5α-Reductase Activities

Sex steroids, ultimately derived from cholesterol, are synthesized predominantly in gonadal tissue, the adrenal gland and placenta as a consequence of enzymatic conversions. The major pathways are shown in Figure 1. After peripheral metabolism, androgenic activity is represented in a variety of steroid molecules that include testosterone. There is accumulating evidence that in a range of tissues the eventual cellular effects of testosterone may not be the result (or not only the result) of direct action of testosterone, but may also reflect the effects of sex steroid metabolites formed as a consequence of local enzyme activities. The most important testosterone metabolites likely to influence bone are 5α-DHT (the result of 5α reduction of testosterone) and estradiol (formed by the aromatization of testosterone). Testosterone and DHT are the major and most potent androgens, with androstenedione (the major circulating androgen in women) and dehydroepiandrosterone (DHEA) as immediate androgen precursors that exhibit weak androgen activity (Mo *et al.,* 2006). DHEA, although a weaker androgen, is still considered anabolic (Labrie *et al.,* 2006). In men, the most abundant circulating androgen metabolite is testosterone, while concentrations of other weaker androgens like androstenedione and DHEA-sulfate are similar between males and females. Downstream metabolites of DHT and androstenedione are inactive at the AR, and include 5α-androstane-3α or 3β,17β-diol (3α/β-androstanediol) and 5α-androstanedione. Data suggest

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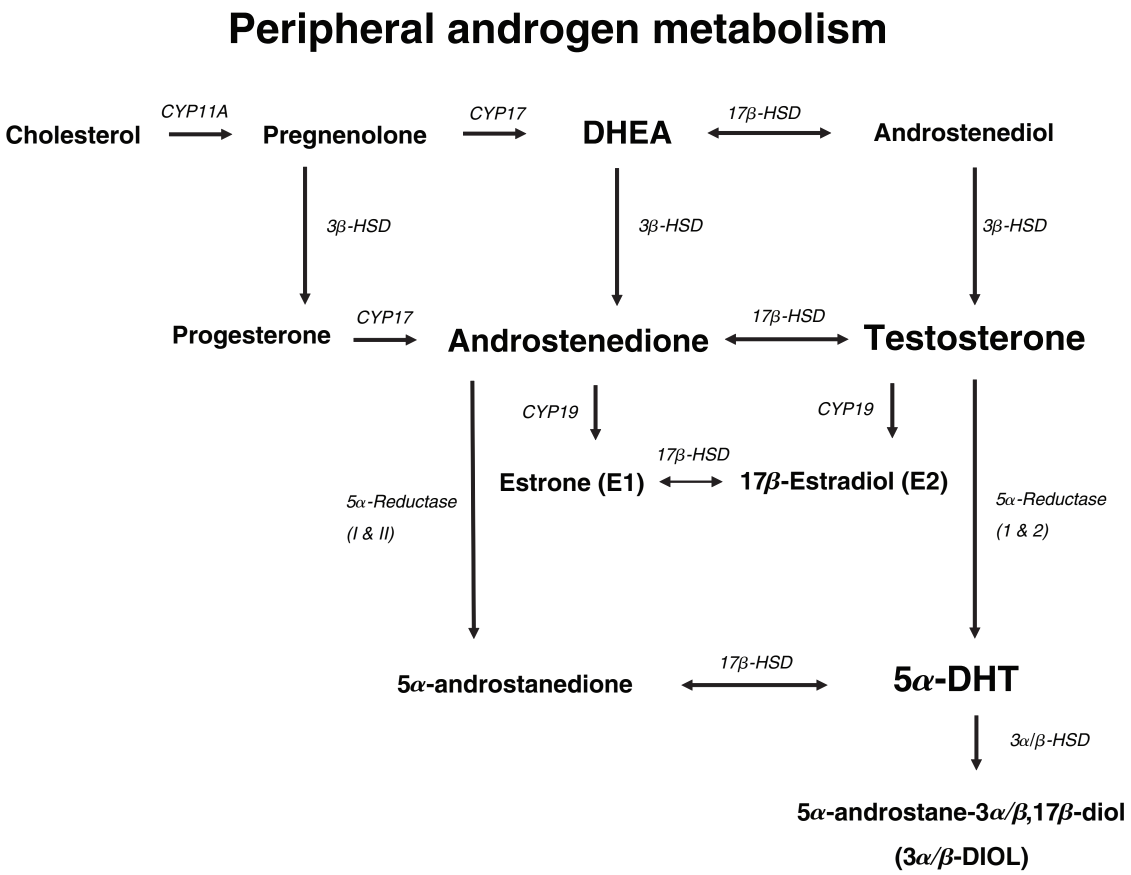
Peripheral androgen metabolism
Cholesterol
CYP11A
Pregnenolone
CYP17
DHEA
17β-HSD
Androstenediol
3β-HSD
3β-HSD
3β-HSD
Progesterone
CYP17
Androstenedione
17β-HSD
Testosterone
CYP19
CYP19
17β-HSD
Estrone (E1)
17β-Estradiol (E2)
5α-Reductase
(I & II)
5α-Reductase
(1 & 2)
5α-androstanedione
17β-HSD
5α-DHT
3α/β-HSD
5α-androstane-3α/β,17β-diol
(3α/β-DIOL)

FIGURE 1 Principle conversions and major enzyme activities involved in androgen synthesis and metabolism. Steroid hormone synthesis involves metabolism of cholesterol, with dehydrogenation of pregnenolone producing progesterone that can serve as a precursor for the other gonadal steroid hormones. DHEA, dehydroepiandrosterone; CYP11A, cytochrome P450 cholesterol side chain cleavage enzyme; CYP17, cytochrome P450 17α hydroxylase/17,20 lyase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; CYP19, aromatase cytochrome P450.

that aromatase cytochrome P450 (the product of the CYP19 gene), 17β-hydroxysteroid dehydrogenase (17β-HSD), and 5α-reductase activities are all present in bone tissue as described later, at least to some measurable extent in some compartments, but the biologic relevance of each remains somewhat controversial.

An important enzymatic arm of testosterone metabolism involves the biosynthesis of estrogens from androgen precursors, catalyzed by aromatase. Aromatase is well known to be expressed and regulated in a pronounced tissue-specific manner (Simpson *et al.,* 1994), and also exhibits species differences, given the low levels in mice. Modest levels of aromatase activity have been reported in bone from mixed cell populations derived from both sexes (Nawata *et al.,* 1995; Sasano *et al.,* 1997; Schweikert *et al.,* 1995) and from osteoblastic cell lines (Nakano *et al.,* 1994; Purohit *et al.,* 1992; Tanaka *et al.,* 1993). Aromatase expression in intact bone has also been documented by *in situ* hybridization and immunohistochemical analysis (Sasano *et al.,* 1997). At least in vertebral bone, the mesenchymal distal promoter I.4 is predominantly utilized (Shozu and Simpson, 1998). The enzyme kinetics in bone cells seem to be similar to those in other tissues, although the V_{max} may be increased by glucocorticoids (Tanaka *et al.,* 1993). Aromatase mRNA is expressed predominantly in lining cells, chondrocytes, and some adipocytes; however, there is little to no detectable expression in osteoclasts, or in cortical bone in mice (Wiren *et al.,* 2004b). Thus, whether the level of aromatase activity in bone is high enough to produce physiologically relevant concentrations of steroids locally remains an open question. Nevertheless, in the male only 15% of circulating estrogen is produced in the testes, with the remaining 85% produced by peripheral metabolism that could theoretically include bone as one site of conversion (Gennari *et al.,* 2004).

Aromatase catalyzes the metabolism of adrenal and testicular C19 androgens (androstenedione and testosterone) to C18 estrogens (estrone and estradiol), thus producing the potent estrogen estradiol (E2) from testosterone, and the weaker estrogen estrone (E1) from its adrenal precursors androstenedione and DHEA (Nawata *et al.,* 1995). Thus, because of aromatase activity, systemically administered testosterone may have effects either at the AR (with either testosterone or DHT as ligands) or at the estrogen receptor (ERα or ERβ). Typically in the circulation, E2 will make up to 40% of total estrogen, E1 will make up an additional 40%, with estriol (E3) comprising the remaining 20% of total estrogen (Lin *et al.,* 2006). In addition to aromatase itself, osteoblasts contain enzymes that are able to interconvert estradiol and estrone (17β-HSD, see following section), and to hydrolyze estrone sulfate, the most abundant estrogen in the circulation, to estrone (steroid sulfatase) (Muir *et al.,* 2004; Purohit *et al.,* 1992). Dexamethasone and 1α,25(OH)$_2$D$_3$ synergistically enhance aromatase activity and aromatase mRNA expression in human osteoblast-like cells (Nawata *et al.,* 1995). In addition, both leptin and 1α,25(OH)$_2$D$_3$ treatment increased aromatase activity in human mesenchymal stem cells during osteogenesis, but not during adipogenesis (Pino *et al.,* 2006). Additional studies are needed to better define expression, given the potential importance of the enzyme, and its regulation by a variety of mechanisms (including androgens and estrogens) in other tissues (Abdelgadir *et al.,* 1994; Simpson *et al.,* 1994).

The clinical impact of aromatase activity, with an indication of the importance of conversion of circulating androgen into estrogen, is shown in reports of women and men with aromatase deficiencies who present with a skeletal phenotype (Jones *et al.,* 2007). The presentation of men with aromatase deficiency is very similar to that of a man with ERα deficiency (Smith *et al.,* 1994), namely an obvious delay in bone age, lack of epiphyseal closure, and tall stature with high bone turnover and osteopenia (Gennari *et al.,* 2004). These findings suggest that aromatase (and likely estrogen action) has an important role to play during skeletal development in the male. Interestingly, natural mutation is remarkably rare with only seven males and seven females reported to date (Jones *et al.,* 2007). In addition, estrogen therapy of males with aromatase deficiency has been associated with an increase in bone mass (Gennari *et al.,* 2004) particularly noted in the growing skeleton (Bouillon *et al.,* 2004). Inhibition of aromatization pharmacologically with nonsteroidal inhibitors (such as vorozole or letrozole) results in modest decreases in bone mineral density and changes in skeletal modeling in young growing orchidectomized males (Vanderschueren *et al.,* 1997), and less dramatically so in boys with constitutional delay of puberty treated for one year (Wickman *et al.,* 2003), suggesting short-term treatment during growth has limited negative consequences in males. Inhibition of aromatization in older orchidectomized males resembles castration with similar increases in bone resorption and bone loss, suggesting that aromatase activity likely does play a role in skeletal maintenance in males (Vanderschueren *et al.,* 1996). Combined, these studies herald the importance of aromatase activity (and estrogen) in the mediation of some androgen action in bone in both males and females. The characterization of these enzyme activities in bone clearly raises the difficult issue of the origin of androgenic effects in the skeleton; do they arise solely from direct androgen effects (as is suggested by the actions of nonaromatizable androgens such as DHT) or also from the local or other-site production of estrogenic intermediates? The results described here would indicate that both steroids appear to be important to both male and female skeletal health.

The 17β-HSDs (most of which are dehydrogenase-reductases, except type 5, which is an aldo-keto reductase) have been shown to catalyze either the last step of sex steroid synthesis or the first step of their degradation. This activity produces weak or potent sex steroids via oxidation or reduction, respectively, and can thus also play a critical role in peripheral steroid metabolism. The oxidative pathway forms 17-ketosteroids while the reductive pathway forms 17β-hydroxysteroids. The enzyme reversibly catalyzes the formation of androstenediol (an estrogen) from DHEA, in addition to the biosynthesis of estradiol from estrone, the synthesis of testosterone from androstenedione, and the production of DHT from 5α-androstanedione all via the reductive activity of 17β-HSD. Of the 13 enzyme isotypes of 17β-HSD activity (Lin *et al.,* 2006), either types 1–4 (Feix *et al.,* 2001) or types 1, 3, and 5 (Miki *et al.,* 2007) have been demonstrated in human osteoblastic cells.

5α-reductase is also an important activity with regard to androgen metabolism, because testosterone is converted to the more potent androgen metabolite DHT via 5α-reductase action (Bruch *et al.,* 1992). 5α-reductase activity was first described in crushed rat mandibular bone (Vittek *et al.,* 1974) with similar findings reported in crushed human spongiosa (Schweikert *et al.,* 1980). Two different 5α-reductase genes encode type 1 and type 2 isozymes in many mammalian species (Russell and Wilson, 1994); mRNAs encoding the type 2 isozyme are more abundant than type 1 mRNAs in most male reproductive tissues, whereas the type 1 predominate in peripheral tissues. Human osteoblastic cells express the type 1 isozyme (Issa *et al.,* 2002). Essentially the same metabolic activities were reported in experiments with human epiphyseal cartilage and chondrocytes (Audi *et al.,* 1984). In general, the K$_m$ values for bone 5α-reductase activity are similar to those in other androgen responsive tissues (Nakano *et al.,* 1994; Schweikert *et al.,* 1980). However, the cellular populations in many of these studies were mixed and hence the specific cell type responsible for the activity is unknown. Interestingly, periosteal cells do not have detectable 5α-reductase activity (Turner *et al.,* 1990a), raising the possibilities that the enzyme may be functional in only selected skeletal compartments, and that testosterone may be the active androgen metabolite at this clinically important site.

From a clinical perspective, the general importance of this enzymatic pathway is uncertain, as patients with 5α-reductase type 2 deficiency have normal bone mineral density (Sobel *et al.,* 2006) and no significant correlation was observed between enzyme activities and bone volume (Bruch *et al.,* 1992). Mutant null mice lacking both 5α-reductase type 1 (and type 2) have been created (Mahendroo *et al.,* 2001), but the effect on the skeleton has not been analyzed. Analysis of the importance of 5α-reductase activity has also been approached with the use of finasteride, an inhibitor of 5α-reductase activity that is selective for type 2 in humans, but comparably inhibits both type 1 and type 2 in rodents (Finn *et al.,* 2006). Finasteride treatment of male animals does not recapitulate the effects of castration (Rosen *et al.,* 1995). Furthermore, inhibition of type 1 5α-reductase using MK-434 does not block the effect of testosterone to reduce bone turnover in orchidectomized rats (Borst *et al.,* 2005). Combined, these studies suggest that reduction of testosterone to DHT is not the major determinant in the effects of gonadal hormones on bone. Consistent with this finding, testosterone therapy in hypogonadal older men, either when administered alone or when combined with finasteride, increases bone mineral density, again suggesting that DHT is not essential for the beneficial effects of testosterone on bone (Amory *et al.,* 2004). Thus, the available clinical data regarding the importance of 5α-reductase activity remain uncertain, and the impact of this enzyme, which isozyme may be involved, whether it is uniformly present in all cell types involved in bone modeling/remodeling, or whether local activity is important in any bone compartment, remain unresolved issues.

The administration of testosterone can stimulate bone formation and inhibit bone resorption, likely through multiple mechanisms that involve both androgen and estrogen receptor-mediated processes. However, there is substantial evidence that some, if in fact not most, of the biologic actions of androgens in the skeleton are mediated by AR signaling in bone. Both *in vivo* and *in vitro* systems reveal the effects of the nonaromatizable androgen DHT to be essentially the same as those of testosterone. In addition, blockade of the AR with the receptor antagonist flutamide results in osteopenia as a result of reduced bone formation (Goulding and Gold, 1993). Consistent with this result, complete androgen insensitivity results in a significant decrease in bone mineral density in spine and hip sites (Sobel *et al.,* 2006) even in the setting of strong compliance with estrogen treatment (Marcus *et al.,* 2000). These reports clearly indicate that androgens, independent of estrogenic metabolites, have primary effects on osteoblast function. However, the clinical reports of subjects with aromatase deficiency also highlight the relevance of metabolism of androgen to biopotent estrogens at least in the circulation, to influence development and/or bone mass maintenance. It thus seems likely that further elucidation of the regulation steroid metabolism, and the potential mechanisms by which androgenic and estrogenic effects are coordinated, will have physiological, pathophysiological, and therapeutic implications.

Synthetic Androgens

In addition to the endogenous steroid metabolites highlighted in Figure. 1, there are also a variety of drugs with androgenic activity. These include anabolic steroids, such as nonaromatizable oxandrolone that can bind and activate AR, albeit with lower affinity than testosterone (Kemppainen *et al.,* 1999). In addition, a class of drugs under extensive development, referred to as selective AR modulators (SARMs), demonstrate tissue-specific agonist or antagonist activities with respect to AR transactivation (Omwancha and Brown, 2006). These orally active nonsteroidal nonaromatizable SARMs are being developed to target androgen action in bone, muscle, fat, and to influence libido but to not exacerbate prostate growth, hirsutism, and acne. Several have recently been identified with beneficial effects on bone mass (Allan *et al.,* 2007; Kearbey *et al.,* 2007; Miner *et al.,* 2007). Thus, this class of drug may provide a new alternative to androgen replacement therapy.

ANDROGEN RECEPTOR AND SKELETAL CELLULAR BIOLOGY

Because there remains confusion clearly interpreting the skeletal actions of circulating sex steroids as noted, the specific mechanisms by which androgens affect skeletal homeostasis are becoming the focus of intensified research (Vanderschueren *et al.,* 2004; Wiren, 2005). As a classic steroid hormone, the biological cellular signaling responses to androgen are mediated through the AR, a ligand-inducible transcription factor. ARs have been identified in a variety of cells found in bone (Abu *et al.,* 1997). Characterization of AR expression in these cells thus clearly identifies bone as a target tissue for androgen action. The direct effects of androgen that influence the complex processes of proliferation, differentiation, mineralization, and gene expression in the osteoblast are being characterized, but much remains to be established. Androgen effects on bone may also be indirectly modulated and/or mediated by other autocrine and paracrine factors in the bone microenvironment. The rest of this chapter will review recent progress on the characterization of androgen action through the AR in bone.

Molecular Mechanisms of Androgen Action in Bone Cells: The AR

Direct characterization of AR expression in a variety of tissues, including bone, was made possible by the cloning of the AR cDNA (Chang *et al.,* 1988; Lubahn *et al.,* 1988). The AR is a member of the class I (so-called classical or steroid) nuclear receptor superfamily, as are the ERα and ERβ isoforms, the progesterone receptor, the mineralocorticoid, and glucocorticoid receptor (Mangelsdorf *et al.,*

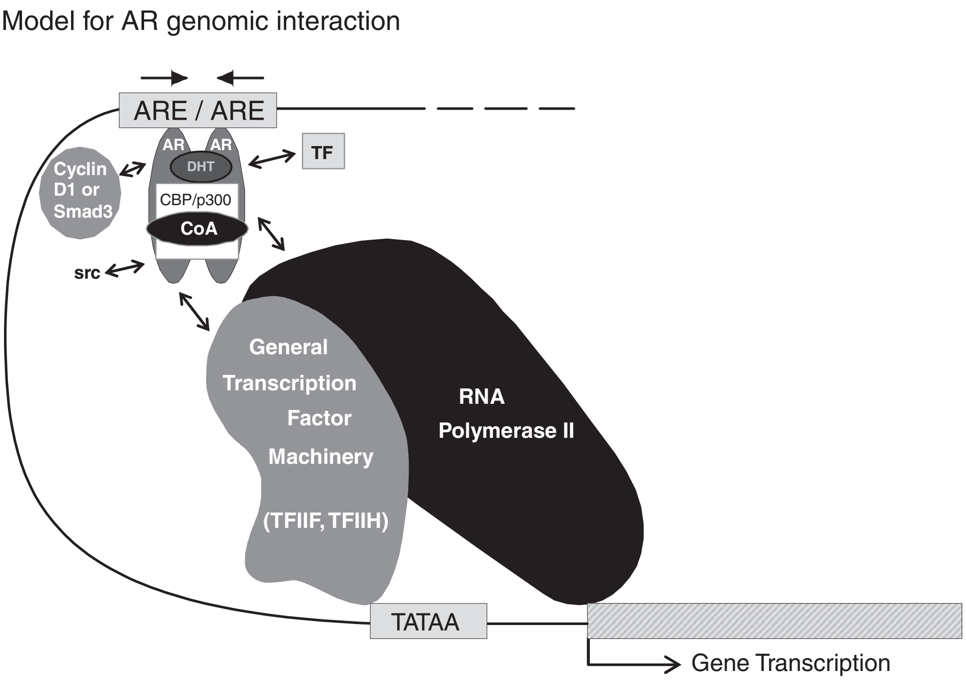

FIGURE 2 Model of AR regulation of gene expression. Binding of androgen promotes high-affinity dimerization, followed by DNA binding at the androgen response element (ARE) in an androgen-responsive gene promoter. Coactivators may remodel/modify chromatin through histone acetylase activity to open chromatin structure (Spencer *et al.*, 1997), or act as a bridge to attract transcription factors (TFs) that target binding of TATA-binding protein to the TATAA sequence (Beato and Sanchez-Pacheco, 1996). Conversely, corepressors act through histone deacetylase activity to reduce accessibility of promoter sequences. Phosphorylation of receptor may result from activation of SRC by growth factors (Kraus *et al.*, 2006). Smad3 can act as either a coactivator or corepressor (Hayes *et al.*, 2001; Kang *et al.*, 2002), while cyclin D1 is a corepressor of AR transactivation (Leader *et al.*, 2006). AR can also directly contact TFIIH and TFIIF (Lee and Chang, 2003) in the general transcription machinery. Such interactions between the AR and the general transcription machinery, leading to stable assembly, results in recruitment of RNA polymerase II and subsequent increased gene transcription. Downregulation of gene expression can also be AR mediated.

1995). Steroid receptors are transcription factors with a highly conserved modular design characterized by three functional domains: the transactivation, DNA binding, and ligand binding domains. In terms of function, the DNA binding domain targets the receptor complex to a specific DNA sequence known as the hormone response element and has high homology among the steroid receptors; the transactivation function mediates transcriptional regulation of gene expression and is localized in both the amino and carboxyterminal of the molecule; the carboxyterminal ligand binding domain mediates not only ligand binding but also receptor dimerization and nuclear translocation, in addition to transcriptional regulation (Mangelsdorf *et al.*, 1995). In the absence of ligand, the AR protein is generally localized in the cytoplasmic compartment of target cells in a large complex of molecular chaperones, consisting of loosely bound heat-shock, cyclophilin, and other accessory proteins (Picard, 2006). Interestingly, in the unliganded form, AR conformation is unique with a relatively unstructured amino-terminal transactivation domain (Shen and Coetzee, 2005). As lipids, androgens can freely diffuse through the plasma membrane to bind the AR to induce a conformational change. Once bound by ligand, the AR dissociates from the multiprotein complex, translocates to the nucleus allowing the formation of homodimers (or potentially heterodimers), and recruits coregulators (coactivators or corepressors), to initiate a cascade of events in the nucleus that influence transcription (Chang *et al.*, 1995). It may be functionally significant that coactivators or corepressors are expressed in a cell type specific manner (Kumar *et al.*, 2006). Bound to DNA, the AR influences transcription and/or translation of a specific network of genes, leading to the specific cellular response to the steroid (see Fig. 2).

A steroid hormone target tissue is generally defined as one that expresses the steroid receptor, at a functional level, and with a measurable response in the presence of the hormone ligand. Bone tissue clearly meets this standard with respect to androgen. AR mRNA and specific androgen binding sites in normal human osteoblastic cells were first reported by these workers (Colvard *et al.*, 1989). The abundance of both AR and ER proteins was similar, suggesting that androgens and estrogens each play important roles in skeletal physiology. Subsequent reports have confirmed AR mRNA expression and/or the presence of androgen binding sites in both normal and clonal, transformed osteoblastic cells derived from a variety of species (Benz *et al.*, 1991; Liesegang *et al.,* 1994; Nakano *et al.,* 1994; Orwoll *et al.,* 1991; Takeuchi *et al.,* 1994; Zhuang *et al.,* 1992). The size of the AR mRNA transcript in osteoblasts (about 10 kb) is similar to that described in prostate and other tissues (Chang *et al.,* 1988), as is the size of the AR protein analyzed by Western blotting (~110 kDa) (Nakano *et al.,* 1994). There are reports of two isoforms of AR protein in human osteoblast-like cells (~110 and ~97 kDa) (Kasperk *et al.,* 1997a) as first described in human prostatic tissue (Wilson and McPhaul, 1994). It appears these isoforms do not possess similar functional activities in bone, particularly with respect to effects on proliferation (Liegibel *et al.,* 2003). The number of specific androgen binding sites in osteoblasts varies, depending on methodology and the cell source, from 1,000–14,000 sites/cell (Kasperk *et al.,* 1997a; Liesegang *et al.,* 1994; Masuyama *et al.,* 1992; Nakano *et al.,* 1994), but is in a range seen in other androgen target tissues. Furthermore, the binding affinity of the AR found in osteoblastic cells ($K_d = 0.5-2 \times 10^{-9}$) is typical of that found in other tissues. Androgen binding is specific, without significant competition by estrogen, progesterone, or dexamethasone (Colvard *et al.,* 1989; Kasperk *et al.,* 1997a; Nakano *et al.,* 1994). Finally, testosterone and DHT appear to have similar though not identical binding affinities for AR (Benz *et al.,* 1991; Nakano *et al.,* 1994). All these data are consistent with the notion that the direct biologic effects of androgenic steroids in osteoblasts are mediated at least in part via classic mechanisms associated with the AR as a member of the steroid hormone receptor superfamily described earlier.

In addition to the classical AR present in bone cells, several other androgen-dependent signaling pathways have been described. Specific binding sites for weaker adrenal androgens (such as DHEA) have been reported (Meikle *et al.,* 1992), thus raising the possibility that DHEA or similar androgenic compounds have direct effects in bone. DHEA can transactivate AR (Mo *et al.,* 2006), but DHEA and its metabolites may also bind and activate additional receptors, including ER, peroxisome proliferator activated receptor-α and pregnane X receptor (Webb *et al.,* 2006). It has been shown that DHEA rapidly inhibited c-*fos* expression in human osteoblastic cells (Bodine *et al.,* 1995), inhibition that was more robust than seen with the classical androgens (DHT, testosterone, androstenedione). In addition, DHEA may inhibit bone resorption by osteoclasts when in the presence of osteoblasts, likely through changes in osteoprotegerin (OPG) and receptor activator of NFγB ligand (RANKL) concentrations (Wang *et al.,* 2006), that are important mediators of osteoclastogenesis. Androgens may also be specifically bound in osteoblastic cells by a novel 63-kDa cytosolic protein (Wrogemann *et al.,* 1991). Finally, androgens may regulate osteoblast activity via rapid nongenomic mechanisms (Kang *et al.,* 2004; Kousteni *et al.,* 2003; Zagar *et al.,* 2004), through membrane receptors displayed at the bone cell surface (Lieberherr and Grosse, 1994). Nongenomic signaling generally involves either changes in intracellular calcium levels or rapid activation of kinase signaling cascades. However, the role and biologic significance of these nonclassical signaling pathways *in vivo* remains controversial (Neill, 2006), and most data suggest that genomic signaling through specific receptors may be the more significant regulator in bone and other tissues (Centrella *et al.,* 2004; Hewitt *et al.,* 2006; Sims *et al.,* 2003; van der Eerden *et al.,* 2002a; Windahl *et al.,* 2006).

Lastly, there are reports of AR polyglutamine tract (CAG/CAA) polymorphisms, leading to shorter or longer glutamine tract lengths in the receptor protein, which likely have a biological impact on androgen responses (Pettaway, 1999). Enhanced androgen action is associated with shorter AR CAG repeats and, conversely, hypoandrogenic traits are seen in patients with an elongation of more than 37 CAG repeats (Zitzmann *et al.,* 2005). Most studies have failed to find an effect of such polymorphisms in AR on bone mass (Kenny *et al.,* 2005; Valimaki *et al.,* 2005; Van Pottelbergh *et al.,* 2001). An exception to these findings is a reported association in postmenopausal women with long CAG AR isoforms, where spinal bone mineral density (BMD) was significantly lower compared to those with AR short alleles (Retornaz *et al.,* 2006). Different AR isoforms have the potential to interact in distinct fashions with other signaling molecules such as c-Jun (Grierson *et al.,* 1999), and may also influence variation in serum testosterone levels (Crabbe *et al.,* 2007). Thus, such AR CAG polymorphisms may have a significant influence on bone mass, although data remain underdeveloped.

Localization of AR Expression in Osteoblastic Populations

Ultimately, bone mass is determined by two biological processes: formation and resorption. Distinct cell types mediate these processes. The bone-forming cell – the osteoblast – synthesizes bone matrix, regulates mineralization, and is responsive to most calciotropic hormones. The osteoclast is responsible for bone resorption. Clues about the potential sequela of AR signaling might be derived from a better understanding of the cell types in which expression is documented. *In vivo* analysis has demonstrated expression of AR in all cells of the osteoblast lineage including osteoblasts and osteocytes, and in osteoclasts (van der Eerden *et al.,* 2002b). In the bone microenvironment, the localization of AR expression has been described in intact human bone by Abu *et al.* using immunocytochemical methods (Abu *et al.,* 1997). In developing bone from young adults, ARs were predominantly expressed in active osteoblasts at sites of bone formation and in bone marrow cells. ARs were also observed in osteocytes embedded in the bone matrix. Interestingly, both the pattern of AR distribution and the level of expression were similar in males and in females. Expression of the AR has also been characterized in cultured osteoblastic cell populations isolated from bone biopsy specimens, determined at both the mRNA level and by binding analysis (Kasperk *et al.*, 1997a). AR levels varied according to the skeletal site of origin and age of the donor of the cultured osteoblastic cells: expression was higher at cortical and intramembranous bone sites, and lower in trabecular bone. This distribution pattern may correlate with androgen responsiveness in various bone compartments. AR expression was highest in osteoblastic cultures generated from young adults, and somewhat lower in samples from either prepubertal or senescent bone. Data indicate preferential nuclear staining of AR in males at sexual maturity, suggesting activation and translocation of the receptor in bone when androgenic steroid levels are elevated, consistent with androgen regulation of AR levels at such times (e.g., Wiren *et al.*, 1999; Wiren *et al.*, 1997). Again, no differences were found between male and female samples, suggesting that differences in receptor number per se do not underlie development of a sexually dimorphic skeleton. Because androgens are so important in bone development at the time of puberty, it is not surprising that ARs are also present in epiphyseal chondrocytes (Abu *et al.*, 1997; Carrascosa *et al.*, 1990). The expression of ARs in such a wide variety of cell types known to be important for bone modeling during development, and remodeling in the adult, provides evidence for direct actions of androgens in bone and cartilage tissue. These results also highlight the complexity of androgen effects on bone. Although bone is a target tissue with respect to androgen action, the mechanisms and cell types by which androgens exert their effects on bone biology remain incompletely characterized. An additional complexity in terms of mechanism is that androgens may influence bone directly by activation of the AR, or indirectly after aromatization of androgens into estrogens with subsequent activation of ER as described earlier.

Regulation of AR Expression

The regulation of AR expression in osteoblasts is incompletely characterized. Homologous regulation of AR mRNA by androgen has been described that is tissue-specific; upregulation by androgen exposure is seen in a variety of mesenchymal cells including osteoblasts (Takeuchi *et al.*, 1994; Wiren *et al.*, 1999; Wiren *et al.*, 1997; Zhuang *et al.*, 1992) whereas downregulation is observed after androgen exposure in prostate and smooth muscle tissue (Lin *et al.*, 1993; Wiren *et al.*, 1997). The androgen mediated upregulation observed in osteoblasts, at least in part, occurs through changes in AR gene transcription (Wiren *et al.*, 1999; Wiren *et al.*, 1997). No effect, or even inhibition, of AR mRNA by androgen exposure in other osteoblastic models has also been described (Hofbauer *et al.*, 1997; Kasperk *et al.*, 1997a). Interestingly, a novel property of the AR is that binding of androgen increases AR protein levels. This property distinguishes AR from most other steroid receptor molecules that are downregulated by ligand binding. At least in part, the elevated AR protein levels may be a consequence of increased stability mediated by androgen binding results from N-terminal and C-terminal interactions (Langley *et al.*, 1998), but the stability of AR in osteoblastic cells has not been determined to date. The mechanism(s) that underlie tissue specificity in autologous AR regulation, and the possible biological significance of distinct autologous regulation of AR, is not established. It is possible that AR upregulation by androgen in bone may result in an enhancement of androgen responsiveness at times when androgen levels are rising or elevated.

Quantitative determination of the level of receptor expression during osteoblast differentiation is difficult to achieve in bone slices. However, analysis of AR, ERα and ERβ mRNA and protein expression during osteoblast differentiation *in vitro* has been described, revealing that each receptor displays differentiation-stage distinct patterns in osteoblasts (Wiren *et al.*, 2002). In contrast to the ERs, AR expression level increases throughout osteoblast differentiation with the highest AR levels seen in mature osteoblast/osteocytic cultures. These results suggest that an important compartment for androgen action may be mature, mineralizing osteoblasts, and also indicate that osteoblast differentiation and steroid receptor regulation are intimately associated. Given that the osteocyte is the most abundant cell type in bone, and a likely mediator of focal bone deposition and response to mechanical strain (Seeman, 2006), it is not surprising that androgens may also augment the osteoanabolic effects of mechanical strain in osteoblasts (Liegibel *et al.*, 2002).

AR expression in osteoblasts can be upregulated by exposure to other steroid hormones, including glucocorticoids, estrogen or 1,25-dihydroxyvitamin D_3 (Kasperk *et al.*, 1997a). Whether additional hormones, growth factors, or agents influence AR expression in bone is not known. Further, whether the AR in osteoblasts undergoes post-translational processing that might influence receptor signaling (stabilization, phosphorylation, etc.) as described in other tissues (Ikonen *et al.*, 1994; Kemppainen *et al.*, 1992), and the potential functional implications (Blok *et al.*, 1996; Wang *et al.*, 1999a), are also unknown. Phosphorylation may be of particular interest in osteoblasts, as it is known to positively or negatively influence receptor interaction with coactivators and corepressors (Weigel and Moore, 2007). Ligand-independent activation of AR has also been described in other tissues (Dehm and Tindall, 2006), but has not been explored in bone.

Steroid receptor transcriptional activity, including that of the AR, is strongly influenced by coactivator or corepressor function (He *et al.*, 2006; Yoon and Wong, 2006).

These coregulators can influence the downstream signaling of nuclear receptors; their levels are influenced by the cellular context, and they can differentially affect specific promoters. AR specific coactivators have been identified (MacLean *et al.,* 1997), many of which interact with the ligand binding domain of the receptor (Yeh and Chang, 1996). Expression and regulation of these modulators may thus influence the ability of steroid receptors to regulate gene expression in bone (Haussler *et al.,* 1997), but this remains underexplored with respect to androgen action. The specific coactivator/corepressor profile present in cells representing different bone compartments (i.e., periosteal cells, proliferating or mineralizing cells) may influence the activity of the selective receptor modulators such as SARMs described earlier.

THE CONSEQUENCES OF ANDROGEN ACTION IN BONE CELLS

Effects of Androgens on Proliferation and Apoptosis

Evidence suggests that androgens act directly on the osteoblast and there are reports, some in clonal osteoblastic cell lines, of modulatory effects of gonadal androgen treatment on proliferation, differentiation, matrix production, and on mineral accumulation (Notelovitz, 2002). Not surprisingly, androgen has been shown to influence bone cells in a complex fashion. As an example, the effect of androgen on osteoblast proliferation has been shown to be biphasic in nature, with enhancement following short or transient treatment but significant inhibition following longer treatment. As a case in point, it was demonstrated (Kasperk *et al.,* 1990; Kasperk *et al.,* 1989) in osteoblast-like cells in primary culture (murine, passaged human) that a variety of androgens in serum-free medium increase DNA synthesis ([^{3}H]thymidine incorporation) and cell counts. Testosterone and nonaromatizable androgens (DHT and fluoxymesterone) were nearly equally effective regulators. Yet the same group (Kasperk *et al.,* 1997a) reported that prolonged DHT treatment inhibited normal human osteoblastic cell proliferation (cell counts) in cultures pretreated with DHT. In addition, prolonged androgen exposure in the presence of serum inhibited proliferation (cell counts) by 15–25% in TE-85, a transformed human osteoblastic line (Benz *et al.,* 1991). Testosterone and DHT again were nearly equally effective regulators. Other workers (Hofbauer *et al.,* 1998) examined the effect of DHT exposure on proliferation in hFOB/AR-6, an immortalized human osteoblastic cell line stably transfected with an AR expression construct (with ~4,000 receptors/cell). In this line, DHT treatment inhibited cell proliferation by 20–35%. Consistent with stimulation of proliferation, Somjen *et al.* have demonstrated after exposure to DHT for 24 hours an increase in creatine kinase specific activity in male osteoblastic cells (Somjen *et al.,* 2006). Although these various studies employed different model systems (transformed osteoblastic cells vs. second to fourth passage normal human cells) and culture conditions (including differences in the state of osteoblast differentiation, receptor number, phenol red-containing vs. phenol red-free, or serum containing vs. serum-free), it appears exposure time is an important variable. Time dependence for the response to androgen has been shown (Wiren *et al.,* 2004a), where osteoblast proliferation was stimulated at early treatment times, but with more prolonged DHT treatment osteoblast viability decreased. This result was AR dependent (i.e., inhibitable by coincubation with flutamide), and was observed in both normal rat calvarial osteoblasts and in stably transfected AR MC-3T3 cells. In mechanistic terms, reduced viability was associated with overall reduction in mitogen-activated (MAP) kinase signaling, and with downstream inhibition of *elk-1* gene expression, protein abundance, and extent of phosphorylation. The inhibition of MAP kinase activity after chronic androgen treatment again contrasts with stimulation of MAP kinase signaling and AP-1 transactivation observed with brief androgen exposure (Wiren *et al.,* 2004a), which may be mediated through nongenomic mechanisms at least *in vitro*.

As an additional component of control of osteoblast abundance, it is important to consider the process of programmed cell death, or apoptosis (Wyllie *et al.,* 1980). Apoptosis is important generally during development and for homeostasis, but it has also been shown that as the osteoblast population differentiates *in vitro*, the mature bone cell phenotype undergoes apoptosis (Lynch *et al.,* 1998). With respect to the effects of androgen exposure, chronic DHT treatment has been shown to result in enhanced osteoblast apoptosis *in vitro* in both proliferating osteoblastic at day 5, and in mature osteocytic mineralizing cultures at day 29 (Wiren *et al.,* 2006). In the same study, the enhancement observed with DHT treatment was opposite to the inhibitory effects on apoptosis seen with E_2 treatment. An androgen-mediated increase in the Bax/Bcl-2 ratio was also observed, predominantly through inhibition of Bcl-2 that was dependent on functional AR. The increase in the Bax/Bcl-2 ratio was at least in part a consequence of reductions in Bcl-2 phosphorylation and protein stability, consistent with inhibition of MAP kinase pathway activation after DHT treatment as noted earlier. Importantly, a similar response was observed *in vivo* with characterization of apoptosis in calvaria harvested from transgenic mice with overexpression of AR targeted throughout the osteoblast lineage. In male transgenic mice with the normal hormonal milieu (i.e., without systemic administration of androgen), enhanced TUNEL staining is observed in bone in both osteoblasts and osteocytes, even in areas of new bone growth (Wiren *et al.,* 2006). This may not be surprising, given an association between new bone growth and apoptosis (Palumbo *et al.,* 2003), as has been observed in other remodeling tissues and/or associated with development and tissue homeostasis (Lanz *et al.,* 2003). Apoptotic cell death could thus be important in making room for new bone formation and matrix deposition, which may have clinical significance by influencing bone homeostasis and bone mineral density (Miura *et al.,* 2004). Thus, mounting evidence suggests that chronic androgen treatment increases neither osteoblast number nor viability in the mature bone compartment. It is interesting to speculate that, given strong androgen-mediated stimulation at the periosteal surface, such inhibitory action by androgens in osteoblasts at the endosteum (also see next section) is important for the maintenance of cortical width that is similar between males and females. Such a response would help pattern a skeleton in males that does not become excessively large and heavy during development or in the adult.

Effects of Androgens on Differentiation of Osteoblastic Cells

Osteoblast differentiation can be characterized by changes in alkaline phosphatase activity and/or alterations in the expression of important extracellular matrix proteins, such as type I collagen, osteocalcin, and osteonectin. Again, the effects of androgens on expression of these marker activities/proteins are poorly described and results are inconsistent between a variety of model systems. For example, enhanced osteoblast differentiation, as measured by increased matrix production, has been shown to result from androgen exposure in both normal osteoblasts and transformed clonal human osteoblastic cells (TE-89). Androgen treatment appeared to increase the proportion of cells expressing alkaline phosphatase activity, thus representing a shift toward a more differentiated phenotype (Kasperk *et al.,* 1989). Furthermore, the same group subsequently reported dose-dependent increases in alkaline phosphatase activity in both high and low-alkaline phosphatase subclones of SaOS2 cells (Kasperk *et al.,* 1996), and human osteoblastic cells (Kasperk *et al.,* 1997b). However, there are also reports employing a variety of model systems of androgens either inhibiting (Hofbauer *et al.,* 1998) or having no effect on alkaline phosphatase activity (Gray *et al.,* 1992; Takeuchi *et al.,* 1994). These various responses may reflect both the underlying complexity and dynamics of osteoblastic differentiation. Androgen-mediated increases in type Iα-1 collagen protein and mRNA levels (Benz *et al.,* 1991; Gray *et al.,* 1992; Kasperk *et al.,* 1996), and increased osteocalcin secretion (Kasperk *et al.,* 1997b), have also been described. Consistent with increased collagen production, androgen treatment has also been shown to stimulate mineral accumulation in a time- and dose-dependent manner (Kapur and Reddi, 1989; Kasperk *et al.,* 1997b; Takeuchi *et al.,* 1994). However, transgenic mice with targeted overexpression of AR in the osteoblast lineage showed decreased levels of most bone markers *in vivo* in RNA extracts derived from long bone samples, including decreased collagen, osterix, and osteocalcin gene expression (Wiren *et al.,* 2004b). These results suggest that, under certain conditions, androgens may enhance osteoblast differentiation and could thus play an important role in the regulation of bone matrix production and/or organization. On the other hand, many positive anabolic effects of androgen may be limited to distinct osteoblastic populations, for example in the periosteal compartment (Wiren, 2005; Wiren *et al.,* 2004b).

Androgen Effects on Other Cell Types in the Skeleton

Within the bone compartment, AR is detected in macrophages, megakaryocytes, and endothelial cells (Mantalaris *et al.,* 2001). Interestingly, ARs are also expressed in bone marrow stromal and mesenchymal precursor cells (Gruber *et al.,* 1999; Sinha-Hikim *et al.,* 2004), pluripotent cells that can differentiate into a variety of tissues including muscle, bone, and fat. Androgen action may modulate precursor differentiation toward the osteoblast and/or myoblast lineage, while inhibiting differentiation toward the adipocyte lineage (Singh *et al.,* 2003). These effects on stromal differentiation could underlie some of the well-described consequences of androgen administration on body composition including increased muscle mass (Herbst and Bhasin, 2004). Although it is an intriguing hypothesis to propose that androgen-mediated increases in muscle would indirectly increase bone mass through enhanced mechanical loading, to date it has not been established how significant such a contribution is. However, the time course required for oxandrolone (a synthetic testosterone analog) to positively effect bone mass in severely burned children is delayed relative to effects on lean mass, suggesting that alterations in muscle are indeed important (Bi *et al.,* 2007; Murphy *et al.,* 2004). In addition, androgen influences immune cells and hematopoiesis, and could play an indirect role in mediating effects on bone via immune cells. Although plausible, additional studies are needed to establish the importance of such indirect regulation of other tissues by androgen, to positively influence the skeleton.

Importantly, AR expression has also been detected in the osteoclast (Michael *et al.,* 2005; Mizuno *et al.,* 1994; van der Eerden *et al.,* 2002b), including human osteoclasts (Michael *et al.,* 2005), but see (Abu *et al.,* 1997; Noble *et al.,*1998), indicating modulation of osteoclast action by androgen. Thus, it has been shown that androgen treatment reduces bone resorption of isolated osteoclasts (Pederson *et al.,*1999), and inhibits osteoclast formation (Huber *et al.,* 2001) including that stimulated by parathyroid hormone (PTH) (Chen *et al.,* 2001). That AR may play a direct role in regulating (inhibiting) osteoclast activity is also suggested by the high turnover osteopenia observed in AR null mice (Kawano *et al.,* 2003). Also of importance, androgens indirectly modulate osteoclastogenesis and osteoclast activity through effects mediated by osteoblasts (Michael *et al.,* 2005), consistent with increased levels of OPG observed following testosterone treatment in cultured osteoblasts (Chen *et al.,* 2004), and in both the serum and in bone derived from skeletally targeted AR3.6-transgenic male mice (Wiren *et al.,* 2004b). Thus, although estrogen also inhibits bone resorption, androgen regulation is distinct compared to estrogen utilizing both direct and indirect pathways (Michael *et al.,* 2005). In addition, DHEA treatment has been shown to increase the OPG/RANKL ratio in osteoblastic cells and inhibit osteoclast activity in coculture (Wang *et al.,* 2006). Androgen may be a less significant determinant of bone resorption *in vivo* than estrogen (Falahati-Nini *et al.,* 2000; Oh *et al.,* 2005), although this remains controversial (Leder *et al.,* 2003).

As with effects noted in osteoblastic populations, androgens also regulate chondrocyte proliferation and expression. Although some of the consequences of androgen action are mediated after metabolic conversion to estrogen, which limits long bone growth, nonaromatizable androgen stimulates longitudinal bone growth (Nilsson *et al.,* 2005). AR expression has been demonstrated in biopsies of proximal tibial growth plate cartilage (Nilsson *et al.,* 2003), and androgen exposure promotes chondrogenesis as shown with increased creatine kinase and DNA synthesis after androgen exposure in cultured epiphyseal chondrocytes (Carrascosa *et al.,* 1990; Somjen *et al.,* 1991). Increased [^{35}S]sulfate incorporation into newly synthesized proteoglycan (Corvol *et al.,* 1987) and increased alkaline phosphatase activity (Schwartz *et al.,* 1994) are androgen mediated. Regulation of these effects is obviously complex, as they were influenced by the age of the animals and the site from which the chondrocytes were derived. Thus, in addition to effects on osteoblasts, multiple cell types in the skeletal milieu are regulated by androgen exposure, and all are likely involved in mediating the effects of androgens on the skeleton.

Interaction with Other Factors to Modulate Bone Activity

The effects of androgens on osteoblast activity must certainly also be considered in the context of the very complex endocrine, paracrine, and autocrine milieu in the bone microenvironment. Systemic and/or local factors can act in concert, or can antagonize, to influence bone cell function. This has been well described with regard to modulation of the effects of estrogen on bone (see for example Horowitz, 1993; Kassem *et al.,* 1996; Kawaguchi *et al.,* 1995). Androgens have also been shown to regulate well-known modulators of osteoblast proliferation or function. The most extensively characterized growth factor influenced by androgen exposure is transforming growth factor-β (TGF-β). TGF-β is stored in bone (the largest reservoir for TGF-β) in a latent form, and has been shown to be a mitogen for osteoblasts (Centrella *et al.,* 1994; Harris *et al.,* 1994). Androgen treatment has been shown to increase TGF-β activity in human osteoblast primary cultures. The expression of some TGF-β mRNA transcripts (apparently TGF-β2) was increased, but no effect on TGF-β1 mRNA abundance was observed (Bodine *et al.,* 1995; Kasperk *et al.,* 1990) but also see (Wang *et al.,* 1999b). At the protein level, specific immunoprecipitation analysis reveals DHT-mediated increases in TGF-β activity to be predominantly TGF-β2 (Bodine *et al.,* 1995; Kasperk *et al.,* 1997b). DHT has also been shown to inhibit both TGF-β gene expression and TGF-β-induced early gene expression that correlates with growth inhibition in this cell line (Hofbauer *et al.,* 1998). The TGF-β-induced early gene has been shown to be a transcription factor that may mediate some TGF-β effects (Subramaniam *et al.,* 1995). These results are consistent with the notion that TGF-β may mediate androgen effects on osteoblast proliferation. On the other hand, TGF-β1 mRNA levels are increased by androgen treatment in human clonal osteoblastic cells (TE-89), under conditions where osteoblast proliferation is slowed (Benz *et al.,* 1991). Thus, the specific TGF-β isoform may determine osteoblast responses. It is interesting to note that *in vivo*, orchiectomy drastically reduces bone content of TGF-β levels, and testosterone replacement prevents this reduction (Gill *et al.,* 1998). Finally, androgen may modulate the levels of certain SMADs (Miki *et al.,* 2007), transcription factors that mediate TGF-β signaling. These data support the findings that androgens influence TGF-β cellular expression or activity, and suggest that the bone loss associated with castration is related to a reduction in growth factor activity induced by androgen deficiency.

Other growth factor systems may also be influenced by androgens. Conditioned media from DHT-treated normal osteoblast cultures are mitogenic, and DHT pretreatment increases the mitogenic response to fibroblast growth factor and to insulin-like growth factor II (IGF-II) (Kasperk *et al.,* 1990). In part, this may be due to slight increases in IGF-II binding in DHT-treated cells (Kasperk *et al.,* 1990), as IGF-I and IGF-II levels in osteoblast conditioned media are not affected by androgen (Canalis *et al.,* 1991; Kasperk *et al.,* 1990). In contrast to effects of estrogen, most studies have not found regulation of IGF-I or IGF-II abundance by androgen exposure (Canalis *et al.,* 1991; Kasperk *et al.,* 1990; Nakano *et al.,* 1994), but see (Gori *et al.,* 1999). Androgens may also modulate expression of components of the AP-1 transcription factor (Bodine *et al.,* 1995) or AP-1 transcriptional activation (Wiren *et al.,* 2004a). Thus, androgens may modulate osteoblast differentiation via a mechanism whereby growth factors or other mediators of differentiation are regulated by androgen exposure.

Androgens may also modulate responses to other important osteotropic hormones/regulators. Testosterone and DHT specifically inhibit the cAMP response elicited by PTH or parathyroid hormone-related protein (PTHrP) in the human clonal osteoblast-like cell line SaOS-2 whereas the inactive or weakly active androgen 17α-epitestosterone had no effect. This inhibition may be mediated via an effect on the PTH receptor-G_s-adenylyl cyclase (Fukayama and Tashjian, 1989; Vermeulen, 1991). The production of prostaglandin E_2 (PGE_2), another important regulator of bone metabolism, is also affected by androgens. Androgens (both DHT and testosterone) were shown as potent inhibitors of both parathyroid hormone and interleukin1 stimulated PGE_2 production in cultured neonatal mouse calvaria (Pilbeam and Raisz, 1990). The effects of androgens on parathyroid hormone action and PGE_2 production suggest that androgens could act to modulate (reduce) bone turnover in response to these agents.

Finally, both androgen (Hofbauer and Khosla, 1999) and estrogen (Kassem *et al.,* 1996; Passeri *et al.,* 1993) (but see Rifas *et al.,* 1995) inhibit production of interleukin-6 by osteoblastic cells. In stromal cells of the bone marrow, androgens have been shown to have potent inhibitory effects on the production of interleukin-6 and the subsequent stimulation of osteoclastogenesis by marrow osteoclast precursors (Bellido *et al.,* 1995). Adrenal androgens (androstenediol, androstenedione, DHEA) have similar inhibitory activities on interleukin-6 gene expression and protein production by stromal cells (Bellido *et al.,* 1995). The loss of inhibition of interleukin-6 production by androgen may also contribute to the marked increase in bone remodeling and resorption that follows orchiectomy, in addition to modulation of osteoclast activity through changes in the OPG/RANKL ratio as noted earlier. Moreover, androgens inhibit the expression of the genes encoding the two subunits of the IL-6 receptor (gp80 and gp130) in the murine bone marrow, another mechanism that may blunt the effects of this osteoclastogenic cytokine in intact animals (Lin *et al.,* 1997). In these aspects, the effects of androgens seem to be similar to those of estrogen, which may also indirectly inhibit osteoclastogenesis via mechanisms that involve interleukin-6 inhibition and/or OPG/RANKL ratio changes.

The Skeletal Effects of Androgen: Animal Studies

The effects of androgens on bone remodeling have been examined fairly extensively in animal models. Much of this work has been in species such as rodents, not perfectly suited to reflect human bone metabolism (but see Kalu, 1991), and certainly the field remains incompletely explored. Nevertheless, animal models do provide valuable insights into the effects of androgens at organ and cellular levels. Many of the studies of androgen action have been performed in male rats, in which rapid skeletal growth occurs until about four months of age, at which time epiphyseal growth slows markedly (although never completely ceases at some sites). Because the effects of androgen deficiency may be different in growing versus more mature adult animals (see Vanderschueren *et al.,* 2004), it is appropriate to consider the two situations independently.

Effects on Epiphyseal Function and Bone Growth during Skeletal Development and Puberty

In most mammals there is a marked gender difference in bone morphology. The mechanisms responsible for these differences are complex, and presumably involve both androgenic and estrogenic actions. Estrogens are particularly important for the regulation of epiphyseal function, and act to reduce the rate of longitudinal growth via influences on chondrocyte proliferation and action, as well as on the timing of epiphyseal closure (Turner *et al.,* 1994). Androgens appear to have opposite effects, and tend to promote long bone growth, chondrocyte maturation, and metaphyseal ossification. Androgen deficiency retards those processes (Lebovitz and Eisenbarth, 1975). Excess concentrations of androgen will accelerate aging of the growth plate and reduce growth potential (Iannotti, 1990), possibly via conversion to estrogens.

Although the specific roles of sex steroids in the regulation of epiphyseal growth and maturation remain somewhat unresolved, there is evidence that androgens do have direct effects independent of those of estrogen. For instance, testosterone injected directly into the growth plates of rats increases plate width (Ren *et al.,* 1989). In a model of endochondral bone development based on the subcutaneous implantation of demineralized bone matrix in castrate rats, both testosterone and DHT increase the incorporation of calcium during osteoid formation (Kapur and Reddi, 1989). Interestingly, in this model androgens reduced the incorporation of [^{35}S]sulfate into glycosaminoglycans early in the developing cartilage. In sum, these data support the contention that androgens play a direct role in chondrocyte physiology, but how these actions are integrated with those of other regulators is unclear.

During childhood and adolescence, skeletal development is characterized by marked expansion of cortical proportions and increasing trabecular density. During this process, the skeleton develops distinctly in males and females, most significantly at the periosteal surface. Thus, sex differences in skeletal morphology and physiology occur at or around puberty. For that reason, it is hypothesized that gender differences, particularly with respect to “bone quality” and architecture, i.e., predominantly bone width, are modulated by the sex steroids estrogen and androgen. Consistent with this, a distinct response to estrogen and androgen has been described *in vivo* especially in cortical bone. At the periosteum, estrogen suppresses while androgen stimulates new bone formation (Matsumoto *et al.,* 2006; Wiren *et al.,* 2004b), yet conversely at the endosteal surface estrogen stimulates but androgen strongly suppresses formation (Wiren *et al.,* 2004b). Thus, estrogen decreases but androgen increases radial growth in cortical bone through periosteal apposition, indicating that these two sex steroids may act in opposition in some situations at distinct bone compartments. These divergent responses to estrogen and androgen during growth likely play an important role in determining sexual dimorphism of the skeleton, i.e., that male bones are wider but not thicker than females (Seeman, 2003). Young men do have larger bone areas than women with increased whole bone cross-sectional area, particularly at peripheral sites (Riggs *et al.,* 2004). Low levels of estrogen in the obligate presence of androgen and AR may also be important for stimulation of periosteal bone formation during development (Bouillon *et al.,* 2004; Rochira *et al.,* 2007), but estrogen is not apparently important after puberty (Matsumoto *et al.,* 2006). Sex steroid effects can be influenced by interaction with the growth hormone IGF-I axis in the coordination of skeletal growth. Growth hormone deficiency in males has no net effect on endosteal growth but reduced by half expansion at the periosteal surface (Kim *et al.,* 2003), and produces greater deficits in females. The effect of growth hormone on periosteal formation may be mediated by increased IGF-I concentrations (Venken *et al.,* 2007). Although androgens did not affect IGF-I expression, both androgen and IGF-I were required for optimal stimulation at the periosteal surface. In contrast, analysis indicates that androgens stimulate trabecular bone modeling independent of growth hormone. Androgens are also essential for the production of peak total-body bone mass in males (Vanderschueren *et al.,* 2005), and a recent study has found that serum testosterone and IGF-1/IGFBP-3 ratio are major determinants of BMD at different stages of puberty in males (Pomerants *et al.,* 2007).

Mature Male Animals

Results from animal studies also support an effect of androgen on bone formation in the mature animal. Experimental strategies such as pharmacological or surgical (gonadectomy) intervention and examination of genetic models have all been employed to characterize androgen signaling in the adult. As expected, the consequences of either androgen administration or conversely withdrawal are complex, with effects in all bone compartments (see Fig. 3). In mature rats, castration eventually results in osteopenia and both cortical and trabecular compartments are affected. At a time when longitudinal growth has slowed markedly, pronounced differences as a consequence of castration appear in cortical bone ash weight per unit length, cross-sectional area, cortical thickness, and bone mineral density (Danielsen, 1992; Vanderschueren *et al.,* 1992; Verhas *et al.,* 1986; Wink and Felts, 1980). Distinct effects of androgen are seen with gonadectomy when comparing the effects of orchiectomy in male vs. ovariectomy in female rats. Generally castration results in changes in both trabecular and cortical bone compartments, and dramatic bone loss in trabecular bone is noted in both males and females, but sex-specific responses are most dimorphic in cortical bone. Ovariectomy and the associated loss of sex steroids in the female generally results in decreased trabecular area with increased osteoclast number. In cortical bone in ovariectomized females, an increase at the periosteal surface is seen with circumferential enlargement but a decrease in endosteal labeling. These results demonstrate that estrogen protects trabecular bone predominantly through inhibition of osteoclast activity/recruitment, but has an inhibitory action at the periosteal surface as noted earlier (for example, see Vandenput *et al.,* 2004). In the male, orchiectomy with the attendant loss of sex steroids also results in decreased trabecular area with increased osteoclast number as in females. However, careful histomorphometric analysis of androgen action in orchiectomized male mice has shown that the bone-sparing effect of AR activation in trabecular bone is distinct from a similar bone-sparing effect of ERα at that site (Moverare *et al.,* 2003). The analysis showed that AR activation does preserve the number of trabeculae, but does not preserve thickness, volumetric density, nor mechanical strength in cortical bone. In cortical bone,

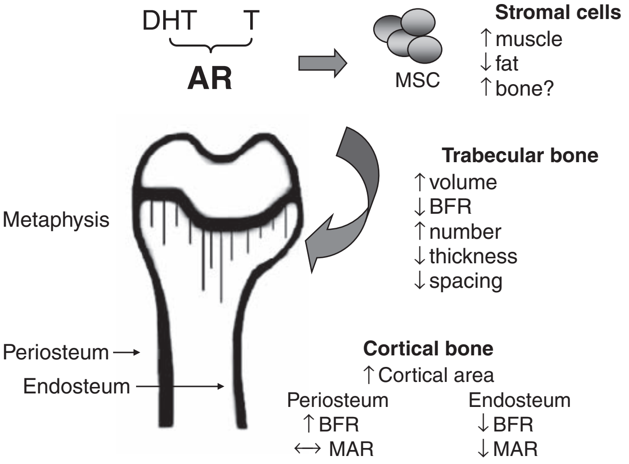

FIGURE 3 Model for androgen-mediated action in the skeleton, derived from androgen deficient or androgen replacement studies. References are cited in the text. AR activation by androgen influences a variety of skeletal compartments or target sites, including stromal cells, trabecular bone, intramembranous bone (not shown), and cortical bone. Arrows indicate the changes induced by androgen action. In general, AR activation in bone cells results in a low turnover phenotype and may also influence body composition.

periosteal formation is reduced with the loss of androgen after gonadectomy in males, in contrast to ovariectomized females. Androgen treatment is effective in suppressing the acceleration of bone remodeling normally seen after orchiectomy (Venken *et al.,* 2005a). This divergent response at the periosteal surface after castration in male and female animals abolishes the sexual dimorphism usually present in radial bone growth. In the intact animal, the stimulation of endosteal formation by estrogen compensates for the lack of periosteal formation, thus leading to no difference in cortical width between the sexes. Nevertheless, factors that influence periosteal apposition may constitute an important therapeutic class because periosteal bone formation is often a neglected determinant of bone strength (Seeman, 2003). Orchiectomy shows little net effect on the endosteal surface in males (Kim *et al.,* 2003) or slight reductions likely due to increased resorption. Consistent with this, increased intracortical resorption cavities are reported to result from orchiectomy (Prakasam *et al.,* 1999; Wink and Felts, 1980). As might be expected in light of these changes, breaking strength (N) is decreased in cortical bone (Kim *et al.,* 2003). Interestingly, DHT as a specific AR agonist is less effective than testosterone in the cortical bone compartment in elderly orchidectomized rats (Vandenput *et al.,* 2002; Wakley *et al.,* 1991). In addition, it appears that orchiectomy affects cranial development more than ovariectomy (Fujita *et al.,* 2004), suggesting that androgen action is particularly important in intramembranous bone.

Changes in the skeleton can occur rapidly after castration, and osteopenia becomes pronounced with time. This bone loss appears to result in part from increased bone resorption, as it is associated with increases in resorption cavities, osteoclasts, and blood flow (Verhas *et al.,* 1986; Wink and Felts, 1980). Initially, dynamic histomorphometric and biochemical measures of bone remodeling increase quickly, with evidence of increased osteoclast numbers only one week after castration (Gunness and Orwoll, 1995; Vanderschueren *et al.,* 1994a; Wink and Felts, 1980). Changes include an increase in osteoblastic activity as well as increased bone resorption, reflecting an initial high turnover state that is followed by a reduction in remodeling rates and low turnover osteopenia. In the SAMP6 mouse, a model of accelerated senescence in which osteoblastic function is impaired, the rise in remodeling following orchiectomy is blunted, which has been interpreted as evidence that the early changes after gonadectomy are dependent on osteoblast-derived signals (Weinstein *et al.,* 1997). As noted earlier, androgens reduce osteoclast formation and activity (Huber *et al.,* 2001), which may be partially indirectly mediated by increased OPG levels (Chen *et al.,* 2004; Wiren *et al.,* 2004b). The initial phase of increased bone remodeling activity subsides with time (Vanderschueren *et al.,* 1994a; Verhas *et al.,* 1986) and by four months there is evidence of a depression in bone turnover rates in some skeletal areas. As in younger animals, indices of mineral metabolism are not altered by these changes in skeletal metabolism (Vanderschueren *et al.,* 1992).

As a potential model for the effects of hypogonadism in humans (see Vanderschueren *et al.,* 2004), animal models therefore indicate an early phase of high bone turnover and bone loss after orchiectomy, followed by a reduction in remodeling rates and osteopenia. The remodeling imbalance responsible for loss of bone mass appears complex, as there are changes in rates of both bone formation and resorption, and patterns that vary from one skeletal compartment to another. Broad changes are similar (but not identical in detail) to those noted in female animals after castration, in which a loss of estrogen signaling has been associated with a stimulation of osteoblast progenitor differentiation, an increase in osteoclast numbers, bone resorption, and bone loss (Jilka *et al.,* 1998).

Androgens in the Female Animal

Of course androgens are present in both sexes and likely also affect bone metabolism in females. Although testosterone serum concentrations are much lower in females than in males, the concentration of other androgens like androstenedione and DHEA-sulfate are in fact similar between the sexes. In castrated female rats, DHT administration suppresses elevated concentrations of osteocalcin and of bone resorption markers (Mason and Morris, 1997). However, alkaline phosphatase activity increases further. Additional evidence to support the contention that androgens play a role in females includes the fact that antiandrogens are capable of evoking osteopenia in intact (i.e., fully estrogenized) female rats (Goulding and Gold, 1993; Lea *et al.,* 1996). This finding suggests that androgens provide crucial support to bone mass in females, in addition to a role for estrogens. Of interest, the character of the bone loss induced by flutamide suggested that estrogen prevents bone resorption whereas androgens may stimulate bone formation. In periosteal bone, DHT and testosterone stimulate periosteal formation after orchiectomy in young male rats, whereas in castrated females they suppress bone formation (Turner *et al.,* 1990b), perhaps reflecting an interaction or synergism between sex steroids and their effects on bone. However, in ovariectomized females with established osteopenia, treatment with DHT-stimulated periosteal bone formation whereas estradiol inhibited formation at the same surface (Coxam *et al.,* 1996). As noted earlier, combination therapy with estrogen and androgen in postmenopausal women is more beneficial than either steroid alone (Castelo-Branco *et al.,* 2000; Miller *et al.,* 2000; Raisz *et al.,* 1996), which has been confirmed in an animal model (Tivesten *et al.,* 2004). There is also some information concerning androgens in additional animal models, including primates. For instance, in young female cynomolgus monkeys, testosterone treatment increased cortical and trabecular bone density as well as biomechanical strength (Kasra and Grynpas, 1995).

Gender Effects

In most mammals, there is a marked gender difference in morphology that results in a sexually dimorphic skeleton. The mechanisms responsible for these differences are obviously complex, and presumably involve both androgenic and estrogenic actions on the skeleton linked to different levels of each steroid between males and females. It is becoming increasingly clear that estrogens are particularly important for the regulation of epiphyseal function and act to reduce the rate of longitudinal growth via influences on chondrocyte proliferation and function, as well as on the timing of epiphyseal closure (Turner *et al.*, 1994). Androgens, on the other hand, appear to have opposite effects to estrogen on the skeleton. Androgens tend to promote long bone growth, chondrocyte maturation, and metaphyseal ossification as noted earlier (Cassorla *et al.*, 1984). Furthermore, the most dramatic effect of androgens is likely on bone size, consistent with gender-specific effects of androgens on periosteal bone formation (Turner *et al.*, 1990b). This difference of course has important biomechanical implications, with thicker bones being stronger bones (Seeman, 2003). Consistent with gender specificity, the response of the adult skeleton to the same intervention results is distinct responses in males and females. For example, in a model of disuse osteopenia, antiorthostatic suspension results in significant reduction in bone formation rate at the endosteal perimeter in males. In females, however, a decrease in bone formation rate occurred along the periosteal perimeter (Bateman *et al.*, 1997). However, DHT has also been shown to enhance periosteal bone formation after ovariectomy in females with established osteopenia (Coxam *et al.*, 1996), similar to the response in males noted earlier. Nevertheless, gender-specific responses *in vivo* and *in vitro* (for example, see Somjen *et al.*, 2006), and the mechanism(s) that underlie such responses in bone cells, may have implications in treatment options for metabolic bone disease.

ANIMAL MODELS OF ALTERED ANDROGEN RESPONSIVENESS

The specific contribution of AR signaling *in vivo* has also been approached in genetic animal models with global AR modulation, including the testicular feminization (Tfm) model of androgen insensitivity (Tozum *et al.*, 2004; Vandenput *et al.*, 2004) and with nontargeted (global) AR knockout mice (Kawano *et al.*, 2003; Yeh *et al.*, 2002). The Tfm male rat is AR signaling deficient, and provides an interesting model for the study of the unique effects of androgens in bone. In these rats androgens are generally incapable of action at the AR, but the model is complicated by the fact that estrogen and androstenedione concentrations are considerably higher than in normal males (Vanderschueren *et al.*, 1994b; Vanderschueren *et al.*, 1993b). Clear increases also exist in Tfm male rats in serum concentrations of osteocalcin, calcium, and phosphorus, whereas IGF-1 concentrations are decreased. Estimates of bone mass suggest that Tfm rats have reduced longitudinal and radial growth rates, but trabecular volume and density are similar to those of normal rats, likely a consequence of high serum estrogen levels. In selected sites, measures of bone mass and remodeling were intermediate between normal male and female values. With castration, bone volume is markedly reduced in Tfm male rats, suggesting a major role for estrogens as well in skeletal homeostasis. This model indicates that androgens do have an independent role to play in normal bone growth and metabolism, but the model as described is complex and not easily dissected. Tfm mice are different from Tfm rats in that Tfm mice develop a high-turnover trabecular bone phenotype (Vandenput *et al.*, 2004), associated with low levels of circulating testosterone and estradiol. Meticulous analysis in Tfm mice has shown that the positive effects of testosterone on cortical bone are generally mediated by stimulation of periosteal bone formation through the AR (Vandenput *et al.*, 2004), whereas testosterone effects on trabecular bone likely involves both AR and ER signaling. Thus, these studies demonstrate that AR-mediated testosterone action is essential for periosteal bone formation (in male mice), while AR contributes to trabecular bone maintenance along with ER. This is very similar to the study of humans with the androgen insensitivity syndrome. Even when compliance with estrogen replacement is excellent, there is a deficit in bone mineral density in women with androgen insensitivity (Marcus *et al.*, 2000) that has been observed at the spine (Danilovic *et al.*, 2007) or at both spine and hip sites (Sobel *et al.*, 2006). In addition, final height was intermediate between what would be predicted for males or males (Danilovic *et al.*, 2007). These results provide evidence for an important role for androgens in normal male growth and bone density that is not replaced by estrogens. However, inadequate estrogen replacement appeared to worsen the deficit (Marcus *et al.*, 2000), and other environmental factors are difficult to quantitate. Thus, in Tfm models that lack functional AR, orchiectomy demonstrates the importance of AR in mediating the positive effects of androgen to contribute to trabecular bone maintenance, and in cortical bone particularly at the periosteal surface (Tozum *et al.*, 2004; Vandenput *et al.*, 2004).

Global AR null mice, developed using a cre/loxP approach, have complete AR disruption from birth (De Gendt *et al.*, 2005; Sato *et al.*, 2002; Yeh *et al.*, 2002). The ARKO null model should not be confused with the aromatase null, or ArKO, mouse. The bone phenotype that develops in male ARKO mice is a high-turnover osteopenia, with reduced trabecular bone volume and a significant stimulatory effect on osteoclast function (Kato *et al.,* 2004; Kawano *et al.,* 2003; Venken *et al.,* 2006; Yeh *et al.,* 2002). As expected, bone loss with orchiectomy in male ARKO mice was only partially prevented by treatment with aromatizable testosterone due to the lack of AR function. Interestingly, orchiectomy in control mice produced a similar trabecular bone phenotype to that observed in the ARKO male, indicating that AR activation is a major determinant of trabecular bone development (Venken *et al.,* 2006). Analysis of the impact of androgens with and without aromatase inhibitors suggests that aromatase inhibition of periosteal bone formation is dependent on AR expression, as aromatase treatment was without effect in ARKO mice but reduced the effect of androgen to stimulate periosteal apposition in controls (Venken *et al.,* 2006). In contrast, aromatase inhibition had no effect on protection of trabecular bone by either testosterone or DHT after orchiectomy, similar to results obtained in Tfm mice described earlier.

Final models for AR modulation are represented by generation of mice with skeletally targeted modification of AR expression, with either overexpression (Wiren *et al.,* 2004b) or deletion using a tissue-specific cre/loxP approach (Notini *et al.,* 2007). In the first model, full-length AR is under the control of the 3.6 kb type I collagen promoter, with AR overexpression in osteoblast stromal precursors and throughout the osteoblast lineage. AR3.6-transgenic mice are the only model with bone-targeted overexpression of AR, and demonstrate enhanced sensitivity to androgen in tissues where AR is overexpressed yet without changes in circulating steroids or the complication of systemic androgen administration (Wiren *et al.,* 2004b). AR overexpression in this model results in a complex phenotype predominantly in males, with increased trabecular bone mass (with increased trabecular number but not thickness) in the setting of inhibition of resorption due to reduced osteoclast activity. In addition, cortical formation is altered in an envelope-specific fashion, with periosteal expansion but inhibition of inner endosteal deposition, in line with the known effects of androgen to stimulate periosteal apposition and opposite to the effects of estrogen on these compartments. Inhibition of osteoclastic resorption may be responsible for altered trabecular morphology, consistent with reduced osteoclast activity and increased trabecular bone volume observed with androgen therapy in rodents and humans. In trabecular bone, AR signaling results in increased volume, increased trabecular number, and decreased spacing with a modest reduction of thickness. The dramatic inhibition of bone formation at the endosteal envelope may underlie the modest decrease in cortical bone area and subsequent reductions in biomechanical properties observed. A second model for specific bone targeting results in disruption of normal AR expression using col 2.3-cre mice crossed with floxed AR mice (Notini *et al.,* 2007). Knockdown of AR expression is confined to mature osteoblasts/osteocytes. During development, there is a progressive loss of trabecular bone with decreased trabecular number but increased spacing and increased width, opposite to the phenotype observed with AR overexpression. There was little effect on cortical bone in this model. Notably, the bone phenotype observed in these models is consistent with many of the known effects of androgen treatment on the skeleton. Combined, these results indicate that AR expressed in bone can be a direct mediator of androgen action to influence skeletal development and homeostasis.

EFFECTS ON THE PERIOSTEUM: THE ROLE OF AR VS. AROMATIZATION OF TESTOSTERONE

As noted earlier, androgen-mediated AR transactivation is likely a key determinant of the sexually dimorphic pattern of periosteal apposition, an effect that is clearly demonstrated in male AR3.6-transgenic mice even in the absence of hormone administration (Wiren *et al.,* 2004b). Furthermore, essentially all of the alterations induced by orchiectomy (in both growing and mature animals) can be prevented at least in part by replacement with either testosterone or nonaromatizable androgens (Kapitola *et al.,* 1995; Prakasam *et al.,* 1999; Schoutens *et al.,* 1984; Somjen *et al.,* 1994; Turner *et al.,* 1990b; Vanderschueren *et al.,* 1993a; Wakley *et al.,* 1991). In sum, these results strongly suggest that aromatization of androgens to estrogens cannot fully explain the actions of androgens on bone metabolism.

However estrogens also seem to play a role in the effects of androgen on apposition, likely through indirect mechanisms. Although AR activity is essential, low levels of estrogens are likely required for optimal stimulation of periosteal growth (Venken *et al.,* 2006), as observed in aromatase deficiency even in males (Bouillon *et al.,* 2004). Estrogens may also help prevent bone loss following castration in male animals. For example, it has been reported (Vanderschueren *et al.,* 1992) that estradiol (and also nandrolone) was capable of not only preventing the increase in biochemical indices stimulated by orchiectomy, but also preventing cortical and trabecular bone loss. In fact, estradiol resulted in an absolute increase in trabecular bone volume not achieved with androgen replacement. Many of these estrogen-mediated responses appear to be indirectly mediated through increased IGF-I levels (Venken *et al.,* 2005b); in contrast androgens stimulate periosteal apposition independent of the growth hormone -IGF axis as noted earlier (Venken *et al.,* 2007). Similarly, estrogen was reported to antagonize the increase in blood flow resulting from castration and to increase bone ash weight more consistently than testosterone.

The gender reversal of androgen administration to female animals is also instructive. Consistent with the stimulatory effect of androgen on the periosteal compartment, treatment with nonaromatizable DHT has been shown to increase periosteal bone formation in ovariectomized females with established osteopenia (Coxam *et al.,* 1996). Nonaromatizable androgens are also capable of preventing or reversing osteopenia and abnormalities in bone remodeling in ovariectomized females (Tobias *et al.,* 1994; Turner *et al.,* 1990b). These actions result from the suppression of trabecular bone resorption as well as stimulation of periosteal bone formation (Tobias *et al.,* 1994). Very similar results have been reported following the treatment of ovariectomized animals with DHEA (Turner *et al.,* 1990b). Moreover, blockage of androgen action with an AR antagonist in female rats already treated with an estrogen antagonist increases bone loss and indices of osteoclast activity more than treatment with an estrogen antagonist alone (Lea and Flanagan, 1999), again indicating that ovarian androgens (apart from estrogens) exert a protective effect on bone in females. Analogously, androstenedione reduces (although does not abrogate) trabecular bone loss and remodeling alterations in ovariectomized animals treated with an aromatase inhibitor. This protective effect was blocked by the addition of an AR antagonist (Lea and Flanagan, 1998; Lea *et al.,* 1998). Finally, although aromatase inhibition in male rats reduces bone mass, the large increase in remodeling induced by orchiectomy does not occur in these animals (Vanderschueren *et al.,* 1997). Also, orchiectomy in ERα null (α-ERKO) mice further reduces bone mass (Sims *et al.,* 2003). The latter observation implicates a role for androgens in the maintenance of bone mass in α-ERKO mice.

SUMMARY

The effects of androgens on bone health are obviously pervasive and complex (see Fig. 3). Androgens are important in the maintenance of a healthy skeleton, and have been shown to stimulate bone formation in the periosteum but reduce formation on the endosteal surface in cortical bone and in trabecular bone and reduce osteoclast activity. Androgens influence skeletal modeling and remolding by multiple mechanisms through effects on osteoblasts, osteoclasts, and even perhaps an influence on the differentiation of pluripotent stem cells toward distinct lineages. The specific effects of androgen on bone cells are mediated directly through an AR-signaling pathway, but there are also indirect contributions to overall skeletal health through aromatization and ER signaling. The effects of androgens are particularly dramatic during growth in boys, but almost certainly play an important role during this period in girls as well. Throughout the rest of life, androgens affect skeletal function in both sexes. Still poorly characterized, more needs to be done to unravel the mechanisms by which androgens influence the physiology and pathophysiology of bone, and there remains much to be learned about the roles of androgens at all levels. The interaction of androgens and estrogens, and how their respective actions in the skeleton and in other tissues can be utilized for specific diagnostic and therapeutic benefit, are important but unanswered issues. With an increase in the understanding of the nature of androgen effects will come greater opportunities to use their positive actions in the prevention and treatment of a wide variety of skeletal disorders.

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Chapter 47

Kinins and Neuro-osteogenic Factors

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INTRODUCTION

Inflammatory processes within soft tissues are characterized by intense vasodilatation, increased blood vessel permeability, and exudation of plasma to the perivascular adjacent tissue, followed by the migration of different leukocytes from the blood into the surrounding tissues. These vascular and cellular reactions are associated with clinical symptoms of inflammation and with altered metabolism in the surrounding milieu. Bone cells can react to a nearby inflammatory process with both anabolic and catabolic reactions. In most cases, this results in osteolytic loss of bone tissue, although in some cases, sclerotic reactions can be seen (Lerner, 2006a). These reactions are induced by inflammatory mediators capable of interacting not only with the inflammatory process, but also with bone cells. In recent years, the understanding of the interactions between cytokines from the immune cells and bone cells has expanded tremendously and the term "osteoimmunology" has been coined (reviewed in Takayanagi, 2007). This research area has been directed not only because of the possible role of cytokines in inflammatory bone disease, but also due to their possible roles in physiological bone remodeling and osteoclast development (reviewed in Takayanagi, 2005; Walsh *et al.,* 2006; Sato and Takayanagi, 2006), as well as their possible pathophysiological role in osteoporosis (reviewed in Pfeilschifter *et al.,* 2002; Lerner, 2006b). The discovery of the pleiotropic cellular effects of cytokines, and their important roles in the communication between different cells in inflammatory processes, has led to the interest in inflammation-induced bone loss, which has been focused on the role of these peptides (Lerner, 2006a). However, it has been known for many years that other peptides and nonpeptides are also involved in nonimmune, or classical, inflammatory reactions, e.g., the kallikrein-kinin system, the coagulation cascade, the fibrinolytic pathway, and prostaglandins are activated in inflammatory processes and play important roles in the tissue inflammatory response. Although these systems are most well known for their effects on vessel permeability and dilatation, pain, extravascular coagulation, and fibrinolysis, it has been demonstrated that they are also involved in cell activation, proliferation, migration, and control of proteolysis. As regarding bone, we and others have shown that kinins and thrombin stimulate bone resorption *in vitro* (reviewed in Lerner, 1994, 1997). Osteoblasts synthesize plasminogen activators and inhibitors of these activators in a manner controlled by stimulators of bone resorption (Leloup *et al.*, 1991); *in vitro* data indicate that this system may be involved in the degradation of noncollagenous bone matrix proteins without having any effect on osteoclast formation (Daci *et al.*, 1999). Interestingly, *in vivo* data show that the lack of plasminogen activator inhibitor 1 protects ovariectomized mice from trabecular bone loss without affecting cortical bone loss (Daci *et al.*, 2000), urokinase receptor knockout mice exhibit enhanced bone mass (Furlan *et al.,* 2007), and polymorphisms in the genes encoding urokinase plasminogen activator and plasminogen activator type I have been associated with increased alveolar bone loss in patients with periodontitis (Decarlo *et al.,* 2007).

The activities of bone cells can be regulated at a local level not only by cytokines and kinins. The immunohistochemical demonstration of nerve fibers containing different neuropeptides in the vicinity of bone tissue (reviewed in Bjurholm, 1989; Konttinen *et al.*, 1996; Lundberg, 2000; Persson, 2005) raises the possibility that neuropeptides, via neuro-osteogenic interactions, may directly or indirectly modulate the activity of bone cells in physiological and pathological conditions (reviewed in Lerner, 2000; Togari, 2002; Togari *et al.,* 2005; Elefteriou, 2005), in line with the view of neuroendocrine and neuroimmune interactions (van Hagen *et al.*, 1999). The presence of receptors for several neuropeptides on osteoblasts (Bjurholm *et al.*, 1992) and the finding that vasoactive intestinal peptide (VIP) can stimulate bone resorption in organ culture (Hohmann *et al.*, 1983), provide evidence for a possible direct effect of neuropeptides on bone. The reports that neuropeptides enhance the production of cytokines from a variety of cell types, including monocytes and bone marrow cells, indicate that signaling molecules from skeletal nerve fibers may indirectly affect the skeleton via a neuroimmune control of bone cells, in line with the view that neuroimmunoendocrine interactions are important

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for the regulation of a variety of cells and tissues. The recent observations demonstrating the importance of signaling from hypothalamus for bone remodeling have elegantly shown the role of central nervous system for bone metabolism (reviewed in Karsenty, 2006; Patel and Elefteriou, 2007; Allison *et al.,* 2007; for further details see Chapter 5).

This chapter summarizes the knowledge of the effects of kinins on bone and the local neuronal influence on bone tissue, as well as the interactions among kinins, neuro-osteogenic factors, and cytokines on bone metabolism.

ACTIVATION OF THE KALLIKREIN-KININ SYSTEM

Kinins are blood-derived short peptides released from kininogens as a result of the enzymatic action of kallikreins, proteolytic enzymes present in most tissues and body fluids (Fig. 1). The biological effects of the kallikrein-kinin system are mainly exerted by bradykinin (BK) and kallidin (Lys-BK) acting on a variety of cells via cell surface receptors of the B2 subtype. In addition, BK and kallidin, without the carboxyterminal arginine residue (des-Arg^9-BK and des-Arg^{10}-Lys-BK; respectively), can exert effects via BK B1 receptors. The kallikrein-kinin system is briefly summarized here, without giving any references to original reports. Readers are referred to extensive reviews in which relevant references can be found (reviewed in Marceau and Regoli, 2004; Moreau *et al.,* 2005; Pesquero and Bader, 2006; Sharma, 2006).

Hageman Factor

Activation of the plasma kallikrein system is initiated by the Hageman factor (coagulation factor XII), a single chain globulin (molecular weight 80,000), which can be activated by exposure to an activating macromolecular anionic surface and by endotoxin, as well as by an autocatalytic mechanism. The kallikrein system is activated by the Hageman factor by the action of this enzyme on plasma prekallikrein (Fig. 1).

Kallikreins

Plasma prekallikrein is a single chain globulin encoded by a single gene and is synthesized and secreted by hepatocytes as an inactive proenzyme. Activated plasma kallikrein acts on high molecular weight (HMW) kininogen at two sites, Lys-Arg and Arg-Ser, to release BK, a peptide consisting of nine amino acids with arginine at both the amino- and the carboxyterminal ends (Fig. 1).

Plasma kallikrein is inactivated rapidly by the C1 inhibitor and may also be inhibited by α_2-macroglobulin and antithrombin III.

Tissue kallikrein is a member of a multigene family with tissue-specific expression. Several of the serine proteases belonging to this family have important roles in the activation of peptide prohormones and growth factors. Tissue kallikrein liberates kallidin (Lys-BK) from both HMW and low molecular weight (LMW) kininogens, but because LMW kininogen is the most abundant kininogen, the enzyme preferentially uses LMW kininogen as substrate (Fig. 1).

As compared to plasma kallikrein, tissue kallikrein is less susceptible to inhibition. In humans, mainly α_1-antiprotease has some inhibitory capacity. In inflammatory processes, tissue kallikrein may be more important for kinin generation, because it seems less susceptible to degradation and because it can use both HMW and LMW kininogen as substrate.

Kininogens

HMW (88–120 kDa) and LMW (50–68 kDa) kininogens are synthesized by hepatocytes as single chain glycoproteins with one amino-terminal heavy chain and one carboxyterminal light chain. The HMW and LMW kininogens are coded for by a single gene, and the different forms are a consequence of alternate splicing of the gene transcript. The heavy chain of both kininogens contains a domain with cysteine proteinase inhibitory capacity, suggesting the possibility that kininogens may possess both pro- and anti-inflammatory activities.

Rats have a unique kininogen, T-kininogen (68 kDa), from which T-kinin (Ile-Ser-BK) is released by T-kininogenase(s). The levels of T-kininogen in plasma seem to be influenced by estrogen, with concentrations being increased in females at puberty and decreased in mature females by ovariectomy.

Kinins

The term kinin is derived from the Greek word *kineo* (to move) and was originally used for substances acting on smooth muscles. Today the term kinin is mainly restricted to peptides related to the nonapeptide BK. In this chapter, the word kinin is used for endogenous mammalian peptides with sequence homology to BK, and the kinin analogs refer to synthetic peptides whose amino acid sequence is modified from that of BK.

The kinins are not synthesized and released by cells, but are bioactive, short, and potent peptides that constitute a small part of large proteins (kininogens) from which they are released extracellularly by kininogenases. Four different, but closely related, primary kinins have been described: BK, kallidin, Hyp^3-BK, and T-kinin. The amino acid sequences of these peptides are shown in Table I.

Kinins released have a very short half-life *in vivo*, being estimated to approximately 30 seconds due to the action of different kininases. An interesting aspect is that one of these kininases, cleaving off the carboxyterminal arginine, gives rise to peptides (des-Arg^9-BK and des-Arg^{10}-Lys-BK) that

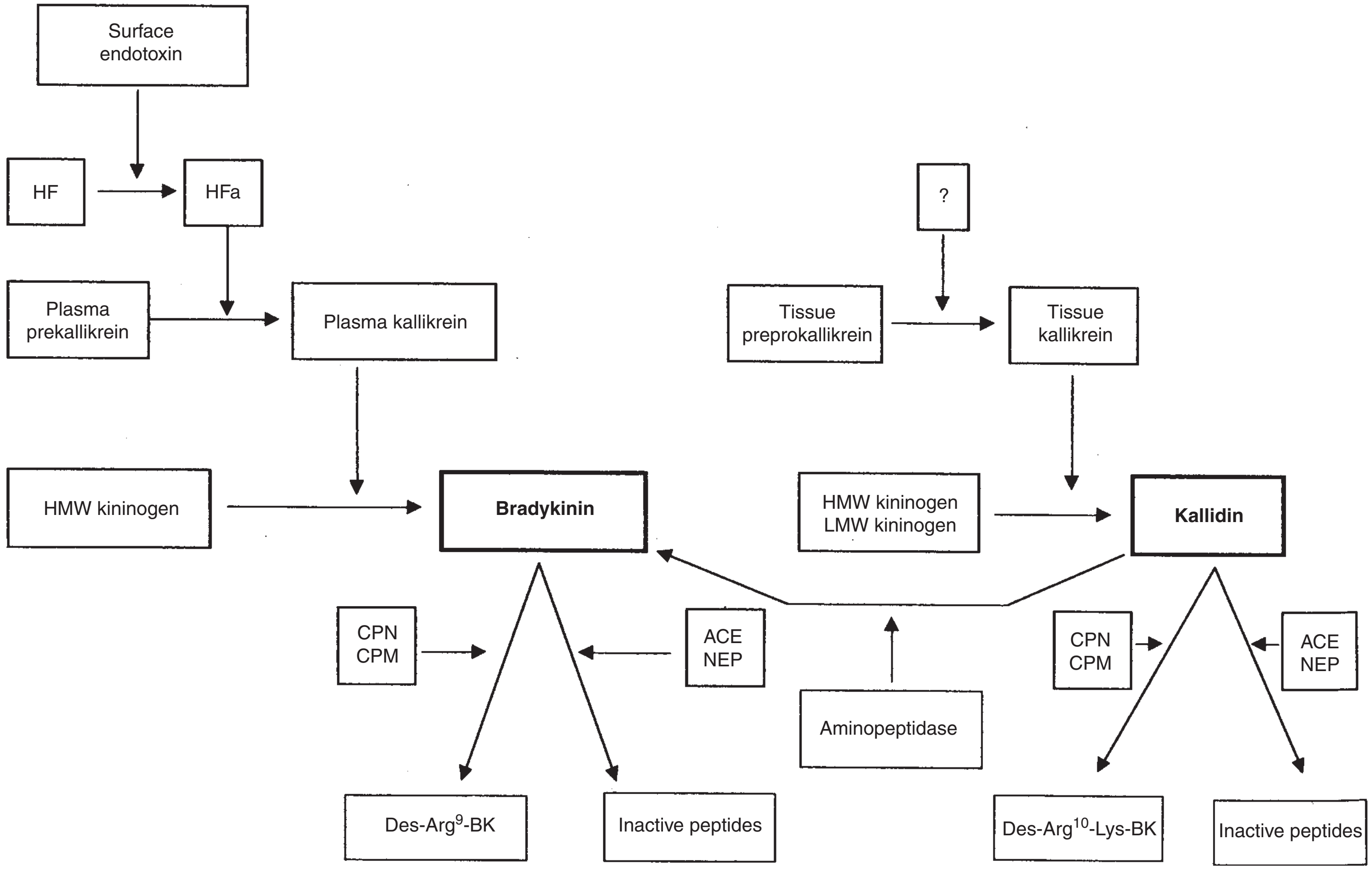
Surface endotoxin
HF
HFa
Plasma prekallikrein
Plasma kallikrein
HMW kininogen
Bradykinin
?
Tissue preprokallikrein
Tissue kallikrein
HMW kininogen
LMW kininogen
Kallidin
CPN
CPM
ACE
NEP
Aminopeptidase
CPN
CPM
ACE
NEP
Des-Arg^9-BK
Inactive peptides
Des-Arg^{10}-Lys-BK
Inactive peptides

FIGURE 1 Schematic representation of activation of the kallikrein-kinin system and the formation of kinins.

TABLE I Amino Acid Sequences of Natural Kinins and Bradykinin Analogs with Receptor Antagonistic Properties

			1	2	3	4	5	6	7	8	9
B2 receptor agonists											
Bradykinin			Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe-	Arg
Kallidin		Lys-	Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe-	Arg
Met-Lys-Bradykinin	Met-	Lys-	Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe-	Arg
Hyp^3-bradykinin			Arg-	Pro-	Hyp-	Gly-	Phe-	Ser-	Pro-	Phe-	Arg
T-kinin	Ile-	Ser-	Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe-	Arg
B1 receptor agonists											
Des-Arg^9-bradykinin			Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe	
Des-Arg^9-Lys-bradykinin		Lys-	Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe	
B1 receptor antagonist											
Des-Arg^9-[Leu^8]-bradykinin			Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Leu	
B2 receptor antagonists											
D-Arg-[Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin		Arg-	Arg-	Pro-	Hyp-	Gly-	Thi-	Ser-	Phe-	Thi-	Arg
Hoe 140		Arg-	Arg-	Pro-	Hyp-	Gly-	Thi-	Ser-	Tic-	Oic-	Arg

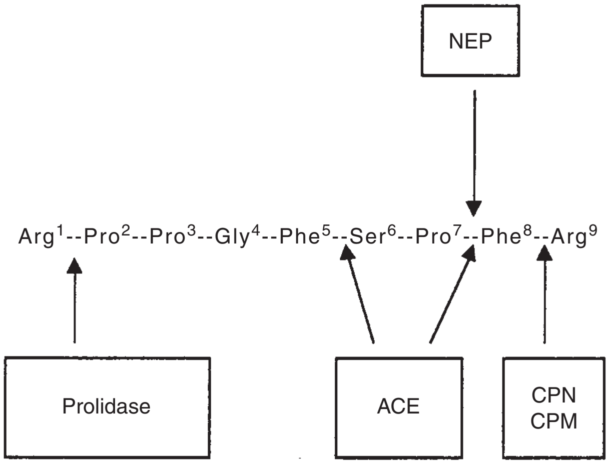
NEP
Arg^1--Pro^2--Pro^3--Gly^4--Phe^5--Ser^6--Pro^7--Phe^8--Arg^9
Prolidase
ACE
CPN
CPM

FIGURE 2 Cleavage sites for different kininases in the bradykinin molecule.

are biologically active in several cell types and therefore des-Arg^9-BK and des-Arg^{10}-Lys-BK are also included in the group of naturally produced kinins with biological activities.

Kininases

Kinins formed have a short half-life because they are destroyed rapidly by the enzymatic action of different proteases, collectively called kininases (Fig. 2). These enzymes are present both as circulating and as cell-bound enzymes. Several kininases have been described, including carboxypeptidase N (CPN) and carboxypeptidase M (CPM), together called kininase-I. Other kininases are two different kininase-II enzymes called angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP). A third type of kininases is prolidase and aminopeptidase.

Kininases have been described in biological fluids and in a variety of cells, but no information on the presence of these enzymes in bone is available. However, the fact that some (but not all) kininase II inhibitors potentiate the bone-resorbing effect of BK (Lerner *et al.*, 1987) provides indirect evidence for the presence of BK-inactivating enzymes in bone.

Bradykinin Receptors

Two different kinin receptors, termed B1 and B2, have been demonstrated using pharmacological methods such as rank order potencies for different agonists, sensitivity to receptor antagonists, and radioligand-binding studies (reviewed in Leeb-Lundberg *et al.*, 2005).

Des-Arg^{9}-BK and des-Arg^{10}-Lys-BK are the natural agonists for B1 receptors with the latter being the more potent ligand. Substitution of phenylalanine at position 8 in des-Arg^{9}-BK by an amino acid with an aliphatic side chain gives rise to B1 receptor antagonists, the classical one being des-Arg^{9}-[Leu^{8}]-BK. Hoe-140-des-Arg^{10} has also been found to be a potent and selective antagonist for B1 receptors.

The natural kinins with affinity for the B2 receptors are BK and Lys-BK. Since the discovery that substitution of proline at position 7 of BK with phenylalanine converts BK agonists to antagonists, several B2 receptor antagonists have been developed, with D-Arg-[Hyp^{3}, Thi^{5,8}, D-Phe^{7}]-BK being a very potent one. Hoe 140 D-Arg-[Hyp^{3}, Thi^{5}, D-Tic^{7}, Oic^{8}]-BK has been shown to be the first totally selective B2 receptor antagonist.

The distribution of kinin receptors in different cells and tissues and the relative expression of B1 and B2 receptors have been studied extensively, mostly by the use of pharmacological methods. B2 receptors seem to be constitutively expressed in a variety of tissues. B1 receptor expression has so far been demonstrated in certain cell types. In most cells, however, B1 receptor expression can be induced by tissue injury and cytokines/growth factors, including interleukin-1 (IL-1), IL-2, oncostatin M, interferon-γ, and epidermal growth factor.

B1 and B2 receptors have been cloned molecularly from human, rabbit, rat, and mice species and found to be highly conserved (70–80% homology). In humans, the predicted sequences of the B1 and B2 receptors show proteins of 357 and 364 amino acids, respectively. However, the homology between the two receptor types is only 36%. Both receptors contain seven transmembrane-spanning domains, typical of G protein-coupled receptors, and are located on chromosome 14q32, in very close proximity to each other. Targeted disruption of the B1 and B2 receptor genes by homologous recombination results in mice who exhibit phenotypes related to the inflammatory response, including decreased extravasation of leukocytes, hypertension, and angiogenic response (e.g., Cayla *et al.*, 2007; Emanueli *et al.*, 2002), that develop normally with normotension, although failing to respond to B1 receptor agonists. No data are available concerning the skeletal phenotype of B1 and B2 receptor knockout mice. Both B1 and B2 receptor genes have been found to express several allelic polymorphisms, although any association to physiological or pathophysiological bone metabolism has not been studied.

EFFECTS OF KININS ON BONE METABOLISM

Studies on the effects of kinins on bone metabolism have been performed *in vitro*, mainly in bone organ cultures. Treatment of mouse calvarial bones with BK for 72–96-hour results in increased bone resorption, as assessed either by the release of ^{45}Ca (Gustafson and Lerner, 1984; Lerner *et al.*, 1987) or by bone matrix degradation (Lerner *et al.*, 1987). BK can stimulate the release of ^{45}Ca also from fetal rat long bones, although the response is less than that seen in mouse calvarial bones (Ljunggren and Lerner, unpublished results). The threshold for action of BK in the mouse calvariae is 3 n*M* and half-maximal stimulation (EC$_{50}$) is obtained at 100 n*M* (Lerner *et al.*, 1987).

Calcitonin, added simultaneously with BK, inhibits the bone-resorptive effect of BK (Lerner *et al.*, 1987). Because calcitonin can inhibit the activity of multinucleated osteoclasts, as well as the recruitment of new osteoclasts, data do not reveal if BK stimulates bone resorption by enhancement of the activity of preformed osteoclasts or by the formation of new osteoclasts. However, morphological studies using both light and electron microscopy have shown that osteoclasts present in the calvariae, when bones are dissected from the mice, disappear after the preculture period. This implies that the stimulation of bone resorption in this system is dependent on proliferation/differentiation/fusion of osteoclast progenitor cells to multinucleated active osteoclasts and thus suggests that BK stimulates bone resorption by enhancing osteoclast recruitment, a hypothesis supported by the fact that the action of BK on bone resorption is delayed, with no effect observed until after 24 hours (Lerner *et al.*, 1987). Because no hematopoetic cells are present in the mouse calvarial explants, the osteoclast precursor cells in these bones are probably late precursor cells in the osteoclastic cell lineage.

Inflammatory bone loss may not only be due to enhanced bone resorption, but also to decreased bone formation. As regarding osteoblast cell proliferation, biosynthesis of bone matrix proteins, and the activity of alkaline phosphatase, very little is known about the possible effects of BK. In the human osteoblastic osteosarcoma cell line MG-63, BK does not stimulate cell proliferation or the biosynthesis of type I collagen and osteocalcin (Rosenquist *et al.*, 1996), although these cells express B2 receptors coupled to a burst of prostanoid formation (Brechter and Lerner, 2002). B1 receptors have been suggested to play a role in fibrinogenesis in fibrotic disorders, and B1 receptor agonists have been shown to stimulate type I collagen biosynthesis in human fibroblasts due to the stabilization of connective tissue growth factor mRNA (Ricupero *et al.*, 2000). In MG-63 cells, however, we have not been able to find any effect on type I collagen biosynthesis by B1 agonists, although these cells express B1 receptors (Rosenquist *et al.*, 1996; Brechter and Lerner, 2002). In agreement with the findings in MG-63 cells, BK has no effect on the proliferation of osteoblast-like cells isolated from human bone (Frost *et al.*, 1999).

Interestingly, whey protein obtained from milk and known to contain a variety of growth factors has been found to increase bone strength in ovariectomized rats (Takada *et al.*, 1997) and to stimulate proliferation and collagen synthesis in MC3T3-E1 cells (Takada *et al.*, 1996). The growth-promoting activity of milk has been purified and found to be a 17-kDa protein with an amino-terminal amino acid sequence very similar to an internal sequence of HMW kininogen (Yamamura *et al.*, 2000). This observation suggests the possibility that kininogens may not only be important for BK formation and cysteine protease inhibition, but also for bone growth.

PROSTAGLANDINS AS MEDIATORS OF BONE RESORPTION INDUCED BY BK

It was noted earlier that bone resorption induced by BK was inhibited by indomethacin, a potent inhibitor of prostaglandin biosynthesis (Gustafson and Lerner, 1984). It was later shown that several inhibitors of the cyclooxygenase pathway of arachidonic acid metabolism, including indomethacin, naproxen, meclofenamic acid, and flurbiprofen, abolish BK-induced mineral mobilization and bone matrix degradation (Lerner *et al.*, 1987). Similarly, all these nonsteroidal anti-inflammatory drugs also completely inhibit the bone resorptive effect of kallidin and Met-Lys-BK (Gustafson *et al.*, 1986; Ljunggren and Lerner, 1988). The glucocorticoids hydrocortisone and dexamethasone, which are potent inhibitors of prostaglandin biosynthesis, also inhibit BK-induced bone resorption (Lerner *et al.*, 1987). These observations indicate that the bone-resorptive effect of BK is totally dependent on the capacity of this peptide to activate prostaglandin formation. Interestingly, most stimulators of bone resorption *in vitro* also stimulate prostanoid formation in bone tissue and bone cells, although the magnitude of the prostaglandin response varies considerably between different stimulators. However, the bone resorptive effect and the biosynthesis of prostaglandins are not necessarily linked to each others. There are stimulators of bone resorption, including PTH, 1,25(OH)$_2$ vitamin D$_3$, TNF-α/TNF-β, and TGF-β, that are totally independent of prostaglandin formation (Palmqvist *et al.*, 2006). Other stimulators, e.g., IL-1, have a larger capacity to stimulate bone resorption in the presence of endogenous prostaglandin production, although a bone-resorptive effect of IL-1 still can be seen in the absence of prostaglandins (Lerner *et al.*, 1991). To a third group of stimulators, being unable to stimulate bone resorption in the absence of prostaglandin production, belong BK, kallidin, and Met-Lys-BK.

In primary cultures of mouse calvarial osteoblasts, BK causes a rapid burst of PGE$_2$ and 6-keto-PGF$_{1\alpha}$ (the stable breakdown product of PGI$_2$) that is maximal after 5–10 minutes (Lerner *et al.*, 1989). The half-maximal effect for the prostaglandin response (10 n*M*) is less than that for the bone-resorptive effect (100 n*M*), probably due to differences in the degradation of BK in short-term cell incubations compared to long-term organ cultures. The nontransformed mouse calvarial osteoblastic cell line MC3T3-E1, which both enzyme-histochemically and biochemically express a significantly lower activity of alkaline phosphatase compared to primary mouse calvarial osteoblasts (indicating that the MC3T3-E1 cells may represent a preosteoblastic phenotype; Lundberg and Lerner, unpublished results), also responds to BK with a burst of prostanoid formation (Lerner *et al.*, 1989). The time course, threshold for action, and EC$_{50}$ value are similar to those found in primary mouse calvarial osteoblasts. A very similar prostanoid response to BK is also obtained in non-enzymatically isolated human bone cells (Ljunggren *et al.*, 1990; Rahman *et al.*, 1992) and in the human osteoblastic osteosarcoma cell line MG-63 (Brechter and Lerner, 2002).

KININ RECEPTORS IN BONE CELLS

BK, Lys-BK, and Met-Lys-BK have been demonstrated to stimulate bone resorption in mouse calvariae, indicating the presence of B2 receptors (Gustafson *et al.*, 1986; Lerner *et al.*, 1987; Ljunggren and Lerner, 1988). This view is further supported by the fact that the B1 receptor antagonist des-Arg^9-[Leu^8]-BK does not affect the bone-resorptive effect of BK (Lerner *et al.*, 1987), an observation that also suggests that the effect of BK is not due to the conversion of BK by kininase-I to the B1 receptor agonist des-Arg^9-BK.

Pretreatment of BK with kininase-I does not affect BK-induced bone resorption, whereas the effect of PTH is reduced significantly (Lerner *et al.*, 1987). This observation suggests that des-Arg^9-BK, a B1 receptor agonist, may be able stimulate bone resorption. In agreement with this view, it has been shown that the addition of des-Arg^9-BK to mouse calvarial bones results in enhanced release of ^{45}Ca (Lerner *et al.*, 1987; Ljunggren and Lerner, 1990), an effect that is inhibited by the B1 receptor antagonist des-Arg^9-[Leu^8]-BK (Ljunggren and Lerner, 1990), indicating that bone cells are also equipped with B1 receptors. The effect of des-Arg^9-BK is abolished by indomethacin, flurbiprofen, and hydrocortisone. In addition, prostanoid biosynthesis in mouse calvarial bones is stimulated by des-Arg^9-BK in 72-hour cultures, but, in contrast to BK, no prostaglandin response is seen in bones incubated for 30 minutes with des-Arg^9-BK.

Using the burst of PGE$_2$ and 6-keto-PGF$_{1\alpha}$ biosynthesis in primary mouse calvarial osteoblasts and in the osteoblastic cell line MC3T3-E1 as parameters, the following rank order potency for different agonist has been shown: BK = Lys-BK > Met-Lys-BK >>>> des-Arg^9-BK, demonstrating the presence of B2 receptors on these osteoblasts (Ljunggren *et al.*, 1991a). The fact that D-Arg^0-[Hyp^3, Thi^{5,8}, D-Phe^7]-BK, but not des-Arg^9-[Leu^8]-BK, inhibits the initial rise of prostaglandins induced by BK further supports the presence of B2, and not B1, receptors on mouse osteoblasts. It has been shown that the human osteosarcoma cell line MG-63 shows a prostanoid burst in response to a wide variety of natural kinins and kinin analog with affinity to BK B2 receptors. The effect of BK in these cells is inhibited by B2 receptor antagonists, but not by B1 receptor antagonists (Brechter and Lerner, 2002). These observations and the finding that [Hyp^3]-BK is a weak agonist and T-kinin is a potent agonist further indicate that osteoblasts are equipped with B2 receptors linked to a burst of prostaglandin biosynthesis. This view is also compatible with the observations that BK and D-Arg^0-[Hyp^3, Thi^{5,8}, D-Phe^7]-BK, but not des-Arg^9-BK, compete with the binding of [^{3}H]BK to osteoblasts (Ljunggren *et al.*, 1991a). Leis *et al.* (1997) have demonstrated that responsiveness to BK, specific binding of [^{3}H]BK, and mRNA expression of BK B2 receptors in subclones of the murine osteoblastic cell line MC3T3-E1 are highest in clones with low alkaline phosphatase activity, indicating that it is mainly osteoblasts at early stages of differentiation that are responsive to BK. This observation is in agreement with findings by Lerner *et al.* (1989) demonstrating that the more confluent mouse calvarial osteoblasts are in cell cultures, the less is the BK responsiveness. Using BK-sensitive MC3T3-E1 cells, it has been shown that these cells express a single category of binding sites for [^{3}H]BK (Windischhofer and Leis, 1997). Radioligand-binding assays in MG-63 cells, using [^{3}H]BK as ligand, have demonstrated specific binding sites that can be competed for by B2 receptor agonists and antagonists. The rank order potency for kinin-induced stimulation of prostaglandin formation and radioligand-binding studies strongly indicate the constitutive expression of B2 receptors in MG-63 cells, a conclusion further supported by RT-PCR analysis showing mRNA expression of B2 receptors (Brechter and Lerner, 2002).

The acute rise of prostaglandin production in osteoblasts in response to BK is preceded by an accumulation of inositol phosphates, a transient increase of intracellular calcium, and an activation of protein kinase C (Ljunggren *et al.*, 1991b, 1993; Leis *et al.*, 1997). The initial, transient rise of intracellular calcium and the sustained influx of extracellular calcium seem to be regulated by different protein kinase C isoenzymes (Sakai *et al.*, 1992). By studying BK-induced release of arachidonic acid from MC3T3-E1 cells, evidence shows that BK receptors are linked to G proteins (Yanaga *et al.*, 1991), well in agreement with cloning data. These findings suggest that activation of BK receptors leads to a phospholipase C-mediated breakdown of phosphatidylinositol 4,5-bisphosphate with subsequent formation of the two putative second messengers: inostiol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG).

In agreement with the observation in mouse calvarial bones, treatment of primary mouse calvarial osteoblasts with des-Arg^9-BK results in a delayed enhancement of PGE_2 formation that can be observed at and after 24 hours (Ljunggren and Lerner, 1990). The effect of des-Arg^9-BK is inhibited by des-Arg^9-[Leu^8]-BK, indicating that des-Arg^9-BK exerts its effect via B1 receptors. Similar observations have been made in MG-63 cells using des-Arg^9-BK, des-Arg^{10}-Lys-BK, [Tyr-Gly-Lys-Aca-Lys]-des-Arg^9-BK, and Sar[D-Phe8]-des-Arg^9-BK as agonists. Stimulation caused by these B1 receptor agonists is inhibited by a variety of B1 receptor antagonists, but not by B2 receptor antagonists. In addition, MG-63 cells display specific binding sites using [^{3}H]-des-Arg^{10}-Lys-BK as ligand and mRNA expression of human B1 receptors (Brechter and Lerner, 2002). The delay in the action of B1 receptor agonists (as compared to B2 receptor agonists) could be due to different postreceptor signal-transducing mechanisms (indicated by findings that des-Arg^9-BK does not stimulate IP3 formation, intracellular calcium, and translocation of protein kinase C) or differences in the mechanism by which prostaglandin biosynthesis is stimulated.

Kinin receptor expression is regulated during inflammation and recently we have found that B1 and B2 receptors are increased when the human osteoblastic cell line MG-63 or intact mouse calvarial bone are stimulated by either IL-1β or TNF-α, with a larger enhancement observed for B1 receptors (Brechter *et al.*, 2008). This response is most likely due to increased gene transcription because mRNA steady-state levels for both B1 and B2 mRNA were increased with no effects by IL-1β on the stability of the transcripts. Electrophoretic mobility shift assay (EMSA) analysis using oligonucleotides specific for different responsive elements in the B1 promoter showed that IL-1β and TNF-α activated both NF-κB and AP-1 in the MG-63 cells and by the use of pharmacological inhibitors we found that NF-κB activity and MAP-kinases are important for the stimulatory effect of IL-1β and TNF-α on both B1 and B2 kinin receptors. Interestingly, TNF-α activated NF-κB via the canonical pathway (p50/p65) whereas IL-1β activated NF-κB via a non-canonical pathway (p52/p65). Differences were also seen for the activation of AP-1 with c-Jun involved for both cytokines but c-Fos only for TNF-α. The stimulatory effect on kinin receptors by IL-1β and TNF-α was not shared with other osteotropic cytokines including IL-6, IL-11, IL-17, leukemia inhibitory factor (LIF), or oncostatin M (OSM).

INTERACTIONS AMONG KININS, CYTOKINES, AND NEUROPEPTIDES

Although much interest has been focused on the role of cytokines, growth factors, hormones, kinins, and neuropeptides in bone metabolism and cell activities in general, the approach has often been to study the effect of these substances in systems in which they have been tested one by one. It is apparent that cells are exposed *in vivo* to several agonists simultaneously and therefore the ultimate cell and tissue response will be dependent on interactions among a variety of hormones, cytokines, growth factors, kinins, and neuropeptides.

The possibility that kinins may interact with cytokines is supported by the findings that BK can stimulate IL-1 and TNF production in murine macrophages (Tiffany and Burch, 1989), cytokine release in rat spleen mononuclear cells (Reissmann *et al.*, 2000), and IL-6 formation in human bone cells (Rahman *et al.*, 1992). The capacity of BK to affect cytokine production has also been demonstrated in human gingival fibroblasts; in these cells, BK does not affect IL-1 production, but potentiates the stimulatory effect of TNF on the biosynthesis of IL-1α and IL-1β (Yucel-Lindberg *et al.*, 1995). Interestingly, hyperalgesia induced by BK is blocked by the IL-1 receptor antagonist and is enhanced by antiserum neutralizing the IL-1 receptor antagonist (Cunha *et al.*, 2000). Such observations should prompt future studies on the possible role of cytokines in BK-induced bone resorption.

It has been shown that BK synergistically potentiates the stimulatory effects of IL-1α and IL-1β on bone resorption and prostanoid biosynthesis in mouse calvarial bones (Lerner, 1991). A similar interaction in mouse calvariae has also been observed between BK and TNF (Lerner *et al.*, unpublished). We have observed that several B2 receptor agonists, as well as B1 receptor agonists, synergistically potentiate IL-1- and TNF-induced biosynthesis of PGE_2 and 6-keto-$PGF_{1\alpha}$ in the human osteoblastic cell line MG-63 (Brechter and Lerner, 2007). The effect is specific for IL-1β and TNF-α because other osteotropic cytokines do not synergistically interact with BK. One important molecular mechanism involved is synergistic potentiation by kinins of IL-1β and TNF-α induced mRNA and protein expression of cyclo-oxygenase-2 due to increased gene transcription. This effect was associated with enhanced phosphorylations of the MAP-kinases p38 and JNK and was inhibited by pharmacological inhibitors of these enzymes with no effect being observed by an inhibitor of ERK. IL-1β enhanced also membrane associated prostaglandin E synthase-1 mRNA and protein, but this response was unaffected by kinins, nor were any effects by IL-1 and kinins on the mRNA expression of other prostaglandin E synthases observed. As mentioned previously, the synergistic interaction is associated also with a cytokine-induced upregulation of binding sites for B1 and B2 receptor-specific ligands. Thus, both enhanced number of kinin receptors as well as potentiation of cytokine-induced cyclo-oxygenase contribute to the synergistic enhancement of prostaglandin formation.

Osteoclast formation and activation are critically dependent on expression of receptor activator of NF-κB (RANK) in osteoclast precursor cells and its activation by RANK ligand (RANKL) in osteoclast supporting cells such as osteoblasts, bone marrow stromal cells, and T-lymphocytes (reviewed in Lerner, 2004; Takayanagi, 2007). Most interestingly, IL-1β induced enhancement of RANKL mRNA and protein was synergistically enhanced by stimulation of either B1 or B2 receptors and this response was inhibited by indomethacin (Brechter and Lerner, 2007). The enhancement of RANKL by co-stimulation with IL-1β and kinins was considerably larger than that caused by maximally effective concentration of 1,25$(OH)_2$-vitamin D3. The interaction between RANKL and RANK is inhibited by the decoy receptor osteoprotegerin (OPG). However, in contrast to 1,25$(OH)_2$-vitamin D3, which decreased OPG, no effect by IL-1β and kinins on OPG mRNA or protein was observed. These observations explain the prostaglandin-dependent potentiation of IL-1β induced bone resorption caused by kinins with affinity for the B1 and B2 receptors.

Pretreatment of human osteoblast-like cells with estrogen upregulates the subsequent stimulation of prostaglandin production induced by BK (Cissel *et al.*, 1996), suggesting the existence of steroid hormone/kinin interactions in bone.

There are indications that the skeleton may be systemically affected in patients with chronic inflammatory diseases and in rats with experimentally induced chronic inflammation (Minne *et al.*, 1984; Motley *et al.*, 1993). The systemic factor involved is not known, but could be related to the demonstration that haptoglobin, one of the acute-phase proteins induced in the liver during chronic inflammation, stimulates bone resorption in neonatal mouse calvariae (Lerner and Fröhlander, 1992). Interestingly, BK synergistically potentiates the stimulatory effect of haptoglobin on PGE_2 formation in mouse calvarial osteoblasts (Fröhlander *et al.*, 1991).

Data indicate that skeletal neuropeptides may play important roles as local mediators regulating bone metabolism (see later). The observations that (i) BK stimulates the expression of β-adrenergic receptors (Yasunaga *et al.*, 2000), (ii) BK enhances the release of calcitonin gene-related peptide (Averbeck *et al.*, 2000), and (iii) kinins participate in neurokinin-1 receptor-dependent neutrophil accumulation in inflamed skin (Cao *et al.*, 2000) raise the possibility of a link between neurohormonal- and kinin-regulated bone metabolism. Our finding that the skeletal neuropeptide VIP regulates the mRNA expression of RANKL, RANK, and OPG in mouse bone marrow cultures and isolated calvarial osteoblasts (Mukohyama *et al.*, 2000; Persson *et al.*, in preparation) and observations showing that the same neuropeptide can affect the expression of IL-6 (Persson *et al.*, 2005; Persson and Lerner, 2005) in mouse calvarial osteoblasts suggest the possibility of a neuroimmune interplay in bone cell activities.

NEURONAL INFLUENCE ON BONE TISSUE

The activities of and interactions between different bone cells are regulated by a variety of systemic hormones, cytokines, growth factors, and inflammatory mediators. Another proposed regulatory element is the nervous system, which, through the release of neuronal messengers, has been suggested to participate in bone metabolism. Although Hohmann *et al.* (1983) reported that the neuropeptide VIP can stimulate bone resorption in mouse calvariae, it has only been since the early 1990s that the possible role of neuroactive substances in the control of bone cell activities has been appreciated. This field of interest has gained tremendously larger interest since the hypothalamic control of bone metabolism was revealed (see Chapter 5).

INNERVATION OF BONE

During the first decades of the twentieth century, the presence of nerve fibers in bone and periosteum was demonstrated using routine histological techniques (reviewed by Hurrel, 1937; Sherman, 1963). The techniques applied were useful in establishing the distribution of nerves and in discriminating between myelinated and unmyelinated nerves in bone, but the information provided was limited by morphology. Three decades ago, a breakthrough in neuroscience occurred when the immunohistochemical technique was developed and made visualization of nerves according to their transmitter content possible. Numerous neuroactive substances have been demonstrated in many different tissues, but the difficulty in bone tissue was to demineralize the bone without destroying the antigenicity of the neuro-related substances. Bjurholm *et al.* (1989) and Hill and Elde (1990) developed techniques making it possible to preserve neuroactive substances in decalcified bone specimens. Following these reports, a number of neuronal messengers and their distribution in bone have been mapped extensively.

In addition to transmitter phenotyping with immunohistochemical techniques, surgical or chemical selective denervation has established the origin of the nerves in bone (reviewed by Lundberg, 2000). Both sensory and autonomic nerve fibers are present in bone tissue. Overall, a substantial part of skeletal nerve fibers are seen along blood vessels, but blood vessel-unrelated and free nerve endings have also been demonstrated. Fibers are spread in all the cell layers of the periosteum of bone and are expressed at a higher density in the epiphysis than in the diaphysis. Small branches of periosteal nerve fibers enter the cortical bone, usually associated with blood vessels located in Volkmann canals or in haversian canals (Bjurholm, 1989; Hill and Elde, 1991a; Hukkanen *et al.*, 1992). Entering the inner compartments of bone, nerve fibers are spread in the bone marrow and richly innervate the osteochondral junction of the growth plate. Interestingly, the epiphyseal part of the growth plate is intensively supplied by peptidergic nerves, whereas the metaphyseal part is innervated more poorly (Hukkanen *et al.*, 1992; Hukkanen, 1994). By injecting green fluorescent protein and β-galactosidase expressing pseudorabies virus into the femoral bone marrow of rats and analyzing the retrograde labeling, the hierarchically organized pathways have been described with virus found in several distinct anatomical areas in the brain and the spinal cord (Dénes *et al.*, 2005).

The immunohistochemical staining of bone tissue sections has demonstrated the presence of a wide variety of neuronal messengers, including both slowly acting transmitters, so-called neuropeptides, and rapidly acting small molecules, so-called classical neurotransmitters, in bone. Presently, the neuropeptides demonstrated in bone are substance P (SP), calcitonin gene-related peptide (CGRP), neurokinin A, and pituitary adenylate cyclase activating peptide 27 (PACAP 27) and 38 (PACAP 38), mainly representing the sensory system; vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY), mainly representing the autonomic system; and met-enkephalin, representing the opioid system. The classical neurotransmitters present in bone are the amines serotonin and the catecholamine noradrenaline (NA) and the excitatory amino acid glutamate (reviewed by Konttinen *et al.*, 1996; Lerner, 2000; Kreicbergs and Ahmed, 1997; Lundberg, 2000). In addition to morphological demonstrations, neuroactive substances in bone have also been quantified biochemically. A technique has been developed to extract and quantify neuropeptides in bone and joints using radioimmunoassay (RIA) (Ahmed *et al.*, 1994). Using this technique, extracts from diaphyseal rat bone tissue, periosteum, and bone marrow have been analyzed for their contents of neuropeptides. SP, CGRP, NPY, and VIP could all be quantitated at all three localizations, with NPY exhibiting the highest concentration at all sites (Ahmed *et al.*, 1994). Moreover, neurotrophic factors such as neurotrophins, known to be important factors required for the development and maintenance of the central and peripheral nerve systems, have been demonstrated in bone tissue. Neurotrophins demonstrated in bone tissue so far are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) (Asaumi *et al.*, 2000).

A neuronal regulation of bone metabolism would not only require the presence and release of neuronal messengers in the vicinity of bone cells, but also the presence of functional receptors for such factors on bone-forming or bone-resorbing cells. Therefore, several attempts have been made to study whether different neuropeptides, neurotransmitters, and neurotrophins can affect receptor-associated functions in osteoblasts and osteoclasts.

RECEPTORS AND EFFECTS BY NEUROPEPTIDES IN BONE

Vasoactive Intestinal Peptide

VIP is a member of the growing family denoted VIP/secretin/glucagon family of neuropeptides, which also includes the structurally related peptides secretin, gastric inhibitory peptide, growth hormone-releasing hormone, peptide histidine isoleucine amide (PHI), PACAP 27 and 38, and the reptilian venom peptides helodermin, helospectin, and exendine.

VIP was first discovered as a vasodilator peptide (Said and Mutt, 1969). Isolation of VIP from porcine gut (Said and Mutt, 1970) revealed an amidated 28 amino acid peptide (Mutt and Said, 1974). VIP is a cleavage product of the 170 amino acid precursor pre-pro VIP, which also is the parent molecule of PHI (Itoh *et al.*, 1983). Using RIA and the immunofluorescence technique, VIP immunoreactivity was discovered in many tissues outside the gastrointestinal tract. In 1976, the finding of VIP in the brain and in peripheral nerves (Said and Rosenberg, 1976) introduced VIP as a neuropeptide.

VIP has been investigated extensively, and a broad range of biological actions has been ascribed to this peptide, both in animal and in human studies. Important actions of VIP in the cardiovascular (Henning and Sawmiller, 2001), reproductive (Fahrenkrug, 2001), pulmonary (Groneberg *et al.*, 2006), immune (Voice *et al.*, 2002; Delgado *et al.*, 2004), and gastrointestinal systems (Schuttleworth and Keef, 1995) have been reviewed. General physiologic effects encompass vasodilatation, bronchodilatation, immunosuppression, hormonal secretion, and increases in gastric motility. In the nervous system, VIP seems to participate in neuronal survival, maturation, and maintenance (reviewed by Gozes and Brenneman, 1993).

VIP is widely distributed in both the central (CNS) and the peripheral nervous systems (PNS). In the brain, VIP immunoreactive neurons are found in the hypothalamus and the cerebral cortex (Hökfelt *et al.*, 1982). In the PNS, VIP immunoreactive nerves, nerve plexus and terminals supplying blood vessels, nonvascular smooth muscles, glandular acinis, and ducts, in a variety of organs, have been described (Hökfelt *et al.*, 1982). VIP immunoreactivity is detected in nerve fibers with both sympathetic and parasympathetic origin and, to a minor extent, in sensory nerve fibers.

In 1986, Tashijan and colleagues initially demonstrated VIP immunoreactive (IR) nerve fibers in bone tissue (Hohmann *et al.*, 1986). VIP-IR nerve fibers with a postganglionic sympathetic origin were localized in the periosteum, associated with vascular elements of bones from different species. In a large study of neuropeptide expression in rat bone nerve fibers, Bjurholm *et al.* (1988a, 1988b) detected VIP-IR nerve fibers preferentially in the periosteum and the epiphysis. These VIP-IR nerve fibers, whose origins are uncertain, were only occasionally associated with blood vessels. In line with these data, Hill and Elde (1991a) also demonstrated VIP-IR in rat periosteum. These nerves were only partially associated with vascular elements. VIP, NPY, and dopamine-β-hydroxylase (DβH) immunoreactive nerves were reduced dramatically in guanethidine-treated animals, strongly indicating a sympathetic origin of theses nerves (Hill and Elde, 1991b).

Tashjian and coworkers provided the first *in vitro* evidence for functional neuropeptide receptors on bone cells, demonstrating that VIP stimulates calcium release in neonatal mouse calvariae (Hohmann *et al.*, 1983). In osteoblasts, the presence of functional receptors for VIP, linked to enhanced formation of cyclic AMP, has been demonstrated in a human osteosarcoma cell line (Saos-2) (Hohmann and Tashjian, 1984; Bjurholm *et al.*, 1992; Lerner *et al.*, 1994), in the rat osteosarcoma cell line UMR 106–01 cells, but not in rat osteosarcoma cell line ROS 17/2.8 cells (Bjurholm *et al.*, 1992; Lerner *et al.*, 1994). In addition, VIP has been found to stimulate cyclic AMP formation in isolated mouse calvarial osteoblasts and in the cloned, nontransformed, osteoblastic cell line MC3T3-E1 (Bjurholm *et al.*, 1992; Lerner *et al.*, 1994).

Since the early 1990s, three different subtypes of VIP receptors have been cloned. These receptors are designated VPAC1, VPAC2, and PAC1 (reviewed in Laburthe *et al.*, 2002). They are all seven transmembrane, G protein-coupled receptors and members of the VIP/secretin/PTH receptor superfamily. All three receptors are distributed in both the CNS and the PNS and can be distinguished by comparing their relative adenylate cyclase activating capacities and by radioligand-binding assays. PAC1 binds PACAP with a much higher affinity (100- to 1,000-fold) than VIP. VPAC1 and VPAC2 bind PACAP and VIP with similar affinities. The fact that VPAC1 binds secretin, and VPAC2 does not, can be used to distinguish between these two VIP receptors.

We have characterized the VIP-binding receptors in mouse calvarial osteoblasts. By comparing the rank order of response of peptides in the VIP/secretin/glucagon family on cyclic AMP formation, we found that PACAP 38 was tenfold more potent than VIP (Lundberg *et al.*, 2001). A similar tenfold difference in potency between PACAP and VIP has also been detected in the rat osteoblast-like tumor cell line UMR 106 (Kovacs *et al.*, 1996) and in the nontransformed murine calvarial cell line MC3T3-E1 (Susuki *et al.*, 1994). By comparing the relative potency of VIP and related peptides to displace ^{125}I-PACAP binding, we found a rank order of response similar to that obtained when cyclic AMP enhancement was quantified. The fact that PACAP-preferring VIP receptors do not bind secretin was confirmed by demonstrating that secretin did not elevate cyclic AMP levels and failed to displace ^{125}I-VIP or ^{125}I-PACAP 38 binding. Using atomic force microscopy (AFM), a novel technique modified to detect specific binding sites on cell surfaces, we have demonstrated specific binding of VIP, but not secretin, on mouse calvarial osteoblasts. Reverse transcriptase PCR further demonstrated that these undifferentiated osteoblasts express mRNA for VPAC2, but not for VPAC1 or PAC1. When these osteoblasts were cultured for 20 days to induce bone noduli formation, VPAC1, in addition to VPAC2, were expressed when the nodules started to mineralize at 12 days

(Lundberg *et al.*, 2001). Taken together, these data demonstrate that mouse calvarial osteoblasts express functional VPAC2, with higher affinity binding for PACAP than for VIP, and that VPAC1 expression is induced during osteoblastic differentiation. Information on mRNA expression of VIP/PACAP receptors in osteoblasts is limited to the observations in mouse calvarial osteoblasts and to a report by Togari *et al.* (1997), demonstrating that primary human osteoblasts and human osteosarcoma cell lines express VPAC1, but not VPAC2 or PAC1. The observed differences in VIP receptor expression in mouse and human osteoblasts may be a matter of differentiation discrepancies, although it cannot be excluded that it is due to species differences.

Interestingly, a differentiation-dependent manner of receptor expression in mouse osteoblasts is not only observed for VIP receptors, but also for vascular endothelial growth factors (VEGF) and their receptors in mouse osteoblasts (Deckers *et al.*, 2000). A role for VEGF in endochondral bone formation has been proposed because inactivation of VEGF inhibits endochondral bone formation via inhibition of angiogenesis (Gerber *et al.*, 1999). Therefore, the increased expression of VEGF receptors and their ligands during osteoblastic differentiation and mineralization supports the theory that VEGF plays an important role in the regulation of bone metabolism. The role of VPAC1 induction during osteoblastic differentiation remains to be elucidated. In contrast to differentiation-induced upregulation of VPAC1 and VEGF receptors, the expression of glutamate transporter in rat osteoblasts declines when mineralization starts in rat osteoblast cultures (Bhangu *et al.*, 2000).

Whether VIP receptors on osteoblasts are coupled to anabolic actions of VIP has been evaluated *in vitro* using mouse calvarial osteoblasts (Lundberg *et al.*, 1999). After six days of culture, VIP stimulates activity of the bone mineralization-associated enzyme alkaline phosphatase (ALP), and the mRNA expression of this enzyme, without affecting cell proliferation. The ALP-staining pattern in histochemical analysis demonstrated that VIP, to a minor extent, increased the number of ALP-stained cells, but mainly increased the staining of individual cells. These morphological analyses suggest that VIP treatment causes an increased differentiation of committed osteoblasts. In line with this, we found that VIP initially causes an increased accumulation of calcium in osteoblast cultures during the formation of mineralized bone nodules, but does not change the total amount of calcium found at the end of the culture. The fact that VIP stimulates ALP activity at six days of culture, a time point when only VPAC2 are expressed (Lundberg *et al.*, 2001), together with the absence of effect by secretin on ALP activity, clearly suggest that VPAC2 receptors mediate the anabolic events in bone caused by VIP. Whether VPAC1 may mediate similar bone-forming effects has to be ascertained.

The first documented *in vitro* effect of a neuropeptide on bone was that of Tashjian and coworkers, demonstrating a catabolic effect by VIP on bone metabolism (Hohmann *et al.*, 1983). Thus, VIP stimulated calcium release in organ-cultured mouse calvarial bones. This stimulation of calvarial bone resorption by VIP may be due either to enhanced activity of osteoclasts or to stimulation of osteoclast formation.

Morphological studies of isolated rat osteoclasts revealed that VIP treatment caused a rapid cytoplasmic contraction along with an associated decrease in motility (Lundberg *et al.*, 2000). Functional studies using an *in vitro* resorption assay showed that VIP caused a transient inhibition of osteoclastic bone resorption. When the osteoclast incubations were extended over time and performed in the presence of marrow-derived stromal cells/osteoblasts, the osteoclasts escaped from the initial inhibition and VIP caused a delayed stimulation of osteoclastic pit formation in bone slices. Similar to VIP, the initial inhibitory effect of calcitonin (CT) was lost over time. However, in contrast to VIP, CT-treated osteoclasts never start to resorb bone more than unstimulated controls. The finding of inhibitory effects, both on osteoclast morphology and on resorptive capacity, suggests that osteoclasts are equipped with VIP receptors and that VIP might be acting directly on osteoclasts.

In order to localize binding sites for VIP on osteoclasts, we took advantage of the newly developed AFM technique. Using AFM and measurements of intracellular calcium, specific VIP-binding sites on osteoclasts were found (Lundberg *et al.*, 2000). Further evidence for the presence of VIP receptors in osteoclasts is our finding of mRNA for VPAC1 and PAC1 in mouse bone marrow osteoclasts isolated by micromanipulation (Ransjö *et al.*, 2000). The late stimulatory effect of VIP is probably the basis of the finding that VIP stimulates resorption in calvarial organ culture (Hohmann *et al.*, 1983). When AFM was used to analyze the presence of VIP receptors in stromal cells/osteoblasts, we found that approximately 20% of the stromal cells/osteoblasts expressed specific binding sites for VIP. This was supported further by the observation that these cells also responded to VIP with a rapid enhancement of intracellular calcium (Lundberg *et al.*, 2000). These receptors might mediate the indirect bone-resorbing effect caused by VIP, both in the resorption pit assay and in the calvariae.

VIP has been reported to stimulate IL-6 production in stromal cells (Cai *et al.*, 1997) and in an osteosarcoma cell line (Greenfield *et al.*, 1996). We have confirmed these observations using mouse calvarial osteoblast. VIP was shown to stimulate IL-6 production in both time- and concentration-dependent manner (Persson *et al.*, 2005). Furthermore, VIP caused enhanced IL-6 promoter activity in cells from the osteoblastic cell line MC3T3-E1 transfected with an IL-6 promoter construct coupled to a luciferase reporter gene. By using several techniques, including electrophoretic mobility shift assay (EMSA), we also showed that the effects by VIP on IL-6 production involved several intracellular pathways. Treatment of osteoblasts with VIP resulted in activation of the cyclic AMP/PKA pathway and increased DNA binding of the transcription factor C/EBP, whereas the DNA binding of the AP-1 complex was decreased by VIP. In contrast, our results indicated that regulation by VIP of IL-6 synthesis did not require activation of the NF-κB pathway (Persson *et al.*, 2005). In addition to the studies on involvement of different intracellular pathways in VIP signaling, we also evaluated which receptor type is responsible for the effects on IL-6 production. Besides VIP, PACAP-38 was also shown to increase IL-6 synthesis, whereas secretin did not have any effect, indicating that the effects by VIP and PACAP-38 are mediated by VPAC2 (Persson *et al.*, 2005).

VIP is a well-known regulator of inflammatory cytokine production in several cell types (reviewed in Delgado *et al.*, 2004). Having shown the stimulatory effects by VIP on IL-6 production in murine osteoblasts, we addressed the question whether VIP may interact with known osteotropic cytokines to regulate the IL-6 production in these cells. Indeed, VIP was found to increase the stimulatory effect by IL-1β, IL-11, IL-17, LIF, OSM, and TGF-β on IL-6 production in a synergistic manner (Persson and Lerner, 2005). In addition, VIP also potentiated the IL-1β-induced stimulation of IL-6 promoter activity in osteoblastic MC3T3-E1 cells transfected with the IL-6 promoter/luciferase construct (Persson and Lerner, 2005).

The stimulation of bone resorption by VIP in organ-cultured mouse calvariae can be explained either by an effect on osteoclast activity or by an effect on osteoclast formation. When osteoclastogenesis was studied in mouse bone marrow cultures, VIP did not enhance the number of osteoclasts (Mukohyama *et al.*, 2000). In contrast, VIP caused an inhibition of osteoclast formation induced either by 1,25(OH)$_2$ vitamin D$_3$ or by PTH. The anti-osteoclastogenic effect of VIP is associated with inhibitory effects on the 1,25(OH)$_2$ vitamin D$_3$-induced upregulation of RANK (receptor activator of NF-κB) and RANK ligand (RANKL). In addition, VIP counteracts the decrease of osteoprotegerin (OPG) caused by 1,25(OH)$_2$ vitamin D$_3$ (Mukohyama *et al.*, 2000). In summary, VIP inhibits osteoclast formation, probably by regulating the expression of RANK, RANKL, and OPG, three molecules known to be important for osteoclast formation. The fact that VIP stimulates osteoclast activity and inhibits osteoclast recruitment suggests that VIP may have a unique role in bone metabolic processes by acting as a fine-tuner of osteoclastic resorption.

In addition to the effects by VIP on bone cell function, recent studies have demonstrated that VIP also has a protective effect on bone destruction in experimentally induced arthritis. Treatment of mice exhibiting collagen-induced arthritis (CIA) with VIP resulted in delayed onset, reduced incidence, and decreased severity of the arthritis, as well as a dramatic decrease of cartilage damage and bone erosion in arthritic joints (Delgado *et al.*, 2001). In addition, VIP decreased levels of IL-1β, IL-6, IL-11, IL-17, and TNF-α, whereas the levels of IL-4 and IL-10 were elevated. VIP also decreased the RANKL/OPG ratio, mainly as a result of increased levels of circulating OPG (Juarranz *et al.*, 2005). In contrast to the effects of VIP on bone cell function and production of osteotropic cytokines by these cells, the effects seen in the arthritis models are believed to be a result of a neuroimmunomodulatory interaction, where VIP regulates the proliferation and differentiation of T lymphocytes, as well as pro-inflammatory cytokine production by T lymphocytes, macrophages, and fibroblasts in the synovial tissue. These findings suggest that VIP, in addition to the effects on bone cell function, may also have a possible role as a therapeutic target for treatment of arthritis.

Pituitary Adenylate Cyclase-Activating Peptide

PACAP was first isolated from ovine hypothalamus and described on the basis of its ability to increase adenylate cyclase activity in rat pituitary cells (Miayata *et al.*, 1989). PACAP occurs in two molecular forms: the 38 amino acid peptide PACAP 38 and the C-terminally truncated form PACAP 27. Both forms of PACAP share 68% amino acid homology with VIP at their N-terminal domains (reviewed by Arimura, 1991, 1992).

In addition to exhibiting extensive molecular similarities to VIP, PACAP partially shares receptors as well as functions with VIP. PACAP has been shown to be a pleiotropic neuropeptide, functioning as a hypothalamic hormone, neurotransmitter, neuromodulator, vasodilator, and a neurotropic factor. Examples of endocrine functions by PACAP are numerous. PACAP (i) stimulates the secretion of adrenaline from the adrenal medulla, (ii) stimulates insulin release from pancreatic β cells, and (iii) causes an increase of $[Ca^{2+}]_i$ in pancreatic β cells. One important developmental biological action of PACAP seems to be as a neurotrophic factor during the development of the brain (reviewed by Arimura, 1998).

PACAP immunoreactivity is detected in nearly all organs and tissues. In the brain, the highest concentration is found in the hypothalamus. In the PNS, the adrenal medulla and testis contain the highest concentrations of PACAP immunoreactivity when compared to those found in the gut and the adrenal gland. PACAP 38 is the predominant form in tissues, making up 90% of the total PACAP (reviewed by Arimura, 1998).

PACAP 27 and PACAP 38 have been demonstrated in cartilage channels in tissue sections from pigs (Strange-Vognsen *et al.*, 1997). Varicose PACAP immunoreactivity fibers were demonstrated in association with blood vessels. Nearly all PACAP immunoreactive fibers contain CGRP and SP, suggesting that these are sensory nerve fibers (Strange-Vognsen *et al.*, 1997).

In mouse calvariae and in mouse calvarial osteoblasts, the presence of functional receptors for PACAP 27 and PACAP 38, linked to the enhanced formation of cyclic AMP, has been demonstrated (Lerner *et al.*, 1994). As described earlier (see VIP), VIP and PACAP share receptors. In addition to VPAC1 and VPAC2, which bind VIP and PACAP with equal affinity, several reports describe the cloning of a high-affinity PACAP receptor (PAC1). PAC1 was, within a year, cloned from rat, human (Ogi *et al.*, 1993), and bovine tissues (Miyamoto *et al.*, 1994). Moreover, PAC1 was demonstrated to exist in six splice variant forms (Svoboda *et al.*, 1993; Journot *et al.*, 1995). PACAP is 100- to 1,000-fold more potent than VIP in binding and stimulating adenylate cyclase activity in cells transfected with PAC1 (Rawlings and Hezareh, 1996). PAC1 has a widespread distribution in the CNS, with the highest levels being found in the olfactory bulb, the dental gyrus of hippocampus, pituitary, cerebellum, thalamus, and hypothalamus. Messenger RNA for PAC1 has been detected in a variety of tissues, including liver, lung, spleen, and intestine (reviewed by Christophe, 1993; Arimura, 1992; Arimura and Shioda, 1995).

Concerning skeletal cells, the presence of PAC1 and VPAC1 mRNA has been demonstrated in microisolated mouse marrow osteoclasts (Ransjö *et al.*, 2000). Using an AFM technique, both VIP and PACAP 38 showed high-affinity binding to rat marrow osteoclasts (Lundberg *et al.*, 2000). If rat osteoclasts express the same VIP/PACAP receptor subtypes as mouse osteoclasts is unknown. However, our preliminary observations that PACAP 38, as well as VIP and secretin, causes a decrease in isolated rat osteoclastic bone resorption, indicate that VPAC1 may have a functional role in the regulation of osteoclast function. Whether PAC1 also has a functional role in the rat osteoclasts is not known.

Similar to VIP, PACAP 38 inhibits 1,25(OH)$_2$D$_3$-stimulated osteoclastogenesis in mouse bone marrow cultures (Mukohyama *et al.*, 2000). PACAP 38 also decreases RANKL and RANK expression and increases OPG in these bone marrow cultures.

Calcitonin Gene-Related Peptide

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide belonging to a superfamily of peptides including CT, α-CGRP, β-CGRP, amylin (AMY), adrenomedullin (ADM), and the recently discovered intermedin (IMD) and calcitonin receptor-stimulating peptide (CRSP; reviewed in Lerner, 2006c). One domain of the insulin β chain also shares homology with these peptides, indicating that they may have diverged from a common ancestral gene during evolution. α-CGRP is produced by tissue-specific alternative splicing of the initial gene transcript encoding the precursor for CT. Consequently, α-CGRP is produced, not only in nerve fibers, but also in thyroid parafollicular C cells, together with CT. However, α-CGRP and CT seem not to be released in parallel, probably due to the fact that plasma levels of α-CGRP have a neurogenic origin. AMY is expressed predominantly in pancreatic β cells, whereas ADM is synthesized in several different tissues and is released from endothelial cells. IMD, initially identified from human and zebrafish expressed sequence tags and using a phylogenetic profiling approach also found in other mammals and teleosts (Roh *et al.*, 2004) has been found to be expressed in the pituitary and gastrointestinal tract. IMD was also in parallel cloned in the pufferfish, *Takifugo ripes* (Takei *et al.*, 2004), and genome-wide analyses in mammalians and teleosts have indicated that five paralogous family genes in humans – *CT, CGRP, AMY, ADM,* and *IMD* – evolved before the emergence of vertebrates and that multiple copies of these coevolved genes are present in teleost genomes (Chang *et al.*, 2004). CRSP has been identified from porcine, pig, bovine, and canine cDNA libraries but not yet in rat and human (Katafuchi and Minamino, 2004). CGRP, AMY, IMD, and CRSP all have an amino-terminal ring created by a disulfide bond; this is lacking in adrenomedullin, which has a linear amino-terminal extension.

CT, CGRP, AMY, and ADM act via related heptahelical receptors. Whereas a CT receptor was cloned already in 1991 (Lin *et al.*, 1991), the receptors for CGRP, AMY, and ADM have been more elusive. CGRP and ADM both bind to a CT receptor-like receptor (CRLR), originally described as an orphan seven transmembrane receptor. Interestingly, CRLR requires interaction with single transmembrane proteins called receptor activity-modifying proteins (RAMP; Foord and Marshall, 1999). Three different RAMPs (RAMP1, RAMP2, and RAMP3) have been cloned and sequenced and found to be expressed in a wide variety of tissues. Cotransfection of CRLR and RAMP1 results in CGRP responsive receptors, whereas cotransfection of CRLR and RAMP2, or RAMP3, leads to expression of ADM responsive receptors (Bühlmann *et al.*, 1999). RAMPs are expressed more abundantly than CRLR, suggesting that RAMPs could be involved in the regulation of other receptors than CRLR. Interestingly, RAMP1, or RAMP3, cotransfection with a CT receptor results in a high-affinity AMY receptor (Muff *et al.*, 1999). The expression of CRLR mRNA and protein have been found in rat and mouse calvarial osteoblasts, and in the rat and mouse osteoblastic cell lines UMR 106-06 and MC3T3-E1 (Pondel, 2000; Uzan *et al.*, 2004, Schinke *et al.*, 2004; Granholm and Lerner, unpublished observations) as well as in multinucleated mouse osteoclasts (Nakamura *et al.*, 2005) and in mononucleated osteoclast precursor cells Granholm *et al.*, 2008). Nakamura *et al.* (2005) reported that mouse multinucleated osteoclasts express RAMP2 mRNA, but not RAMP1 and RAMP3. We have found that mouse mononucleated osteoclast precursor cells from bone marrow express all three RAMPs both at the mRNA and protein level (Granholm *et al.*, 2007).

Receptors for CGRP, as assessed by a cyclic AMP response, have been demonstrated on the rat osteosarcoma cell line UMR 106–01 (but not on ROS 17/2.8), the human osteosarcoma cell line Saos-2, the mouse calvarial osteoblastic cell line MC3T3-E1, and enzymatically isolated osteoblastic cells from chick, rat, and mouse (Michelangeli *et al.*, 1986, 1989; Thiebaud *et al.*, 1991; Bjurholm *et al.*, 1992). Expression of receptors for CGRP seems to be a feature of the osteoblastic phenotype, as the degree of cyclic AMP formation in primary osteoblasts correlates with the activity of alkaline phosphatase and to the responsiveness to PTH (in terms of cyclic AMP formation; Michelangeli *et al.*, 1989). Using RT-PCR, CGRP receptors have been demonstrated in human periosteum-derived osteoblastic cells and in human osteosarcoma cell lines (Togari *et al.*, 1997). The presence of receptors recognizing AMY and ADM in osteoblasts is suggested by the observations that amylin stimulates cyclic AMP formation and that both peptides stimulate [^{3}H]thymidine incorporation into osteoblasts (Tamura *et al.*, 1992; Cornish *et al.*, 1995, 1997).

CGRP receptors are coupled to the stimulation of osteoblast proliferation and enhanced bone colony formation *in vitro* (Shih and Bernard, 1997a; Cornish *et al.*, 1999). An anabolic effect of CGRP *in vivo* is demonstrated by the findings that the targeted expression of calcitonin gene-related peptide to osteoblasts, under the control of the osteocalcin promoter, results in enhanced trabecular bone density, trabecular bone volume, and increased bone formation rates in mice (Ballica *et al.*, 1999). The same group has also reported that injection of CGRP into ovariectomized rats can partly prevent the bone loss caused by estrogen deficiency (Valentijn *et al.*, 1997).

Occupancy of AMY receptors in osteoblasts stimulates anabolic activities both *in vitro* and *in vivo*. Thus, AMY stimulates cell proliferation in osteoblast cell cultures (Cornish *et al.*, 1995, 1998; Villa *et al.*, 1997); AMY is more potent than CGRP but seems to act via a common CGRP/AMY receptor (Cornish *et al.*, 1999). Treatment of mice or rats with AMY leads to enhanced trabecular bone volume as a consequence of both increased trabecular thickness and number (Romero *et al.*, 1995; Cornish *et al.*, 1998). The increase of bone volume can also be achieved by injection of the amino-terminal fragment amylin 1–8, although the effect is less than that obtained by full-length amylin (Cornish *et al.*, 2000). Treatment with amylin leads to enhanced mechanical bone strength (Cornish *et al.*, 2000).

The stimulatory effects of CGRP and AMY on osteoblast cell proliferation are shared by ADM, which in fact is more potent than the other two peptides (Cornish *et al.*, 1997).

The interest of CGRP in bone resorption was initially prompted by the findings that CGRP shows amino acid sequence homology to CT in the amino-terminal region and that CGRP is costored with CT in thyroid C cells. Injection of CGRP into rats and rabbits causes a hypocalcemic reaction (Tippins *et al.*, 1984; Roos *et al.*, 1986). The fact that CGRP inhibits bone resorption in fetal rat long bones (Roos *et al.*, 1986; Tamura *et al.*, 1992) and in neonatal mouse calvariae (Yamamoto *et al.*, 1986) indicates that the decrease of serum calcium in intact animals is due to CGRP-induced inhibition of bone resorption. In the mouse calvarial system, rat β-CGRP is slightly more potent than rat α-CGRP, which is slightly more potent than human CGRP (Lerner, unpublished results). Similar to CT, the effect of CGRP is transient in both fetal rat long bones and in neonatal mouse calvariae (Roos *et al.*, 1986; Lerner, unpublished results). Inhibition of bone resorption can be due to inhibition of osteoclast activity and/or recruitment. An inhibitory effect of osteoclast activity has been observed in isolated rat osteoclasts as assessed by decreased pit formation (Zaidi *et al.*, 1987a, 1987b). In this system, α-CGRP and β-CGRP are equipotent. The hypocalcemic effect of CGRP, as well as the inhibitory effects on bone resorption in organ culture and on osteoclastic pit formation, is mimicked by AMY (Datta *et al.*, 1989; MacIntyre, 1989; Zaidi *et al.*, 1990; Tamura *et al.*, 1992; Pietschmann *et al.*, 1993). Interestingly, ADM, for reasons unknown, does not inhibit PTH-stimulated bone resorption in neonatal mouse calvariae (Cornish *et al.*, 1997; Lerner *et al.*, unpublished results).

Calcitonin-induced inhibition of bone resorption by isolated rat osteoclasts is associated with increased levels of intracellular Ca^{2+} and cyclic AMP, as well as with retraction and ceased motility of osteoclasts (Alam *et al.*, 1992a). At variance, inhibition caused by CGRP and AMY is associated with enhanced cyclic AMP and ceased motility, but not with increased intracellular Ca^{2+} and retraction (Alam *et al.*, 1991, 1992a, 1992b). These observations have prompted the speculation that the effects of CT are mediated via two separate receptors and that CGRP and AMY act only via one of these receptors (the one linked to cyclic AMP and motility). Further support for this hypothesis are the observations that CGRP 8–37 (i) inhibits the effects of AMY and CGRP on motility and (ii) inhibits the effect of CT on motility, but not on retraction. This would imply that retracted osteoclasts are still capable of motility. In contrast, Cornish *et al.* (1998) could not observe any antagonistic effect by CGRP 8–37 (or AMY 8–37) on the AMY-induced inhibition of bone resorption in neonatal mouse calvariae (an observation confirmed in the authors' laboratory), although the antagonists blocked the stimulatory effect by AMY on cell proliferation in the calvarial bones. The issue of which receptors in osteoclasts (CT receptor, CRLR/RAMP1, CRLR/RAMP2, CRLR/RAMP3) are used by CGRP (and AMY) and how these receptors are linked to the mechanism causing inhibition of bone resorption still remains an open question. The observation made by Cornish *et al.* (1998) indicates that separate AMY receptors are present on osteoblasts and osteoclasts.

The degree of bone loss is not only dependent on osteoclast activity but also on osteoclast formation. Very few studies deal with effects of peptides in the CT family on osteoclastogenesis. CT has been reported to inhibit osteoclast formation in PTH stimulated feline marrow-derived mononuclear cell cultures (Ibbotson *et al.*, 1984) as well as D3 stimulated osteoclastogenesis in primate marrow cell cultures (Roodman *et al.*, 1985) and mouse bone marrow cultures (Cornish *et al.*, 2001). Also AMY and CGRP inhibited D3 stimulated osteoclast formation in the mouse bone marrow system. Recently, it has been found that CGRP can inhibit isoproterenol-induced osteoclast formation in mouse bone marrow cultures without affecting isoproterenol-stimulated RANKL mRNA expression, indicating that the effect was due to an effect directly on osteoclast precursor cells (Ishizuka *et al.*, 2005). CRSP-1 has been reported to decrease osteoclast formation in mouse bone marrow and spleen cell cultures stimulated by M-CSF and RANKL (Notoya *et al.*, 2007), further supporting the view that the effect is directly on osteoclast precursor cells. We have been able to demonstrate that CT acts directly on highly purified osteoclast precursor cells stimulated by M-CSF/RANKL and, interestingly, the inhibitory effect on osteoclast formation is independent on c-Fms and RANK-induced transcription of very many osteoclast genes involved in differentiation, fusion, and function (Granholm *et al.*, 2007).

Deletions of the genes for ligands in the CT family of peptides and for one of the receptors have revealed unexpected findings that have changed our view on the role of these peptides in bone remodeling. Because CT is such a well-known inhibitor of bone resorption *in vitro* and *in vivo*, it was assumed that deletions of the *CT/α-CGRP* and *CTR* genes would lead to bone loss. Surprisingly, it was found that *CT/α-CGRP*$^{-/-}$ and *CTR*$^{+/-}$ mice have increased bone mass due to increased bone formation (Hoff *et al.*, 2002; Dacquin *et al.*, 2004). *CT/α-CGRP*$^{-/-}$ mice have normal serum calcium and exhibit enhanced bone mass due to increased trabecular bone volume in both vertebral bodies and tibias. The histomorphometric analysis showed that *CT/α-CGRP*$^{-/-}$ mice had increased trabecular number. The number of osteoblasts was not affected by deletion of the *CT/α-CGRP* gene, but dynamic histomorphometry, using calcein double labeling, showed a 1.5- to 2-fold enhancement of bone formation rate. Interestingly, *CT/α-CGRP*$^{-/-}$ mice were resistant to ovariectomized-induced osteopenia because of increased bone formation rate. Confirming that lack of CT signaling via CTR leads to enhanced bone mass, it has recently been reported that heterozygous *CTR* knockout mice also exhibit increased bone mass due to enhanced bone formation rate (Dacquin *et al.*, 2004). It is not likely that the enhanced bone formation is caused by a lack of an inhibitory effect by CT or *α-CGRP* on osteoblasts, because the CTR is not expressed on osteoblasts and mice with a selective deletion of *α-CGRP* have low bone mass (Schinke *et al.*, 2004).

Because CTR are also present in the brain (Sexton, 1992), and because the CNS has been found to be able to regulate bone remodeling (see earlier discussion), it cannot be ruled out that the skeletal phenotype in *CT/α-CGRP*$^{-/-}$ mice is mediated by the CNS. Mapping of CTR in the brain has demonstrated the expression of CTR in very many areas, but, interestingly, ventromedial, lateral, and posterior hypothalamus are three sites in which expression of immonoreactive CTR has been observed (Becskei *et al.*, 2004).

Although it remains to be proven that it is the role of α-CGRP as a signaling molecule in skeletal nerve fibers that causes the skeletal phenotype in α-CGRP deficient mice, it is most likely the explanation. It should, however, be kept in mind that *α-CGRP*$^{-/-}$ mice still express β-CGRP and it will be interesting to know which will be the phenotype of *β-CGRP*$^{-/-}$ mice and of *α-CGRP*$^{-/-}$/*β-CGRP*$^{-/-}$ mice. It was recently shown that capsaicin treatment destroys the unmyelinated sensory axons expressing CGRP and that the reduced CGRP signaling was associated with decreased bone mass (Offley *et al.*, 2005), similar to patients with familial dysautomia, which lacks unmyelinated sensory neurons and exhibits decreased bone mass and increased fracture rate.

Under certain experimental conditions, the deletion of the *CT/α-CGRP* gene resulted in, as expected, increased bone resorption. Thus, *CT/α-CGRP*$^{-/-}$ mice showed significantly higher serum calcium after injection of either PTH or 1,25(OH)$_2$-vitamin D3, a response that could be reversed by CT, but not by α-CGRP (Hoff *et al.*, 2002).

Mice with deletion of the AMY gene, in contrast to *CT/α-CGRP*$^{-/-}$ and *CTR*$^{+/-}$ mice, exhibit bone loss due to enhanced bone resorption (Dacquin *et al.*, 2004). The low bone mass in amylin-deficient mice was observed after 12 weeks of age, but not earlier and was caused by decreased trabecular and cortical thickness. The loss of bone was not associated with any change in osteoblast number, or bone formation rate *in vivo*, nor in any differences in alkaline phosphatase activity, biosynthesis of type I collagen, and mineralization of bone noduli *in vitro*. In line with the observations that amylin can inhibit bone resorption *in vitro*, the urinary excretion of deoxypyridinoline was significantly enhanced already at 8 weeks of age and persisted at least for 24 weeks, which indicates that AMY deficiency leads to enhanced bone resorption, which was supported by the observation that osteoclast number was enhanced 1.6-fold.

These data suggest that CT may be a regulator of bone formation, wheras AMY might be a more physiological regulator of bone resorption. It is apparent that our understanding of the role of the CGRP and other members of the CT family of peptides has been changed dramatically and much more data have to be gained before we fully understand the roles these peptides have in bone biology.

Substance P

The knowledge about the role of Substance P (SP) in bone is very limited. Neurokinin-1 (NK-1) and NK-2, but not NK-3, receptors recognizing SP have been immunolocalized in rat bone osteoblasts and osteocytes (Goto *et al.*, 1998; Fristad *et al.*, 2003). Osteogenesis, as assessed by bone colony formation in bone marrow cell cultures and in rat calvarial osteoblasts, can be stimulated by SP (Shih and Bernard, 1997b; Goto *et al.*, 2007). Interestingly, the NK-1 receptors were not expressed initially in the osteoblast cultures but induced during culture (day 14). The enhanced bone formation in rat osteoblasts was associated with increased expression of osteocalcin, runx2/cbfa1, and type I collagen. For reasons not understood, Azuma *et al.* (2004) report contrasting data showing that SP decreases the mRNA expression of alkaline phosphatase, bone sialoprotein, osteocalcin, and runx2 in fetal rat calvarial osteoblast cultures.

The abundance of NK-1 receptors in rat bone osteoclasts (Goto *et al.*, 1998) can be reconciled with observations that SP causes an acute rise of intracellular Ca^{2+} in rabbit osteoclasts and an increased pit area (but not an increase of pit number) excavated by osteoclasts when incubated on dentine slices (Mori *et al.*, 1999). The effects were blocked by two different NK-1 receptor antagonists. These observations indicate that SP may stimulate osteoclast activity by a direct effect on terminally differentiated osteoclasts. It has recently been shown that SP can stimulate osteoclast formation in mouse bone marrow cultures stimulated by M-CSF/RANKL and that this effect is associated with enhanced translocation of NF-κB to the nuclei (Sohn, 2005). SP not only can directly affect osteoclast progenitor cells but also indirectly stimulate osteoclastogenesis by enhancing the expression of RANKL and decreasing that of OPG in rat synovial fibroblasts (Matayoshi *et al.*, 2005). Coculture of SP stimulated synovial fibroblasts with rat blood monocytes resulted in enhanced osteoclast formation. Most interestingly, SP-deficient mice exhibit decreased bone loss and significantly fewer osteoclasts in the calvariae of mice locally treated with polyethylene particles (Wedemeyer *et al.*, 2007), in accordance with the *in vitro* findings, indicating that SP is a stimulator of osteoclastic bone resorption.

Neuropeptide Y

NPY mediates its action through the activation of five Y receptor subtypes (Y1, Y2, Y4, Y5, and in mouse, y6), which differ not only in their binding profiles but also in their distribution in the central nervous system and the periphery (Blomqvist and Herzog 2004; Lin *et al.*, 2005). There has been some controversy over whether or not bone tissue cells express Y receptors. Togari and collaborators showed expression of a Y receptor mRNA corresponding to the Y1 receptor in human osteoblastic and human osteosarcoma-derived cell lines (Togari *et al.*, 1997). In line with these findings, another group reported the expression of Y1 receptors in mouse bone marrow cells (Nakamura *et al.*, 1995). Recently, the expression of Y1 receptor mRNA in mouse bone marrow stromal cells and in mouse calvarial osteoblast has been reported (Lundberg *et al.*, 2007; Baldock *et al.*, 2007). Moreover, evidence for Y1 receptors *in situ* in mouse bone was provided (Lundberg *et al.*, 2007). In contrast, an earlier study by Baldock *et al.* (2002) did not detect transcripts for any of the Y receptors in primary murine osteoblastic cultures or whole long bone preparations using RT-PCR. Amano and collaborators recently demonstrated that crude bone marrow cell cultures express Y1 receptors but it was not elucidated which cells expressed the receptors (Amano *et al.*, 2007). To conclude, the presence of Y1 receptors in mouse bone marrow cells, osteoblasts, and stromal cells seems to be established but so far it is uncertain if any of the other Y receptors are present in bone.

The importance of the neuropeptide Y receptor system in the regulation of bone was first discovered in 2002 with the demonstration that germ line deletion of Y2 receptors resulted in increased bone formation in the distal femur of mice. The increased bone formation was shown to be due to increased osteoblast activity, resulting in a twofold greater trabecular bone volume and a significantly elevated cortical bone mass compared to wild-type mice (Baldock *et al.*, 2002; Baldock *et al.*, 2006). Intriguingly, conditional knockout of Y2 receptors solely in the hypothalamus of adult mice caused a comparable increase in bone volume and the study by Baldock *et al.* (2002) was the first indicating a centrally mediated pathway for NPY in the control of bone formation. This hypothesis was supported by a lack of change in levels of insulin-like growth factor-1, free T4, calcium, leptin, or testosterone.

Ducy *et al.* (2000) were the first to demonstrate an *in vivo* model in which bone formation was under the control of the central nervous system, with the discovery that in the absence of leptin (*ob/ob* mice), or its signaling receptor (*ObRb*), trabecular bone mass was increased. The similarity between *ob/ob* and $Y2^{-/-}$ mice pointed toward a mechanistic link between NPY and leptin for the regulation of bone. However, distinctions between the ObRb and Y2 pathways were initially revealed following central administration of NPY to both $Y2^{-/-}$ and wild-type mice. This resulted in markedly elevated body weight and subsequently elevated leptin levels, leading to reduced osteoblast activity in wild-type mice. Importantly, however, $Y2^{-/-}$ mice were able to maintain their elevated rate of osteoblast activity, revealing distinct pathways regulated by Y2 receptors and leptin with increasing leptin levels (Baldock *et al.*, 2005). Furthermore, subsequent studies demonstrated that the effects of the Y2 and leptin-deficient pathways were opposite in cortical bone, with Y2 deletion consistently stimulating bone formation, again indicating that the actions of these two pathways may be separate (Baldock *et al.,* 2006).

In contrast to the Y2 knockout model, ablation of Y4 receptors does not alter bone mass from wild-type levels (Sainsbury *et al.,* 2003). Because deletion of Y4 receptors did not result in an altered bone phenotype, it was unexpected that *Y2*$^{-/-}$/*Y4*$^{-/-}$ double knockout male mice exhibited a synergistic threefold increase in trabecular bone volume, an effect that was not detectable in female mice (Sainsbury *et al.,* 2003). Interestingly, the female mice did not show any changes in plasma leptin, whereas the male mice levels were decreased by 60%, suggesting that the anabolic synergistic increase in bone volume observed in males could be the result of additive effects of the Y2- and leptin-associated anti-osteogenic pathways in the absence of functional Y4 receptors (Sainsbury *et al.,* 2003).

Interestingly, conditional deletion of hypothalamic neuropeptide Y2 receptors can prevent ongoing bone loss in sex hormone-deficient adult mice (Allison *et al.,* 2006). This effect was attributed solely to an osteoblastic bone formation response that counterbalanced the increased bone resorption due to sex hormone deficiency. Thus, the bone anabolic actions of the Y2 receptor pathway are independent of the presence of sex hormones.

Until now the mechanism by which Y2 receptor deletion causes a doubling in bone volume has not been revealed. Culturing bone marrow stromal cells from Y2-deficient mice demonstrated an increased mineralization *in vitro,* in line with the *in vivo* findings (Lundberg *et al.,* 2007). Following collagenase digestion of compact mice bones and depletion of hematopoietic cells, the collected cells were divided into immature mesenchymal stem cells (MSCs) and mature progenitor cells based on the presence or absence of stem cell antigen-1 (Sca-1). Sca-1 is a cell surface glycoprotein commonly used as a marker for the isolation of hematopoietic stem cells (HSCs) from mouse bone marrow. Sca-1 is also expressed on non-hematopoietic bone marrow stromal cells, and mice lacking Sca-1 develop late onset osteoporosis due to a deficiency in osteoprogenitor cells (Bonyadi *et al.,* 2003). Therefore, Sca-1 expression also appears to be required for the appropriate self-renewal of mesenchymal progenitors and identifies a population of immature mesenchymal cells with the ability to undergo osteoblast differentiation. The Sca-1+ cell population isolated from bone following depletion of hematopoietic cells, which represent an immature multipotential cell type (P. Simmons, personal communication, unpublished data) did not differ between *Y2*$^{-/-}$ mice and wild-type mice. Furthermore, additional sorting of the Sca-1^{-} population of cells using an antibody recognizing CD51, or the αV subunit of the vitronectin receptor, eliminated CD51^{-} erythroid precursor cell types and yielded a more mature "osteoprogenitor" cell population. The number of Sca-1^{-}CD51^{+} mature progenitor cells was twofold greater in bones from Y2$^{-/-}$ mice relative to wild-type (Lundberg *et al.,* 2007). The greater number of these more mature progenitor cells provides a likely mechanism by which mineralization and adipogenesis were increased in stromal cell cultures from *Y2*$^{-/-}$ mice compared with wild-type. These findings support the likelihood that an increase in the progenitor population may contribute to the increased bone formation and volume of *Y2*$^{-/-}$ mice *in vivo*.

Analysis of Y receptor transcripts in cultured stromal cells from wild-type mice revealed high levels of Y1 but not Y2, Y4, Y5, or y6 receptor mRNA (Lundberg *et al.,* 2007). One probable explanation to this event is that germ line Y2 receptor deletion causes Y1 receptor downregulation in stromal cells and bone tissue due to the lack of feedback inhibition of NPY release and subsequent overstimulation of Y1 receptors. It is a well-known phenomenon that Y2 receptors, which usually are expressed on the pre-synaptic side of sympathetic nerve terminals, act in an autoinhibitory fashion to regulate the release of NPY and other neurotransmitters (King *et al.,* 2000). Interestingly, in contrast to the germ line and hypothalamic Y2 receptor or germ line Y1 receptor deletion, conditional deletion of hypothalamic Y1 receptors in adult mice did not alter bone homeostasis, an indication of a peripheral action of Y1 receptors in bone (Baldock *et al.,* 2007). Furthermore, deletion of both Y1 and Y2 receptors did not produce additive effects in bone or adiposity pointing at a common signaling pathway for these Y receptors (Baldock *et al.,* 2007). Together, these findings indicate that the greater number of mesenchymal progenitors and the altered Y1 receptor expression within bone cells in the absence of Y2 receptors are a likely mechanism for the greater bone mineralization *in vivo* and *in vitro.* It is not known if the hypothalamic Y2 knockout mouse model also shows an increase in osteoprogenitor numbers and a downregulation of Y1 receptors in stromal cells. This possible regulatory route is supported by a recent study that used pseudorabies virus-based transneuronal tracing to map transsynaptically connected neurons from rat bone, providing direct evidence that nerve fibers within bone tissue are under the control of synaptic transmission from the hypothalamus (Dénes *et al.,* 2005). However, it cannot be excluded that part of the effect on Y1-receptor downregulation is due to NPY produced by cells locally in the bone marrow such as megakaryocytes (Ericsson *et al.,* 1987).

The effects by NPY on osteoclast formation and activity is limited to the study by Amano *et al.* (2006) demonstrating that NPY inhibits the isoprenaline-induced osteoclastogenesis in mouse bone marrow cultures, an effect associated with decreased cyclic AMP formation and reduced RANKL expression. The fact that NPY did not inhibit D3 or RANKL stimulated osteoclast formation in the bone marrow cultures demonstrates that NPY did not inhibit isoprenaline induced osteoclast formation by an effect directly on osteoclast progenitor cells. The observation that NPY inhibited isoprenaline induced cyclic

AMP formation, together with the finding that NPY inhibited osteoclast formation caused by forskolin, an activator of adenylate cyclase, but not that caused by a cyclic AMP analog activating PKA, shows that NPY affects osteoclast indirectly by inhibiting the synthesis of cyclic AMP in supporting stromal cells. This view is supported by the observation that NPY can inhibit noradrenaline induced cyclic AMP formation in the osteoblastic UMR106-01 cell line (Bjurholm *et al.*, 1988c).

Opioid Peptides

The opioid family of peptides is synthesized from three different precursor molecules: proopiomelanocortin (POMC), proenkephalin (PENK), and prodynorphin (PDYN). Due to tissue-specific processing, different opioid peptides are produced, not only in the nervous and endocrine systems, but also in several other tissues, including the skeleton (Rosen *et al.*, 1998). Opioid peptides exert their opiate-like activity via three different receptors – μ-opioid, δ-opioid, and κ-opioid receptors – all of which are seven transmembrane G proteincoupled receptors linked to inhibition of adenylate cyclase.

Prompted by their initial observation that PENK mRNA is expressed highly and transiently during embryonic development in mesenchymal tissues, including bone and cartilage (Keshet *et al.*, 1989), Rosen *et al.* (1991) reported that PENK mRNA, but not POMC or PDYN mRNAs, is highly expressed in rat calvarial osteoblasts, in rat osteosarcoma cell lines ROS 17/2.8 and ROS 25/1, and weakly in mouse MC3T3-E1 osteoblastic cells and in human Saos-2 osteosarcoma cells. The expression of PENK mRNA in osteoblasts is decreased by $1{,}25(OH)_2D_3$, PTH, and TGF-β (Rosen *et al.* 1991, 1995, 1998). In addition, it has been shown that osteoblasts synthesize enkephalin-containing peptides, including Met-enkephalin. Using immunohistochemistry, Met-enkephalin has been demonstrated not only in bone cells and bone marrow cells, but also in skeletal nerve fibers (Elhassan *et al.*, 1998).

The fact that Met- and Leu-enkephalin, as well as Met-enkephalin-Arg-Phe, decrease alkaline phosphatase activity in ROS 17/2.8 cells indicates that osteoblasts are equipped with opioid receptors and that opioid peptides may act as local regulators of bone cell differentiation in an auto- or paracrine manner (Rosen *et al.*, 1991). The reciprocal interrelationships between osteoblast maturation (as assessed by alkaline phophatase) and PENK expression further indicate that opioid peptide expression is linked to osteoblast differentiation. Based on these observations, Rosen and Bar-Shavit (1994) have proposed the hypothesis that the retained capacity in the adult skeleton to synthesize PENK-derived peptides, in a defined population of undifferentiated cells, may be important in local remodeling of the skeleton, including fracture repair.

In contrast to the observations in rat osteoblasts, opioid receptor agonists such as morphine and DAMGO do not affect alkaline phosphatase activity in the human osteosarcoma cell line MG-63 (Pérez-Castrillón *et al.*, 2000). This was not due to an absence of opioid receptors, as $1{,}25(OH)_2D_3$-stimulated secretion of osteocalcin was decreased by morphine and DAMGO, an effect that was abolished by naloxone. It was also shown that MG-63 cells express all three opioid receptors and that stimulation of μ-opioid receptors, but not of δ-opioid receptors, decreased osteocalcin expression. Also, human osteoblast-like cells isolated from cancellous bone seem to be equipped with opioid receptors; Met-enkephalin inhibits cell proliferation by a mechanism sensitive to inhibition by the opioid receptor antagonist naltrexone (Elhassan *et al.*, 1998). No analysis of alkaline phosphatase was performed in these cell cultures and therefore it is not known if Met-enkephalin affects osteoblast differentiation in human bone cells.

The effect of opioid peptides on osteoclast activity is indicated by the observation that the synthetic analgesic opioid buprenorphine inhibits rat osteoclast activity, as assessed by the pit formation assay (Hall *et al.*, 1996). However, the effect seems unrelated to opioid receptors, as it was not shared by other opioid receptor agonists nor blocked by the opioid antagonist naloxone.

Interestingly, Elhassan *et al.* (1998) found that Met-enkephalin levels are decreased significantly in ankle joints from Lewis rats with adjuvant arthritis. Using immunohistochemistry, a significant decrease was observed in synovial type A cells. If Met-enkephalin levels were affected also in bone cells, it was not reported.

Somatostatin

Somatostatin receptors have been immunolocalized to metaphysis immediately adjacent to hypertrophic cartilage (Mackie *et al.*, 1990). Somatostatin-binding cells stain positive for alkaline phosphatase and are probably osteoblast precursor cells, suggesting that somatostatin may be involved in the regulation of osteoblastic differentiation during enchondral ossification. Mature osteoblasts, as well as osteoclasts and chondrocytes, are negative for somatostatin receptors, which are also lacking in membranous bones. Somatostatin receptor agonists do not affect basal or PTH-stimulated bone resorption in neonatal mouse calvariae (Lerner and Feyen, unpublished observations).

RECEPTORS AND EFFECTS BY NEUROTRANSMITTERS IN BONE

Catecholamines

The presence of adrenergic receptors on osteoblasts has been reviewed (Lerner, 2000) and the important role of

β-adrenergic receptors on osteoblasts for the sympathetic control of bone formation is reviewed in a separate chapter (Chapter 5).

Glutamate, Glutamate Receptors, and Glutamate Transporter

The activity of excitatory amines released into synapses is controlled by a family of homologous excitatory amino acid transporters (EAATs), which are responsible for the reuptake of such amines into presynaptic terminals, a mechanism for the termination of synaptic transmission. To this group belong the glutamate/aspartate transporter (GLAST; EAAT1), Glu transporter-1 (GLT-1; EAAT2), excitatory amino acid carrier (EAAC1; EAAT3), EAAT4, and EAAT5. Moreover, vesicular glutamate transporters (VGLUT1, VGLUT2) are also involved in glutamate signaling. The expression of these transporters in the nervous system is well known, but their presence in non-neural tissues has also been recognized. The expression of GLAST, dopamine, and serotonin transporters has been demonstrated in bone cells. Mason *et al.* (1997) reported the expression of GLAST in osteocytes and osteoblasts, both at the mRNA and protein level, and the downregulation of its expression by mechanical loading using differential RNA display in samples from rat ulnae. Immunohistochemistry demonstrated that the GLAST expression in osteocytes disappeared after loading, whereas it was upregulated in periosteal osteoblasts at sites showing enhanced cellular proliferation and bone formation. The expression of GLAST is regulated *in vitro*, being downregulated during rat osteoblastic differentiation (Bhangu *et al.*, 2000). Osteoblasts are capable of taking up glutamate and releasing glutamate by calcium-sensitive mechanisms, similar to those used by neuronal cells. Skerry (1999) has put forward the hypothesis that glutaminergic signaling may be involved in the coupling between mechanical loading and anabolic events in the skeleton. The origin of glutamate in bone is, however, not fully known. Human and mouse osteoblasts are able to actively release glutamate (Genever and Skerry, 2000), but the possibility of glutamatergic innervation in bone is indicated by the immunolocalization of glutamate in skeletal nerve fibers (Serre *et al.*, 1999).

The action of glutamate is mediated by two different types of receptors: ionotropic receptors, which use the regulation of transmembrane ion fluxes as a signal-transducing mechanism, and metabotropic receptors, which are seven transmembrane-spanning domain, G protein-coupled receptors, using either stimulation of phospholipase C or inhibition of adenylate cyclase as intracellular signaling mechanisms (Chenu, 2002; Mason, 2004; Spencer *et al.*, 2007). Ionotropic receptors can be subdivided into three groups based on their sensitivities to *N*-methyl-D-aspartate (NMDA), AMPA, and kainate. Metabotropic receptors are classified into three groups: group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3), and group III (mGluR4, mGluR6, mGluR7, and mGluR8).

Using immunohistochemistry, *in situ* hybridization, and radio-labeled ligand binding, NMDA receptors have been demonstrated in mouse, rat, rabbit, and human osteoclasts (Chenu *et al.*, 1998; Patton *et al.*, 1998; Itzstein *et al.*, 2000; Merle *et al.*, 2003). The presence of functional NMDA receptors in osteoclasts has been confirmed using electrophysiological patch-clamp techniques (Espinosa *et al.*, 1999; Peet *et al.*, 1999). However, controversy exists as to whether these receptors can regulate the activity of terminally differentiated osteoclasts. Chenu and collaborators have reported that an antibody directed to the NMDA receptor 1 subunit, as well as four different specific NMDA receptor antagonists (D-AP5, MK 801, DEP, and L-689,560), inhibits rabbit osteoclastic bone resorption and decreases the percentage of osteoclasts with actin rings, while having no effect on osteoclast adhesion or apoptosis (Chenu *et al.*, 1998; Itzstein *et al.*, 2000). In contrast, Peet *et al.* (1999) reported that the NMDA receptor antagonist MK 801 inhibits glutamate-induced current in rabbit mature osteoclasts, but has no effect on actin ring formation in these cells nor inhibits pit formation by mature rabbit and rat osteoclasts or basal ^{45}Ca release from neonatal mouse calvarial bones.

The possibility that glutamate NMDA receptors may be involved in the regulation of osteoclastogenesis has been suggested by Peet *et al.* (1999). MK 801 inhibits osteoclast size, nuclearity, TRAP expression, and resorptive activity in cocultures of mouse bone marrow cells and calvarial osteoblasts stimulated by 1,25(OH)$_2$D$_3$, suggesting that osteoclastogenesis is dependent on constitutive glutamate signaling. In agreement with these observations, Merle *et al.* (2003) reported that two different NMDA antagonists decreased RANKL stimulated osteoclast formation in mouse bone marrow cultures, as well as in the monocytic cell line RAW264.7. Furthermore, activation of NMDA stimulated the translocation of NF-κB to osteoclast nuclei.

Osteoclast has been shown to express VGLUT1 and, most interestingly, *VGLUT1*$^{-/-}$ mice exhibit low bone mass (Morimoto *et al.*, 2006).

NMDA receptor subunit 1 knockout mice do not seem to have any skeletal phenotype (Peet *et al.*, 1999), which could be due to redundancy given the expression of the many different glutamate receptor subtypes.

In contrast to previous observations in osteoclasts, Hinoi *et al.* (2007) have recently reported that osteoclasts do not express any of the ionotropic or metabotropic glutamate receptor subtypes, nor GLAST, EAAC1, EAAT5, or VGLUTs. However, GLT-1, EAAT4, and the cystine/glutamate antiporter were expressed in the purified mouse bone marrow osteoclasts. Glutamate inhibited RANKL induced osteoclastogenesis in a manner sensitive to an antiporter antagonist but not to EAAT inhibitors. In contrast, a large variety of agonists for ionotropic or metabotropic glutamate receptors did not affect osteoclastogenesis.

Moreover, intraperitoneal injection of glutamate prevented ovariectomy-induced cancellous bone loss in mice due to decreased number of osteoclasts on bone surfaces. The divergent results obtained by different groups may be explained by the techniques used to purify the osteoclast precursors although this remains to be proven. Clearly, much more work has to be done to clarify the role of glutamate in osteoclast formation and activity.

Immunohistochemistry and *in situ* hybridization have revealed the expression of ionotropic NMDA receptors in rat and human osteoblasts (Chenu *et al.*, 1998; Patton *et al.*, 1998; Gu and Publicover, 2000). NMDA glutamate receptors have also been demonstrated in the human osteoblastic cell lines MG-63 and Saos-2 using radioligand binding and electrophysiological assessments (Laketic-Ljubojevic *et al.*, 1999). Activation by glutamate resulted in increased levels of intracellular Ca^{2+} via a receptor sensitive to inhibition by MK 801, suggesting the presence of active NMDA receptors in these osteoblastic cell lines. Similarly, NMDA was found to increase intracellular Ca^{2+} in rat osteoblasts (Gu and Publicover, 2000). Bone lining osteoblasts in young rats express several ionotropic glutamate receptors and mechanical loading *in vivo* resulted in decreased expression of several of the receptor subtypes (Szczesniak *et al.*, 2005). The function of osteoblastic glutamate receptors is still elusive but glutamate has been found both to inhibit (Uno *et al.*, 2007) and to stimulate (Fatokun *et al.*, 2006) proliferation of osteoblastic MC3T3-E1 cells. NMDA antagonist suppresses rat calvarial osteoblast differentiation via decreased expression of runx2 (Hinoi *et al.*, 2003).

Gu and Publicover (2000) reported the presence of metabotropic GluR1 (but not mGluR2-6) in primary rat osteoblasts using RT-PCR analysis. Activation of these receptors resulted in an elevation of intracellular Ca^{2+}. Interestingly, electrophysiologic analysis and fluorometric studies on intracellular Ca^{2+} showed interactions between ionotropic and metabotropic glutamate receptors in these cells, suggesting a complex glutamatergic signaling in bone cells.

Serotonin Receptors and Serotonin Transporter

Using RT-PCR and radiolabeled ligand binding, Bliziotes and colleagues demonstrated the expression of the serotonin (5-HT) transporter (5-HTT) in several different rat osteoblastic cell lines and primary rat osteoblasts (Bliziotes *et al.*, 2001). The functional expression of 5-HTT was confirmed by studies on [^{3}H]5-HT uptake in ROS 17/2.8 cells. In addition, it was shown that rat osteoblasts express four different 5-HT receptors: 5-HT1A, 5-HT1D, 5-HT2A, and 5-HT2B. Interestingly, the expression of 5-HTT increased during the differentiation of rat osteoblasts (Bliziotes *et al.*, 2000a), in contrast to that of GLAST (Bhangu *et al.*, 2000). Also the osteocytic cell line MLO-Y4 expresses serotonin transporter and serotonin receptors (Bliziotes *et al.*, 2006). Similarly, Westbroek *et al.* (2001) demonstrated serotonin receptors in chicken osteoblasts and osteocytes. In addition, serotonin receptors and 5-HTT have been demonstrated in osteoclasts (Gustafsson *et al.*, 2006). 5-HTT expression is enhanced in RANKL stimulated RAW264.7 cells and the SSRI fluoxetine reduces osteoclast formation (Battaglino *et al.*, 2004). These findings show that all three bone cells can both respond to and regulate 5-HT activity.

Most interestingly, mice deficient of serotonin transporter ($5\text{-}HTT^{-/-}$) exhibit reduced cancellous bone volume, decreased cortical area, and reduced ultimate force of three-point bending (Warden *et al.*, 2005a). The phenotype was due to reduced bone formation rate with no effect on osteoclast surface/bone surface. Similarly, a selective serotonin reuptake inhibitor (SSRI) caused decreased bone mineral accrual in mice. These data indicate that treatment with SSRIs may enhance the risk of developing secondary osteoporosis (Warden *et al.*, 2005b). In agreement with this view, the use of SSRIs has been associated with lower bone mineral density in older men (Haney *et al.*, 2007). In contrast to this finding, fluoxetine treatment of mice has been found to enhance bone mass due to increased cancellous bone volume without affecting ovariectomy-induced bone loss (Battaglino *et al.*, 2007).

Although the data on effects by serotonin on the skeleton are not conclusive, the available information clearly demonstrates that serotonin can influence bone cell activities *in vitro* and bone metabolism *in vivo*.

Dopamine Transporter

The dopamine transporter (DAT) is believed to control the activity of released dopamine (DA) into presynaptic terminals (or possibly other DAT-expressing cells). Mice deficient in DAT exhibit decreased bone mass due to diminished cancellous bone volume, increased trabecular spacing, and reduced trabecular volume (Bliziotes *et al.*, 2000b). The skeletal phenotype includes a reduction in cortical thickness, cortical strength, and decreased femur length. It is not yet known whether the osteopenic phenotype is due to DAT deficiency in bone cells or is mediated by indirect mechanisms. RT-PCR in UMR 106–01 and ROS 17/2.8 cells has failed to demonstrate mRNA for DAT in these osteoblastic cell lines. No data are available regarding the possible expression of DA in skeletal nerve fibers or bone cells. The fact that serum and urinary calcium and phosphorus, as well as circulating PTH, are normal indicates that the mechanism does not involve abnormalities in calcium and phosphorus homeostasis. The possibility may exist that the pathogenesis may, at least partly, be related to the decreased body weight or the anterior pituitary hypoplasia observed in $dat^{-/-}$ mice.

RECEPTORS AND EFFECTS BY NEUROTROPHINS IN BONE

Neurotrophic factors, including the neurotrophins NGF, BDNF, and NT-3, are known to play important roles in development of the central and peripheral nervous systems. These factors are also known to promote the differentiation and survival of various types of neurons. The *trk* proto-oncogenes *trk*A, *trk*B, and *trk*C have been identified as receptors, linked to the activation of tyrosine kinase for these neurotrophins. Thus, the neurotrophins selectively recognize these receptors and NGF, BDNF, and NT-3 bind to the products of *trk*A, *trk*B and *trk*C, respectively. Several neurotrophins have been found to be expressed in bone, and it has been suggested that these factors may have a role not only in bone-associated neuronal biology, but also in bone metabolism.

In the case of bone tissues, there are several reports of osteoblastic expression of neurotrophins and neurotrophin receptors. Nakanishi *et al.* (1994a) reported that the mouse osteoblastic cell line MC3T3-E1 expresses mRNA for NGF, BDNF, and NT-3 and that the expression levels were upregulated during differentiation. The rat ROS 17/2.8 cell line expresses mRNA for NGF, but not for BDNF, and NGF levels are increased by 1,25$(OH)_2D_3$ (Jehan *et al.*, 1996). MC3T3-E1 cells have also been demonstrated to express mRNA encoding *trk*C, the receptor for NT-3 (Nakanishi *et al.*, 1994b). A functional role of this receptor was suggested by the observations that NT-3, but not NGF, stimulated the proliferation of MC3T3-E1 cells and calcium incorporation in the cell layers. Also, ROS 17/2.8 cells have binding sites for NGF (Jehan *et al.*, 1996), although the regulatory role of NGF receptors in these cells has not been assessed. Furthermore, it has been shown that exogenous NT-3 induces DNA-binding activities in MC3T3-E1 cells at several sites, including the cyclic AMP responsive element, partly due to activation of c-fos and c-jun (Iwata *et al.*, 1996). In addition, NGF enhances cell proliferation and the biosynthesis of proteoglycans during chondrogenesis in organ culture (Kawamura and Urist, 1988). These *in vitro* findings suggest that neurotrophins may participate in the regulation of bone formation as auto- or paracrine factors.

Studies of neurotrophins and neurotrophic receptor expression during fracture healing further support the idea of neurotrophic effects in bone. Increased sensory and sympathetic innervation during fracture healing has been reported in animal experiments (Hukkanen *et al.*, 1993). NGF has been immunolocalized in normal rat bone preferentially in osteoprogenitor cells. During fracture healing, however, osteoprogenitor cells, as well as bone marrow stromal cells, osteoblasts, young osteocytes, and most of the chondrocytes in the callus, are expressing NGF protein (Grills and Schuijers, 1998). No NGF was seen in osteoclasts. In a large study including 70 rib-fractured mice, NGF, BDNF, and NT-3 were demonstrated in bone-forming cells at the fracture callus (Asaumi *et al.*, 2000). Interestingly, expressions of the three neurotrophins were increased during the process of healing, especially those of NGF and NT-3. Messenger RNA encoding their respective receptors, *trk*A and *trk*C, were also detected in the bone-forming cells at the fracture callus. An interesting speculation made by the authors is that the expression of NT-3 and *trk*C in osteoblast-like cells at the fracture callus and the increasing expression of NT-3 mRNA during the week after fracture indicate autocrine loop functions for the neurotrophic factor during fracture healing (Asaumi *et al.*, 2000). Such a view is supported by the findings that local application of NGF, at the site of fractured rat ribs, results in dramatically increased levels not only of norepinephrine and epinephrine, but also in increased healing rate and bone strength (Grills *et al.*, 1997). A possible local regulation of NGF expression in fracture sites by bone-derived molecules is suggested by the observation that BMP-2 (in the presence of TNF-α) strongly upregulates NGF in fibroblasts (Hattorl *et al.*, 1996).

RECEPTORS AND EFFECTS BY CANNABINOIDS IN BONE

The members of the cannabinoid system have, besides the classical psychotropic effects, diverse physiological functions including regulation of analgesic, immunomodulatory, and neurophysiological processes. The effects by both synthetic and endogenous cannabinoids are mainly exerted through the cannabinoid receptor type 1 and 2 (CB_1 and CB_2, respectively; for review, see Howlett *et al.*, 2002). Interestingly, recent publications have demonstrated that the cannabinoid system also plays a role in the regulation of bone metabolism. Ralston and colleagues demonstrated the presence of both CB_1 and CB_2 on osteoclasts. They also reported that mice lacking CB_1 developed a higher bone mass than wild-type littermates, and the knockout mice were also protected from ovariectomy-induced bone loss (Idris *et al.*, 2005). Furthermore, CB_1-selective antagonists were shown to inhibit osteoclast formation, whereas treatment with the endogenous cannabinoid receptor agonist anandamide stimulated osteoclast formation (Idris *et al.*, 2005). In contrast to this report, Bab and colleagues recently reported that their CB_1 knockout mice developed a low bone mass phenotype with decreased bone formation rate and increased osteoclast number (Tam *et al.*, 2006). The two studies were conducted using different mouse strain backgrounds, and the opposing results reported may be explained by the fact that the CB_1 deletion results in several different intra-species phenotypes.

In addition to the studies on the CB_1 receptor in regulation of bone metabolism, the importance of signaling through the CB_2 receptor has also been evaluated. Bab and colleagues reported that deletion of the *CNR2* gene induced an increase in bone turnover resulting in a low bone mass phenotype (Ofek *et al.*, 2006). Expression of CB_2 mRNA was detected in both osteoblasts and osteoclasts, as well as in osteocytes. By the use of the synthetic CB_2 agonist HU-308, it was shown that CB_2 signaling in wild-type mice resulted in an increased number of osteoblast precursors and differentiation of osteoblasts. In contrast, the number of osteoclasts was decreased, which was suggested to be a consequence of both direct inhibitory action on the osteoclast precursors, as well as a decrease in RANKL expression after treatment with HU-308 of stromal cells/osteoblasts. Because CB_2 is expressed on many different cell types, including immune cells, indirect effects on bone metabolism by other cell types present in the bone microenvironment cannot be excluded.

Besides the animal studies on the importance of the cannabinoid system in bone metabolism, several reports have implicated that cannabinoids also play a role in human osteoporosis. Linkage analysis showed an association of single nucleotide polymorphisms on chromosome 1p36, which contains the *CNR2* gene, with low spine and hip BMD (Devoto *et al.*, 1998). Furthermore, a study by Karsak and colleagues demonstrated a significant association of single nucleotide polymorphisms encompassing the *CNR2* gene with low BMD in patients with postmenopausal osteoporosis (Karsak *et al.*, 2005).

In summary, animal studies have shown that members of the cannabinoid system function as regulators of bone mass and bone cell function, and that together with the human studies suggesting an important role of CB_2 in human osteoporosis further strengthen the role of cannabinoids as members of the growing network of known factors regulating bone metabolism.

EXPERIMENTAL DENERVATION

The idea that there is a close interaction among the bone neural network, the regulation of bone cell activity, and skeletal turnover is supported by experimental denervation in animals. It has been shown that developmental skeletal growth in the rat hind foot is reduced after surgical denervation. In denervated animals, CGRP and SP immunoreactive nerve fibers were not observed in the perichondrium or periosteum of the metatarsal bones. Metatarsal bones on the contralateral unoperated side exhibited a normal pattern of innervation. The skeletal phenotype could not be due to decreased physical activity, as tendectomized control rats exhibited normal metatarsal bone lengths (Edoff *et al.*, 1997). These results indicate that sensory nerve fibers have growth-promoting effects on immature limb bones.

The possibility that neuropeptides may also influence the metabolism of adult skeleton is suggested by studies demonstrating a significant change in osteoclast numbers in jawbones as a consequence of sensory and sympathetic denervation (Hill and Elde, 1991b). Treatment with guanethidine results in a dramatic decrease of the immunohistochemical staining for VIP, NPY, and DβH in the periosteum of mandible and calvariae in rats, indicating a sympathetic origin of these nerve fibers. This resulted in no change of bone formation, as assessed by periosteal apposition rate in tibiae, but a 50% increase of bone surface in mandible covered by osteoclasts. This could indicate that VIP, NPY, and/or catecholamines may have an inhibitory effect on osteoclast formation and/or activity. These results are in line with our findings that both VIP and PACAP decrease osteoclastogenesis in mouse marrow cultures (Mukohyama *et al.*, 2000). However, capsaicin treatment results in a 20% decrease of bone surface occupied by osteoclasts and, again, no effect on the periosteal apposition rate (Hill and Elde, 1991b). In contrast, Offley *et al.* (2005) reported that capsaicin treatment of rats reduced SP and CGRP content in nerves and caused reduced bone mass associated with increased number of osteoclasts and decreased osteoblast activity and bone formation rate.

In line with the effects of sympathetic depletions demonstrated by Hill and Elde (1991b), deprived sympathetic innervation of rat mandibular alveolar bones showed an increase of osteoclast number per sockets (Sandhu *et al.*, 1987). Moreover, the periosteal and endosteal apposition and mineralization rate was reduced in the sympathectomized jawbones (Sandhu *et al.*, 1987). Because the jawbones are unloaded, these effects cannot be due to decreased loading. Together, the data indicate that sympathetic neurons modulate bone resorption and bone remodeling *in vivo*.

CLINICAL OBSERVATIONS

Skeletal pain in patients with inflammatory and neoplastic disorders clearly suggests the existence of an extensive sensory nervous system in bone tissues. An increased fracture rate in paraplegic children due to myelomeningocele, subdural hematoma, spinal fractures associated with cord lesions, lumbrosacral root avulsion, transverse myelitis, and cord tumors indicates a role of the nervous system also in skeletal metabolism. Excessive callus formation during fracture healing in paraplegic patients further suggests a role of skeletal nerve fibers in bone metabolism (for references, see Lundberg, 2000). The fact that the neurotoxin thalidomide induces skeletal malformation further implicates the nervous system, not only in bone turnover and fracture healing, but also in embryonic skeletal development (McCredie and McBride, 1973).

Patients with tumors producing an excess of circulating VIP may develop hypercalcemia (Dohmen *et al.*, 1991; Lundstedt *et al.*, 1994). Although the pathogenesis is not known, the possibility may exist, given the capacity of VIP

TABLE II Receptor Expression and Effects on Bone Cell Functions by Neuro-osteogenic Factors

	Receptors[a]		Bone cell functions		
Neuro-osteogenic factor	Osteoblasts	Osteoclasts	Bone formation	Bone resorption	Osteoclastogenesis
VIP	+	+	↑[b]	↓↑[c]	↓[d]
PACAP	+	+	↑[b]	↓[e]	↓[d]
CGRP	+	+	↑[f]	↓[g]	↓[h]
SP	+	+	↓[i]	↑[j]	↑[k]
NPY	+	?	↓[l]	?	-[m]
Met-enkef.	+	?	↓[n]	?	?
Somatostatin	+	−	?	?	?
NA/A	+	?	↓[o]	↑[p]	↑[q]
Glutamate	+	+	?	↑[r]	↑↓[s]
Serotonin	+	+	↓[t]	?	?
BDNF[u]	?	?	?	?	?
NGF[u]	+	?	↑[w]	?	?
NT-3[u]	+	?	↑[x]	?	?

[a] The presence of receptors has been indicated by mRNA expression, a rise of cyclic AMP/Ca^{2+} i in individual cells, or immunohistochemistry.
[b] VIP and PACAP stimulate ALP; VIP increases calcium accumulation in bone nodules.
[c] VIP causes an initial, transient "calcitonin-like" inhibition followed by delayed stimulation of rat osteoclasts; VIP stimulates calcium release from mouse calvariae.
[d] VIP and PACAP inhibit osteoclast formation in mouse bone marrow cultures.
[e] PACAP inhibits rat osteoclast pit formation; a possible delayed stimulation has not been assessed.
[f] CGRP stimulates osteoblast proliferation and increases bone mass in vivo; α-$CGRP^{-/-}$ mice exhibit low bone mass.
[g] CGRP inhibits bone resorption in vitro and causes hypocalcemia in vivo.
[h] CGRP inhibits osteoclast formation in human and mouse bone marrow cultures.
[i] SP inhibits expression of extracellular bone matrix proteins in osteoblast cultures.
[j] SP stimulates rabbit osteoclast pit resorption area.
[k] SP stimulates osteoclast formation in bone marrow cultures.
[l] NPY receptor knockout mice have increased bone mass most likely due to enhanced number of osteogenic progenitor cells.
[m] NPY does not affect RANKL stimulated osteoclast formation.
[n] Met-enkephalin, Leu-enkephalin, and Met-enkephalin-Arg-Phe decrease alkaline phosphatase activity in ROS 17/2.8 cells; Met-enkephalin inhibits human osteoblast proliferation.
[o] Mice deficient of β-adrenergic receptor exhibit enhanced bone formation.
[p] Mice deficient of β-adrenergic receptors exhibit decreased bone resorption; norepinephrine stimulates calcium release in mouse calvariae.
[q] Epinephrine stimulates osteoclast formation in bone marrow cultures.
[r] Glutamate receptor antagonists inhibit rabbit osteoclast resorption.
[s] Glutamate receptor antagonist inhibits osteoclast formation in mouse bone marrow cultures; glutamate inhibits RANKL stimulated osteoclast formation.
[t] $5\text{-HTT}^{-/-}$ mice exhibit reduced bone formation rate.
[u] BDNF, NGF, and NT-3 are expressed by osteoblasts.
[w] NGF increases fracture healing.
[x] NT-3 stimulates cell proliferation in MC3T3-E1 cells.

to stimulate osteoclast activity (Lundberg *et al.*, 2000), that VIP-induced enhanced bone resorption may be involved.

It is well known that a high proportion of patients with hip fractures previously have had a stroke (Ramnemark, 1999). Skeletal fractures are also a frequent complication in paraplegic patients during rehabilitation. Most of these poststroke fractures are on the paretic side. Although a high incidence of falls may contribute to the high incidence of hip fractures, it has been suggested that decreased bone mass in the paretic side may be an important factor. Cross-sectional studies have all demonstrated reduced bone mass in the paretic side as compared to the nonparetic side (reviewed in Ramnemark, 1999). A prospective study found a time-dependent enhanced loss of bone mineral density in the paretic side during the first year after stroke (Ramnemark *et al.*, 1999a). Another prospective study for four months showed similar results (Hamdy *et al.*, 1995). The development of hemiosteoporosis is independent on weight changes after stroke (Ramnemark *et al.*, 1999b).

The loss of bone in paraplegic patients is highest during the first 12 months but continues at least for 36 months (Ramnemark, 1999). The fact that the bone resorption marker carboxyterminal telopeptide of type I collagen (ICTP) is increased in patients with hemiosteoporosis

(Fiore *et al.*, 1999; Ramnemark *et al.*, in manuscript) and that osteocalcin, carboxyterminal propeptide of type I collagen (PICP), and alkaline phosphatase are normal (Ramnemark *et al.*, in manuscript) indicate that the loss of bone in the paretic side is mainly due to enhanced bone resorption. Interestingly, both osteocalcin and PICP are increased significantly over a 12-month poststroke period (Ramnemark *et al.*, in manuscript), indicating the presence of high turnover osteoporosis in the paretic skeleton.

Patients with spinal cord injuries, similar to stroke patients, lose bone mineral contents in paralyzed areas of the skeleton (Biering-Sörensen *et al.*, 1988, 1990; Garland *et al.*, 1992; Wilmet *et al.*, 1995; Dauty *et al.*, 2000). The osteopenia is fastest in trabecular bone, showing a total loss of 50% in 18 months and then reaching a plateau phase. The decrease is slower in cortical bones, but continues for longer periods of time. The loss of bone in traumatic paraplegia is associated with an increase in urinary calcium, phosphate, hydroxyproline, and deoxypiridoline (Bergmann *et al.*, 1977–1978; Dauty *et al.*, 2000), indicating that enhanced bone resorption is an important pathogenetic mechanism, similar to the observations in stroke patients. No differences in serum levels of calcium, alkaline phosphate, or osteocalcin were observed. However, serum phosphate was increased.

It may be argued that hemiosteoporosis in paretic patients is due to disuse. However, the population of stroke patients studied by Ramnemark (1999) suffered from severe stroke and was therefore substantially immobilized and still developed local bone loss. Biering-Sörensen *et al.* (1988) reported that the decrease of bone mineral content seen in the lower extremities in patients with spinal cord injuries could not be prevented by spasticity or daily use of long leg braces. In the study by Dauty *et al.* (2000), patients with spinal cord lesions showed a 41% loss of bone mineral density in sublesional areas of the skeleton. However, there was no correlation among daily duration of sitting, daily verticalization, use of long leg braces, or bone mineral density. These observations suggest that osteoporosis in paretic patients cannot simply be classified as disuse osteoporosis. Thus, hemiosteoporosis may be caused by factors unrelated to lack of loading. This raises the possibility that loss of innervation and local control of bone metabolism by skeletal neuro-osteogenic factors may play a role. Most interestingly, Demulder *et al.* (1998) showed that osteoclast formation in 1,25$(OH)_2D_3$-stimulated cultured bone marrow from iliac crest (below the lesional level) is increased significantly as compared to osteoclast formation in sternal bone marrow cultures (above the lesional level) established from paraplegic patients with spinal cord injuries. No such differences were seen in quadriparetic, quadriplegic, or healthy patients. Differences in the *ex vivo* osteoclast formation rate were observed in cultures established both 6 weeks and 12 months after the spinal cord lesion. The authors speculated that the deficiency of skeletal neuropeptides may be responsible for the enhanced osteoclastogenesis seen in bone marrow from paralyzed skeletal areas of paraplegic patients and have demonstrated that CGRP inhibits human osteoclast formation induced by 1,25$(OH)_2D_3$ (Akopian *et al.*, 2000).

Clinical observations, together with findings using experimental denervation to knockout signaling molecules in the nervous system and *in vitro* and *in vivo* data showing effects of and receptor expression for neuropeptides, neurotransmitters, and neurotrophins (see Table II), strongly suggest that skeletal metabolism is controlled by neuro-osteogenic factors. In addition, the nervous system has been suggested to play an important role in the pathogenesis of osteoarthritis as well as rheumatoid arthritis (Vilensky and Cook, 1998; Cerinic *et al.*, 1998). Interestingly, mild mental stress, such as cage change or cold exposure, similar to injection of corticosterone, decreases plasma osteocalcin in rats (Patterson-Buckendahl *et al.*, 1988), suggesting that not only dramatic changes of the neuronal influence on the skeleton, but also more subtle fluctuations, may influence skeletal metabolism.

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Chapter 48

Regulation of Bone Remodeling by Central and Peripheral Nervous Signals

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INTRODUCTION

The 1990s have seen the explosion of mouse genetics driving skeletal biology toward a detailed understanding of the patterning and cell differentiation processes occurring during development. More recently, taking advantage of the availability of a growing number of genetically modified mouse models but also of the possibility to delete one or several genes at will in a defined time frame or in specific tissue(s), this research effort has been extended to skeletal physiology. This *in vivo* approach has been especially successful in uncovering the genetic bases of the regulation of bone remodeling. Among the most surprising findings several regulatory pathways linking the nervous system with bone formation and/or resorption have been uncovered. Although in most cases these pathways are still far from being fully defined, their identification has already attracted much attention from the basic and sometimes clinical science communities. One can therefore predict that their in-depth analysis will be one of the main challenges of the next decade.

Hints of a connection between the nervous system and the regulation of bone mass have long been present in the literature. For instance, osteoporosis is a known complication of spinal cord injury and experimental models of sensory or sympathetic denervation have shown that these two neuronal systems could be involved in bone development and remodeling (Chenu, 2004; Jiang *et al.,* 2006). Likewise, major depression is associated with low bone mass and increased incidence of osteoporotic fractures and the use of several types of central nervous system-active drugs, such as anticonvulsants and opioids, have been associated with increased risk of fracture (Kinjo *et al.,* 2005). Remarkably, these examples all point at a regulation of bone mass by the nervous system, i.e., at signals efferent from the brain (or nerves) to bone cells. This might explain why most of the earlier studies have focused on peripherally produced neuromediators (Chenu, 2002; Spencer *et al.,* 2007). Yet, if one accepts that either or both arms of bone remodeling are centrally controlled, it is legitimate to consider that one or several afferent signals may exist that influence(s) the brain's control of bone mass. The identification of such signal(s), which can be viewed as an archetype of the current effort of integrative biology, might bring into light connections between the skeleton and organs previously unsuspected to play a role in its biology. This emerging field of investigation may therefore become critical to develop novel concepts in drug development.

AFFERENT SIGNALS REGULATING BONE REMODELING VIA THE CENTRAL NERVOUS SYSTEM: LEPTIN AS A MEDIATOR BETWEEN FAT AND BONE METABOLISMS

At the present time the only signal known, *in vivo*, to affect bone mass via a central nervous system (CNS) relay is leptin. Leptin is a 16 kDa peptide hormone synthesized specifically by adipocytes that was originally identified by positional cloning of the mutation present in *ob/ob* mice, a natural mutant presenting morbid obesity and sterility (Zhang *et al.,* 1994). Leptin inhibits appetite and favors reproduction by binding to a receptor present on hypothalamic neurons; consequently, mice lacking the leptin receptor (*db/db* mice) are also obese and sterile (Tartaglia *et al.,* 1995). In spite of their hypogonadism, bone mass is

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increased in leptin signaling-deficient (*ob/ob* and *db/db*) mice compared to wild-type mice (Ducy *et al.,* 2000). Importantly, *ob/ob* mice fed a low-fat diet have a normal weight at 1 month of age but they already display a high bone mass phenotype. The leptin-signaling deficient bone phenotype is owing to the absence of leptin not to the obesity of the *ob/ob* and *db/db* mice because mice lacking fat as the result of transgenic expression of a dominant-negative protein inhibiting the activity of b-ZIP adipocyte differentiation factors also display a high bone mass phenotype (Ducy *et al.,* 2000). Moreover, a leptin transgene can correct the high bone mass of these lipodystrophic mice indicating that leptin is the adipocyte-derived gene product responsible for their bone phenotype (Elefteriou *et al.,* 2004). It is worthwhile to note that glucocorticoid serum levels are elevated in *ob/ob* mice but not in lipodystrophic mice (or patients). Yet, they display an increase in bone formation activity, thus demonstrating that the bone phenotype caused by an absence of leptin signaling is not linked to their level of circulating glucocorticoids. Lastly, it is remarkable that the high bone mass phenotype observed in leptin-signaling deficient mice exists despite an increase in bone resorption caused by both leptin-independent (hypogonadism) and leptin-dependent mechanisms (see below).

Regulation of Both Arms of Bone Remodeling by Leptin

Histomorphometric analyses, which allow the most objective evaluation of bone parameters because their results do not need to be adjusted for differences of lean/fat mass between samples, have shown a nearly twofold increase in trabecular bone volume in *ob/ob* (or *db/db*) mice compared to wild-type littermates (Ducy *et al.,* 2000). This phenotype is observed in both sexes, in growing and remodeling animals, and in long bones as well as in vertebrae. Remarkably, it affects only trabecular bone, where it is marked by the presence of many more thick trabeculae; cortical bone is not affected (Ducy *et al.,* 2000). The high bone mass phenotype of leptin-signaling deficient mice is owing to a massive increase in bone formation rate. As for the increase in bone volume, an increased bone formation rate is already present in fat-restricted 1-month-old *ob/ob* mice and heterozygote *ob/+* mice, which are not obese, indicating that it is the absence of leptin, not an increase in fat mass, that is responsible for this phenotype in leptin-signaling deficient animals (Ducy *et al.,* 2000).

In addition to its negative action on bone formation, leptin also regulates bone resorption. Both *ob/ob* and *db/db* mice show an increase in the number of osteoclasts and increased elimination of deoxypyridinoline cross-links (Dpd) in the urine. Although some of this phenotype can be attributed to their hypogonadism (Ducy *et al.,* 2000) subsequent studies, presented later, demonstrated that leptin exerts a dual influence on bone resorption: On the one hand, it favors it through the sympathetic nervous system, and on the other hand, it inhibits it through another molecular relay, cocaine- and amphetamine-regulated transcript (CART) (Elefteriou *et al.,* 2005). Based on the existence of a high bone mass phenotype in *ob/ob* and *db/db* mice one can nevertheless conclude that leptin's antiosteogenic action on bone formation is dominant over its effect on bone resorption (Fig. 1).

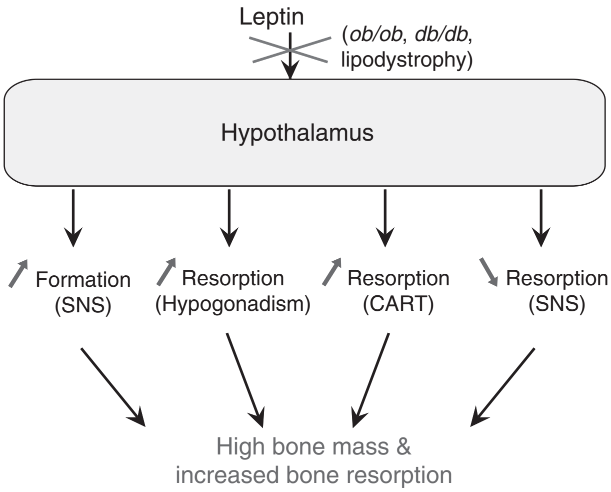
Leptin
(*ob/ob, db/db,* lipodystrophy)
Hypothalamus
↗ Formation (SNS)
↗ Resorption (Hypogonadism)
↗ Resorption (CART)
↘ Resorption (SNS)
High bone mass & increased bone resorption

FIGURE 1 Dual effect of leptin on bone remodeling. The absence of leptin signaling in the hypothalamus causes a high bone mass phenotype despite an overall increase in bone resorption: disruption of the leptin-SNS dependent cascade both increases bone formation and decreases bone resorption while hypogonadism and a CART-dependent mechanism increase bone resorption.

Evidence of Leptin's Antiosteogenic Function in Human

Several observations provide pivotal evidence that the absence of leptin affects the function of osteoblasts in the same manner in human and mice. First, a patient harboring an inactivating mutation of the leptin gene was shown to display high bone mass (Elefteriou *et al.,* 2004). Second, lipodystrophic patients, whose serum leptin levels are extremely low because of the absence of adipocytes, display advanced bone age, an objective evidence of increased osteoblasts function, and osteosclerotic lesions (Westvik, 1996; Elefteriou *et al.,* 2004). Third, although they are based on less straightforward arguments, epidemiologic evidence also indicates that leptin antiosteogenic activity is conserved between mice and human. An early study noted an inverse association between serum leptin levels adjusted for fat mass and BMD in a group of 221 Japanese men (Sato *et al.,* 2001). Likewise, in a large cohort of young men (the Gothenburg Osteoporosis and Obesity Determinants [GOOD] cohort), leptin was found to be a negative independent predictor of areal BMD (aBMD), at several measured sites, and of cortical bone size of both the non–weight-loaded bone (radius) and the weight-loaded bone (tibia) (Lorentzon *et al.,* 2006). Importantly, this study was performed on a primarily healthy population with normal body mass indexes sparing the need to adjust BMD readings for differences in lean and fat mass. In agreement with the absence of cortical phenotype in leptin-signaling deficient mice, this same study found that serum leptin levels were not independent predictors of cortical vBMD (Lorentzon *et al.,* 2006). Other cross-sectional studies have failed to show such negative association between serum leptin levels and areal aBMD (Goulding and Taylor, 1998; Martini *et al.*, 2001; Thomas *et al.*, 2001; Papadopoulou *et al.*, 2004). Most likely, some of the differences between these and other studies can be attributed to the way data are evaluated and presented either adjusted for body weight or unadjusted. For instance, in a large North American population-based study including a high representation of the elderly, non-Hispanic blacks, and Mexican Americans, BMD increases with increasing leptin concentration in men. However, after adjustment for body mass index (BMI) and other bone-related factors, an inverse association emerged, being most evident in men younger than age 60 (Ruhl and Everhart, 2002). Similarly, in a few recent studies in men, leptin was inversely correlated to aBMD, an association that became apparent only after adjustment of aBMD for body weight (Sato *et al.*, 2001; Morberg *et al.*, 2003). Additionally, one must be cautious when reporting the results of clinical studies including obese participants. Indeed, they are likely to show results shifted toward a positive association between serum leptin levels and BMD in response not to peripheral leptin levels but to central leptin resistance; the mere fact that obese individuals are overweight despite high levels of serum leptin demonstrates such leptin loss-of-function effect.

Anatomical Targets of Leptin's Action on Bone Remodeling

It is generally admitted that leptin's action on appetite and reproduction depends of its binding to a specific receptor located on hypothalamic neurons (Ahima and Flier 2000; Ahima, 2004). In agreement with this central mode of action intracerebroventricular (ICV) infusion of low doses of leptin in *ob/ob* mice, at a rate that does not result in any detectable leak of leptin in the general circulation, corrects their high bone mass (Ducy *et al.,* 2000). This rescue is complete, and occurs even at minimal doses that do not influence body weight (Elefteriou *et al.,* 2004), arguing that the central, presumably hypothalamic, action of leptin is its only physiological mode of action in terms of bone biology. Likewise, low doses of leptin administered by ICV infusion in wild-type animals induce a potent anti-osteogenic effect resulting in catastrophic bone loss within 1 month of infusion (Ducy *et al.,* 2000). This is an important point as peripheral injection of supra-physiological amounts of leptin to wild-type animals is correlated with a gain in bone mass (Cornish *et al.,* 2002; Martin *et al.,* 2005). However, as in obese individuals the presence of high levels of leptin in the serum of these treated animals is likely to induce central resistance and therefore induce in these animals a phenotype functionally similar to leptin deficiency i.e., bone gain.

Other experimental arguments also indicate that leptin, physiologically, does not act directly on osteoblasts. First, Stat3 phosphorylation cannot be detected following treatment of primary osteoblast cultures with physiological doses of leptin (Ducy *et al.,* 2000); an effect of leptin was observed only when high doses of leptin were used on osteoblastic cells differentiated *in vitro* (Thomas *et al.,* 1999). Second, a local action of leptin implies that the high bone mass observed in *db/db* mice, which lack the leptin receptor, is because of an osteoblast defect. Yet, cultured osteoblasts from *db/db* mice do not produce any more extracellular matrix than wild-type osteoblasts (Ducy *et al.,* 2000). Third, transgenic mice overexpressing leptin in osteoblasts have no overt bone abnormalities (Takeda *et al.,* 2002). Thus, at the present time there is no convincing evidence of a direct action of leptin on osteoblasts in physiological conditions *in vivo*.

Hypothalamic leptin receptors are known to be mainly present in two locations: neurons of the arcuate (ARC) nuclei, identified as most important in controlling leptin's function on appetite, and neurons of the ventromedial hypothalamic (VMH) nuclei. Lesioning neurons of the ARC nuclei in wild-type mice using monosodium glutamate (MSG) can increase appetite and induces obesity but does not affect bone formation parameters (Takeda *et al.,* 2002). In contrast, lesioning neurons of the VMH nuclei using gold thioglucose (GTG) induces an increase in bone mass because of an increase in bone formation parameters similar to the one observed in *ob/ob* mice (Takeda *et al.,* 2002). Moreover, in *ob/ob* mice whose VMH neurons have been lesioned leptin ICV infusion decreases body weight but does not affect bone formation parameters or bone mass. Conversely, leptin ICV fails to decrease body weight but decreases bone mass and bone formation in *ob/ob* mice whose ARC neurons have been lesioned (Takeda *et al.,* 2002). Thus, VMH neurons (or more precisely GTG-sensitive neurons) regulate bone formation under the control of leptin (Fig. 2). Importantly, if one considers the physiological implications of modulating leptin signaling to target bone formation disorders, this VMH rather than ARC mediation of leptin antiosteogenic effect dissociates this hormone's activity on appetite and bone mass.

EFFERENT SIGNALS AFFECTING BONE REMODELING

Cocaine- and Amphetamine-Regulated Transcript (CART)

CART is a neuropeptide precursor protein encoded by a gene expressed in hypothalamic neurons, in other parts

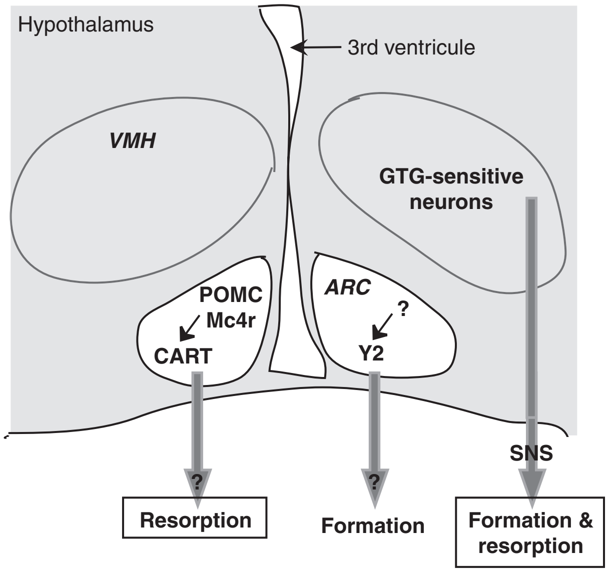

FIGURE 2 Role of hypothalamic regulations in the control of bone remodeling. The GTG-sensitive neurons present in the VMH nuclei mediate the leptin-dependent regulation of both arms of bone remodeling by the SNS. In addition, leptin, this time presumably in the ARC nuclei, controls a melanocortin (POMC/Mc4r)-dependent regulation of CART expression to act, via an unknown receptor, on the regulation of bone resorption by osteoblasts. Also presumably in the ARC nuclei signaling via the Y2 receptor has been proposed to regulate bone formation through an unknown cascade. At the present time only the CART and SNS pathways have been shown of relevance to human bone biology (boxed labels).

of the nervous system, in peripheral organs such as the pancreas and the adrenal glands but not in bone cells (Couceyro *et al.*, 1997; Elias *et al.*, 1998; Wierup *et al.*, 2004; Elefteriou *et al.*, 2005). That *Cart* expression is virtually undetectable in hypothalamic neurons of *ob*/*ob* mice suggested that it is positively regulated by leptin and could therefore act as a mediator of its function(s) (Douglass *et al.*, 1995; Kristensen *et al.*, 1998). Yet, Cart-deficient mice do not present a body weight or reproduction phenotype (Asnicar *et al.*, 2001). Instead, they display, in both sexes, a low bone mass phenotype at 6 months of age (Elefteriou *et al.*, 2005). Osteoblast numbers and bone formation rates are normal in these mice but osteoclast surface and number are nearly doubled in Cart-/- bones leading to a significant increase in urinary Dpd elimination. Considering the absence of *Cart* expression in *ob/ob* mice, this negative regulation of bone resorption by CART explains, at least in part, the increase in bone resorption observed in these mice. It was subsequently shown that *CART* polymorphism affects bone mass in postmenopausal women indicating that CART also regulates bone remodeling in humans (Guerardel *et al.*, 2006).

In agreement with the absence of *Cart* expression in bone cells, the Cart-/- phenotype is not bone cells autonomous as Cart-deficient bone marrow macrophages differentiate normally into osteoclasts and Cart-deficient bone marrow stromal cells can normally support osteoclastogenesis in coculture experiments (Elefteriou *et al.*, 2005). Analysis of gene expression in Cart-/- bones detected an increase in *Rankl* expression suggesting that Cart regulates bone resorption by ultimately modulating *Rankl* signaling (Elefteriou *et al.*, 2005; Ahn *et al.*, 2006). In absence of any identified CART receptor one can only speculate whether this factor acts directly on osteoblasts or uses one or several relays to signal to bone cells.

Melanocortin 4 Receptor

Melanocortins, a family of peptides produced by post-translational processing of pro-opiomelanocortin (POMC), regulate food intake and energy expenditure via binding to two melanocortin receptors expressed in the central nervous system (Mc3R, Mc4R). Although these receptors show a fairly widespread presence in the rodent brain (Mountjoy *et al.*, 1994; Kishi *et al.*, 2003), POMC has a limited distribution, with only two neuronal populations described: one in the arcuate nucleus of the hypothalamus (ARC) and the other in the nucleus of the tractus solitarius (NTS) of the brainstem (Palkovits *et al.*, 1987; Bronstein *et al.*, 1992). The POMC neurons of the ARC are known to be responsive to leptin via leptin receptors expressed on their surface and MC4R has thus been implicated in leptin's control of appetite (Cheung *et al.*, 1997; Cone, 1999). It was first reported that patients lacking Mc4R have high bone mineral density and advanced bone age (Farooqi *et al.*, 2000). Subsequently, Mc4r-/- mice were shown to have an increase in bone mass (Elefteriou *et al.*, 2005). This high bone mass phenotype is caused by an isolated decrease in osteoclast number and function while, throughout their life, these mice show normal bone formation parameters. Consistent with this phenotype, *Rankl* expression is lower in Mc4r-/- bones than in wild-type bones (Elefteriou *et al.*, 2005). The same decrease in bone resorption activity was also noted in MC4R-deficient patients (Ahn *et al.*, 2006).

Among the many abnormalities caused by Mc4R inactivation there is a nearly twofold increase in hypothalamic *Cart* expression (Elefteriou *et al.*, 2005). Likewise, serum CART levels are significantly increased in patients heterozygous for inactivating mutations of Mc4R (Ahn *et al.*, 2006). Analysis of Mc4R-deficient mice lacking either one or two copies of the *Cart* gene demonstrated that removing one allele of *Cart* is sufficient to normalize their bone resorption parameters and thereby their bone mass (Ahn *et al.*, 2006). Accordingly, *Rankl* expression is normalized in such compound homozygote mutant mice. In contrast, removing either one or two alleles of *Cart* from Mc4r-/- mice does not correct their obesity and their endocrine abnormalities (Ahn *et al.*, 2006). These results indicate that a high level of *Cart* expression, and thereby of CART signaling, is the main molecular event accounting for the low-bone-resorption/high-bone-mass phenotype observed in absence of Mc4R but that it does not account for the accompanying metabolic abnormalities (Fig. 2).

Interleukin-1 (IL-1)

IL-1 is a polypeptide product that mediates several components of the acute-phase response to infection and injury. Its main sites of expression are the peripheral immune system and bone cells, as well as glia and neuron cells in the CNS (Lorenzo *et al.*, 1990; Dinarello, 1997). IL-1 is a potent stimulator of bone resorption; when injected subcutaneously it increases osteoclasts number and bone resorption surfaces in mice (Sabatini *et al.*, 1988). A natural IL-1 receptor antagonists has been identified, IL-1ra, that binds but does not activate the two known IL-1 receptors, IL-1RI and IL-1RII (Arend *et al.*, 1990; Dinarello, 1997). A role for peripheral IL-1 in regulating estrogen depletion-induced bone loss has been suggested as peripheral IL-1ra administration decreases osteoclast formation and bone resorption in ovariectomized mice (Kitazawa *et al.*, 1994), and IL-1RI-deficient mice do not lose bone after ovariectomy (Lorenzo *et al.*, 1998).

A recent study has implicated a central response to IL-1 in regulating bone remodeling (Bajayo *et al.*, 2005). Targeted overexpression of human IL-1ra to the central nervous system of mice using the murine glial fibrillary acidic protein promoter results in a low bone mass phenotype caused by a doubling of osteoclast numbers. Bone formation parameters are also increased, although moderately, in these transgenic mice. The relevance of this regulation to human physiology as well as the pathway connecting this central IL-1 signaling to bone remodeling is still unknown.

Y Receptors Signaling

The Y signaling system is complex, consisting of at least five receptors (Y1, Y2, Y4, Y5, and Y6) with different binding profiles and sites of expression in the central nervous system and the periphery, and of multiple endogenous ligands, Neuropeptide Y (NPY), peptide YY (PYY), and pancreatic polypeptide (PP) (Lin *et al.*, 2004). NPY is widely expressed in the central and peripheral nervous system and has been shown to play an important role in numerous physiological processes (Hokfelt *et al.*, 1998). Notably, increased NPY expression in the hypothalamus leads to increased food intake and obesity in mice if they are put on a high sucrose diet (Kaga *et al.*, 2001). In agreement with this observation, NPY fibers project from the ARC nuclei, which are known to participate in the control of appetite. The related family members peptide YY (PYY) and pancreatic polypeptide (PP) are produced in the small intestine and colon or in the pancreas, respectively (Hazelwood, 1993) where they affect gut motility, pancreatic and gall bladder secretion. PYY and PP can also access specific Y receptors in the hypothalamus and the brainstem to further influence pancreatic and gastric secretion. NPY and PYY have identical affinity for all known Y receptors (Y1, Y2, Y4, Y5, and in mouse also Y6), with PP preferring the Y4 receptor (Larhammar, 1996).

Although it is not clear which ligand(s) is/are involved because NPY-deficient mice show a normal bone mass (Elefteriou *et al.*, 2003), studies of genetically engineered mouse models have begun to shed some light on the role played by several Y receptors on bone biology in this species. Indeed, deletion of the Y2 receptor either germ line or hypothalamus-specific (via brain stereotaxic injection of Cre-expressing adenovirus) causes an increase in cancellous bone volume and cortical thickness secondary to accelerated bone formation (Baldock *et al.*, 2002; Baldock *et al.*, 2006). The Y2-deficient mice do not show an effect on bone resorption, as measured by osteoclast surface, but they have significantly reduced osteoclast numbers (Baldock *et al.*, 2002). More recently, it was shown that Y1-deficient mice also display a high bone mass phenotype characterized by increased cancellous bone volume and cortical density despite increased osteoclast surfaces (Baldock *et al.*, 2007). However, this phenotype cannot be observed when the Y1 deletion was restricted to the hypothalamus, indicating that this receptor does not act centrally to regulate bone mass (Baldock *et al.*, 2007). Instead, treatment of wild-type mouse calvarial osteoblasts with NPY markedly reduces cell numbers and this effect is abolished in Y1-deficient culture suggesting that Y1 regulates bone mass by acting directly on bone cells (Baldock *et al.*, 2007). Interestingly, the germ line deletion of Y2 causes a downregulation of Y1 expression in bone (Lundberg *et al.*, 2007) suggesting that part of the bone phenotype displayed by these mice could be caused, at least partly, by peripheral effects.

In contrast, bone mass is not altered in Y4-deficient mice (Sainsbury *et al.*, 2003). However, bone volume is increased threefold in Y2-/-;Y4-/- double mutant mice because of an increase in the bone formation rate (Sainsbury *et al.*, 2003). These changes are more pronounced than those observed in Y2-deficient mice, suggesting that the Y2 and Y4 signaling pathways act synergistically. Although these mice show an increase in circulating levels of PP, the preferred Y4 ligand, this abnormality is not responsible for their bone phenotype because neither Y4-deficient mice, which also show an increase in PP levels, nor transgenic mice overexpressing PP have a bone phenotype (Sainsbury *et al.*, 2003). In contrast, because the Y2 deletion results in elevated NPY levels in the hypothalamus (Sainsbury *et al.*, 2003) it is conceivable that the more severe phenotype observed in Y2/Y4-deficient mice compared to Y2 or Y4 single mutants results from an enhanced signaling by this neuropeptide on Y4 receptors that is disrupted in the double mutant mice. Another possibility could be that the decrease in serum leptin levels observed in double Y2/Y4-deficient mice but not in single mutant mice (Sainsbury *et al.,* 2003) contributes to their phenotype.

NeuromedinU

NeuromedinU (NMU) is a neuropeptide produced by nerve cells in the small intestine and by structures in the brain, including the dorsomedial nucleus of the hypothalamus and the pituitary (Brighton *et al.,* 2004). It is generally assumed that NMU regulates various aspects of physiology including appetite, stress response, and SNS activation (Brighton *et al.,* 2004). Although NMU expression appears regulated by leptin (Howard *et al.,* 2000), NMU-deficient mice develop a leptin-independent obesity phenotype (Hanada *et al.,* 2004). However, NMU-deficient mice present a high bone mass phenotype associated with an increase in bone formation similar to the one displayed by *ob/ob* mice (Sato *et al.,* 2007). This phenotype is not cell autonomous as NMU-deficient osteoblasts in culture are indistinguishable from wild-type osteoblasts and treatment of wild-type osteoblasts with NMU do not affect their proliferation or differentiation. In contrast, ICV infusion of NMU in both NMU-/- mice and wild-type mice decreases bone formation and bone volume (Sato *et al.,* 2007).

Most importantly, ICV infusion of NMU in *ob/ob* mice decreases bone volume as in wild-type mice but ICV infusion of leptin in NMU-/- mice cannot decrease bone mass, demonstrating that NMU is a mediator for leptin's action on bone formation (Sato *et al.,* 2007). Furthermore, treatment of NMU-deficient mice with the β-agonist isoproterenol does not decrease their bone mass, indicating that NMU is involved in the SNS regulation of bone remodeling. Additional analyses demonstrated that hypothalamic NMU only affects the leptin-dependent negative regulation of bone formation by the molecular clock (Sato *et al.,* 2007). Given the lack of an obesity phenotype in mice deficient for the NMU receptor (NMUR2) (Zeng *et al.,* 2006), and provided that this pathway is conserved in human NMU antagonists might become good candidates to treat bone-loss disorders without inducing undesirable effect on body weight.

Sympathetic Nervous System

One of the many abnormalities displayed by the *ob/ob* mice is a low sympathetic activity (Bray and York 1998) suggesting that the sympathetic nervous system (SNS) could mediate leptin's regulation of bone mass. In support to this hypothesis restoring sympathetic activity in *ob/ob* mice by treating them with an adrenergic agonist leads to a dramatic decrease in bone mass (Takeda *et al.,* 2002). Also consistent with a role of the sympathetic nervous system in mediating leptin's antiosteogenic action on bone mice deficient in dopamine β-hydroxylase (DBH), an enzyme necessary to produce norepinephrine and epinephrine, the catecholamine ligands produced by the SNS, have a high bone mass and this high bone mass persists upon leptin ICV infusion (Takeda *et al.,* 2002). A further demonstration that the central effect of leptin on bone remodeling is mediated by the sympathetic nervous system instead of a circulating factor is based on cross-circulation experiments between *ob/ob* animals. In pairs of parabiosed *ob/ob* mice where one mouse was implanted with a pump infusing leptin centrally, bone mass dropped significantly only in the mouse receiving leptin ICV (Takeda *et al.,* 2002). Although this result does not rule out the existence of a short-lived humoral mediator, it strongly suggests the existence of a neuronal mediation of leptin antiosteogenic function.

An extensive gene expression analysis determined that the only adrenergic receptor expressed in osteoblasts is the β2 adrenergic receptor (Adrβ_2) (Takeda *et al.,* 2002). In the light of this specificity and of the fact that they have normal body and fat pad weights and none of the endocrine abnormalities observed in *ob/ob*, *db/db,* or Dbh-/- mice Adrβ2-/- mice are the most accurate model to elucidate whether and how sympathetic signaling in bone cells regulates bone mass. Histomorphometric analysis of Adrβ2-deficient mice revealed that they have high bone mass phenotype (Takeda *et al.,* 2002). This phenotype is cell-autonomous as transplantation of bone marrow cells (BMCs) from wild-type mice into irradiated Adrβ2-/- mice normalizes their bone formation parameters whereas transplantation of Adrβ2-/- BMCs into irradiated wild-type mice significantly increases bone formation (Elefteriou *et al.,* 2005). Importantly the bone phenotype observed in Adrβ2-deficient mice cannot be rescued by leptin ICV infusion demonstrating that the sympathetic nervous system, via Adrβ2 mediates leptin regulation of bone mass (Takeda *et al.,* 2002; Elefteriou *et al.,* 2005). Because Adrβ2-/- mice have no overt endocrine abnormalities this experiment also formally establishes that the high bone mass observed in absence of leptin signaling is not secondary to any metabolic perturbations.

The bone phenotype of the Adrβ2-/- mice originates from both an increase in bone formation and a decrease in bone resorption. Indeed, these mice have an increase in osteoblast number because of an increase in these cells' proliferation ability. At the molecular level this phenotype is caused by a dual mechanism: On the one hand, sympathetic signaling acts on AP-1 genes to favor osteoblast proliferation, and on the other hand, it inhibits osteoblast proliferation by affecting, through the molecular clock, both *D type cyclin* and *AP-1* gene expression (Fu *et al.,* 2005). The Adrβ2-/- mice also show a significant decrease in number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts caused by a defect in osteoclast differentiation, and therefore a decrease in urinary elimination of Dpd (Elefteriou *et al.,* 2005). This phenotype can also be observed in wild-type mice transplanted with Adrβ2-/- bone marrow cells indicating that it is cell autonomous. It is caused by an increase in *Rankl* expression by osteoblast progenitor cells following protein kinase A phosphorylation of ATF4, a cell-specific CREB-related transcription factor essential for osteoblast differentiation and function (Yang *et al.*, 2004; Elefteriou *et al.*, 2005). Neither bone formation nor bone resorption phenotypes can be corrected by leptin ICV infusion, indicating that leptin signaling can regulate both arms of bone remodeling via the SNS (Takeda *et al.*, 2002; Elefteriou *et al.*, 2005).

As for leptin's regulation of bone remodeling, several clinical observations indicate that the SNS function on bone mass is conserved in human. For instance, patients with reflex sympathetic dystrophy, a disease characterized by localized high sympathetic activity, develop a severe and localized osteoporosis that can be treated by β-blockers (Kurvers, 1998). Likewise, multiple epidemiologic studies reported beneficial effects of β-blocker administration on BMD and/or fracture risk in women. In a population of 1,344 postmenopausal women (Geelong Osteoporosis Study) it was found that a higher BMD at the total hip (2.5%, $p = 0.03$) and ultradistal forearm (3.6%, $p = 0.04$) as well as a decreased fracture risk (odds ratio for fracture associated with β-blocker use = 0.68) associates with β-blockers use (Pasco *et al.*, 2004). Subsequent analyses also reported that β-blocker use was associated with a decrease in serum CTx levels (Pasco *et al.*, 2005). A case-control analysis using the UK General Practice Research Database (GPRD) showed that compared to patients who did not use them, the odds ratio for current use of β-blockers was 0.77 (Schlienger *et al.*, 2004). Another study of 158 women taking β-blockers and 341 age-matched controls showed that the odds ratio for fracture (at all sites) in the β-blocker user group was 0.56. In this study β-blocker use was associated with a higher BMD at the femoral neck (+4.2%, $p < 0.05$) and L1–L4 (+3.2%, $p < 0.05$) while proximal femur scans revealed significantly higher cortical width (+3.6%, $p < 0.05$) at the femoral neck of patients under β-blockers (Bonnet *et al.*, 2007). A small prospective case-control study in elderly patients showed that bone mineral density was significantly greater at the total hip and spine sites of beta-blocker users compared with nonusers (Turker *et al.*, 2006). More generally, a systematic computerized search of MEDLINE, EMBASE, and LILACS databases from 1966 to December 2005 for case-control and cohort studies of associations between exposure to antihypertensive agents and fracture outcomes showed that β-blockers induce a statistically significant protection against fractures (pooled relative risk = 0.86), especially relevant to hip fractures (28% relative risk fracture reduction); in contrast, the use of α-blockers did not appear to protect against fractures (Wiens *et al.*, 2006).

A few studies also reported less conclusive evidence of a beneficial effect of β-blockers on bone mass in human patients. For instance, no difference in BMD could be found between men with severe myocardial infarction taking or not taking cardioselective β-blockers. Likewise, in U.S. women enrolled in the Study of Osteoporotic Fractures (SOF) total hip BMD was greater among β-blocker users but adjustment for body weight or other parameters eliminated the difference. Nevertheless, there was a protective effect of β-blockers against hip fracture (hazard ratio for hip fracture associated with β-blocker use was 0.76) (95% CI 0.58–0.99) (Reid *et al.*, 2005). The discrepancy between the results of these and the studies cited earlier could have many origins. Among the most likely is the specificity of the β-blocker used. Indeed, it was recently shown that although mouse osteoblasts do not normally express the adrenergic receptor 1 (Adrβ1) and Adrβ1-deficient mice do not show a bone phenotype, Adrβ1-/-; Adrβ2-/- double mutant mice deficient display a low bone mass phenotype (Pierroz *et al.*, 2006). This result suggests that the Adrβ1 receptor could, via an osteoblast-independent mechanism, counteract the effect of Adrβ2 on bone remodeling. If this mechanism is conserved in human this observation implies that patients treated with non-Adrβ2 specific β-blockers could display either a low or a normal BMD. Differences of doses of β-blockers could be another significant factor of variability between studies. Administration of 0.1, or 5, or 20 mg/kg of the β-blocker propranolol in ovariectomized rats showed that the best preventive effect against bone loss was obtained with the lowest dose, the highest dose being ineffective (Bonnet *et al.*, 2006). It is therefore most likely that only long-term prospective randomized trials taking into account at least the specificity and dosage of the β-blocker(s) evaluated will provide the most accurate demonstration of the protective effect of this class of molecules in human.

Cannabinoid Receptors

An unexpected role of the endocannabinoid system, which is known to regulate analgesia, energy balance, and appetite, in the regulation of bone mass has been revealed in mice and some of these findings have been validated in human (Di Marzo *et al.*, 2004). There are two highly homologous cannabinoid receptors, CB1 and CB2, which are similarly activated by endocannabinoids but yet show functional differences (Bisogno *et al.*, 2005). CB1 is predominantly expressed in the brain and peripheral neurons, where it is responsible for the psychotropic action of cannabinoids, but it is also expressed in peripheral tissues including immune cells, the reproductive system, the gastrointestinal tract, and the lung (Di Marzo *et al.*, 2004). CB2 is not expressed in the brain; it is mainly expressed in immune cells (Di Marzo *et al.*, 2004).

Mice with a targeted deletion of CB1 have an increase in bone mass in both an ADH and a CD1 genetic background (Idris *et al.*, 2005; Ofek *et al.*, 2006; Tam *et al.*, 2006), although bone formation and resorption parameters are normal in these latter mice (Tam *et al.*, 2006).

Surprisingly, the same mutation, this time in the C57Bl/6J genetic background, causes a low bone mass phenotype associated with a decrease in bone formation rate and, in females only, an increase in osteoclast number (Tam *et al.,* 2006). The bases of this difference of phenotype between strains of mice are still unclear. Four single nucleotide polymorphisms (SNPs) have been identified in the human CB1 gene but no statistically significant differences of allele or genotype distributions were found between postmenopausal osteoporotic patients and age- and sex-matched controls (Karsak *et al.,* 2005).

In contrast, CB2 appears to be involved in regulating bone remodeling in both rodent and human species. CB2-deficient mice display a low bone mass phenotype characterized by an increase in bone formation rate as well as a sharp increase in osteoclast number (Ofek *et al.,* 2006). In a population of 168 postmenopausal osteoporotic women and 220 ethnically, age- and sex-matched controls, several CB2 SNPs show a significant difference of allele frequencies between cases and controls (Karsak *et al.,* 2005). These results are consistent with an involvement of the CB2 locus in human osteoporosis.

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Chapter 49

Insulin-like Growth Factors and the IGF Binding Proteins: Implications for Bone Biology

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INTRODUCTION

It has been fifty years since Salmon and Daughaday first reported the presence of a soluble factor induced by growth hormone (GH) that had insulin-like properties and mediated somatic growth. The subsequent identification of the insulin-like growth factors (IGFs) has led to remarkable discoveries and therapeutic advances, particularly in relation to the skeleton. IGFs are 7 kDa peptides that are ubiquitous in nature and regulate cell growth and death (Daughaday *et al*., 1972). Outside the liver, bone is the richest source of IGF-I and IGF-II in the mammalian body, and these 'local' IGFs play a predominant role in skeletal modeling and acquisition. IGFs are also highly conserved across species; hence the physiology of these peptides can be studied in numerous animal models. Although GH has been recognized as the major determinant of circulating IGF-I, the balance between skeletal and serum IGF-I has also been clarified, particularly with the relatively recent introduction of genomic engineering.

The role of the GH/IGF system in aging has undergone intense scrutiny in the last two decades. Biological aging is a normal physiological process, part of the continuum from growth to death. Like other organ systems, skeletal homeostasis is maximized during the second and third decades of life, and IGFs are essential for that process. However, trabecular bone loss also begins by the third decade, even though we still do not understand how this happens. The hypothalamic-pituitary axis is profoundly affected by aging. GH secretion is reduced, resulting in lower levels of circulating IGF-I and IGF-II, but higher levels of IGF binding proteins, IGFBP-1, -2, and -4 (Kelijman, 1991; Rudman *et al*., 1981). Early attempts to link age-related bone loss to a suppressed GH-IGF-I axis, or enhanced IGFBP expression, spawned considerable interest in GH and/or IGF-I as therapeutic tools for osteoporosis. The advent of recombinant gene technology propelled synthetic growth factors into an ever-expanding therapeutic domain, particularly in regard to short stature. Another therapeutic venue for GH has been in its potential utility for the frail elderly. The paradigm that a "somatopause," or GH deficiency state with aging, produces discrete musculoskeletal changes, has never been firmly defined, nor whether these changes can be reversed with GH. On the other hand, GH is indicated for individuals with pituitary insufficiency, and long-term studies suggest a major benefit can be realized in respect to bone mineral density (BMD) and quality of life. Regardless, more attention has focused on understanding the IGF regulatory system (i.e., IGFs, IGFBPs, Type I, II IGFR, and proteases that cleave IGFBPs) in relation to both bone acquisition and maintenance (Rudman *et al*., 1990). In this chapter, we will review the cellular and systemic actions of the IGFs, in order to more fully understand the functional integration of IGF regulatory components with other skeletal and systemic factors. As such, a thorough overview of the physiology of the IGF system, from GH to receptor signaling, is warranted.

THE IGF REGULATORY SYSTEM AND ITS RELATIONSHIP TO THE SKELETON

IGF-I, -II, and Their Receptors (IGFRs)

The IGFs are single-chain polypeptides. IGF-I consists of 70 amino-acid residues and IGF-II has 67 amino acids.

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They have B, C, and A domains similar to proinsulin, but a D domain that is not found in proinsulin. This D domain may sterically hinder the interaction of the IGFs with the insulin receptor (IR), leading to only weak ligand binding of the IGFs to the IR. A number of post-transcriptional and post-translational variants of the IGFs have also been described (Slootweg *et al.*, 1990). These IGFs have variable affinities for IGFBPs and the IGFRs. *In vitro,* these growth factors may have significantly greater activity than native IGF-I or IGF-II, especially those that exhibit weak binding to the IGFBPs.

IGF-I and IGF-II differ in their abilities to promote tissue growth due in part to the presence of distinct IGFRS, IGF-IR and IGF-IIR (Le Roith *et al.*, 1997) (see Fig. 1). IGF-IR is a tetramer consisting of two identical extracellular α-subunits (conferring ligand binding specificity) and two identical transmembrane β-subunits (possessing tyrosine kinase activity). IGF-IR resembles the IR, and shares amino acid sequence homology (Zeslawski *et al.*, 2001). IGF-II and insulin also bind to the IGF-IR but with 2- to 15-fold and 1,000-fold lower affinities, respectively (D'Ercole, 1996). It has an intrinsic tyrosine kinase activity critical for specific second message generation and indeed, ligand binding to the extracellular domain of the IGF-IR results in autophosphorylation and tyrosine phosphorylation of IGF-IR substrates. Tyrosine-phosphorylated IRS-1 and SHC bind different effector proteins involved in interconnecting pathways, including Ras/Raf-1/mitogen-activated protein kinase (MAPK, also known as extracellular signal-regulated kinase, ERK) and phosphatidylinositol 3-kinase (PI3K)/ phosphoinositide-dependent kinase-1 (PDK-1)/Akt (Maroni *et al.*, 2004). Activation of the Ras/Raf-1/MAPK pathway is considered to be critical for cell proliferation whereas the PI3K/PDK-1/Akt pathway is considered to be important for cell survival. The protein encoded by the phosphatase and tensin homologue gene deleted on chromosome 10 (PTEN) is a lipid phosphatase that decreases the activation of Akt by dephosphorylating phosphatidylinositol-3,4,5-triphosphate (PIP3) and phosphatidylinositol-3,4-biphosphate (PIP2). Therefore, PTEN acts as an off switch for the PI3K/PDK-1/Akt pathway as well as a tumor suppressor. This tumor suppressor gene is deleted or mutated in various types of human cancers (Zhao *et al.*, 2004; Sansal *et al.*, 2004). On the other hand, the monomeric IGF type II receptor (IGF-IIR) does not bind insulin but binds IGF-II, with a 500-fold increased affinity compared to IGF-I (Khandwala, 2000).

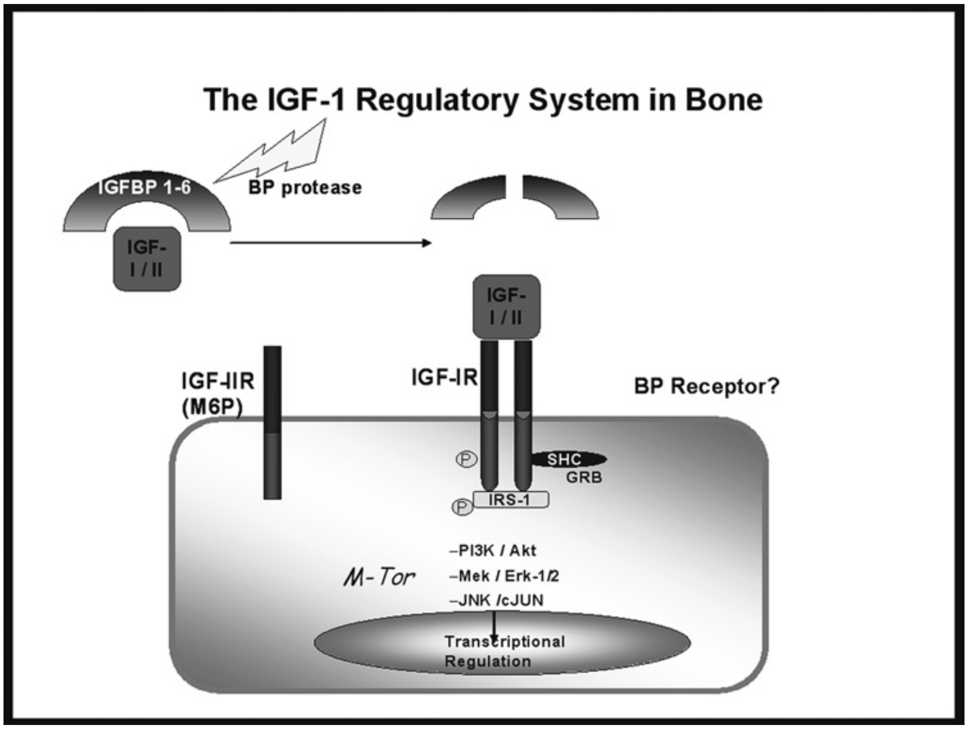


FIGURE 1 The IGF regulatory system contributes to bone formation through activation of the IGF type I receptor on osteoblasts. The downstream signaling is via three pathways 1-PI3 kinase/Akt/mTor, 2-MAP kinase/ERK, and 3-JNK/Jun. PI3K/mTor regulates cell differentiation, while the MAP/ERK and JNK/Jun impact cell growth. The IGFBPs carry IGFs to the receptor; several proteases can cleave IGFs for binding to the type I receptor. The type II IGF receptor can bind IGF-II and generally is a clearance mechanism for IGF-II and serves also as mannose 6 phosphate binding site. Finally, IGFBP receptors have been theorized as possible IGF independent signaling factors but none have been proven. However, it is likely that IGFBPs could bind to other receptors (e.g., EGF) and affect cell growth and differentiation.

The IGF-IIR exhibits no intrinsic kinase activity but is structurally very similar to the mannose 6-phosphate receptor, which is involved in targeting lysosomal enzymes intracellularly (Le Roith *et al.*, 1997; Sell *et al.*, 1995). Interestingly, it is now clear that in most tissues, except the liver, there are hybrid IGF-IR/IR receptors leading to significant cross-utilization between two rather distinct peptides. Hybrid receptors may also explain the growth-promoting activity of insulin, even in skeletal tissue, as well as the hypoglycemic effects of recombinant human IGF-I (rhIGF-I) when administered parenterally.

The IGFs possess tremendous growth potential as endocrine, autocrine, and paracrine factors. However, adequate nutrition is required for the full expression of IGFs' biologic activity, including its critical role in linear growth. For example, during states of malnutrition, GH production increases but hepatic IGF-I generation is severely impaired. Resistance at the hepatic GH receptor (GHR) reduces serum IGF-I and impairs GH bioactivity. The mechanism surrounding these nutrient-sensitive changes has not been clarified although message stability is clearly reduced by undernutrition. For malnourished children, the result is cessation of linear growth. GH resistance, to lesser degrees, occurs in other conditions such as diabetes mellitus, acute catabolic stresses, and renal insufficiency.

IGFs are produced in virtually every tissue (Rosen *et al.*, 1994). However, the main source of circulating IGFs is the liver. Other sources of IGF-I include the skeleton and adipose tissue (Rosen *et al.*, 1994; Mohan *et al.*, 1990). Together these three sites contribute more than 95% to the circulating IGF-I pool. With acute or chronic hepatic insufficiency, both serum IGF-I and IGF-II levels are markedly decreased. In the circulation, IGFs are bound to serum IGFBPs, with a relatively small but detectable amount of 'free' IGF-I, which does circulate, but has a very short half-life. GH treatment increases total and free IGF-I levels in a dose-dependent manner, but rhIGF-I increases 'free' IGF-I considerably more than rhGH. The clinical significance of increases in 'free' IGF-I, versus that bound to the IGFBPs is not known.

The distribution of IGFs in the serum pool is determined by the relative saturation of the IGFBPs. This may explain why treatment with IGF-I may have different tissue effects than treatment with GH. Infusions of IGF-I produce a transient rise in free IGF-I and suppression of IGF-II, insulin, and endogenous GH (Ebeling *et al.*, 1993). During the course of an IGF-I infusion, however, IGF-I is partitioned into several pools. This is due to the unsaturated nature of the lower molecular weight IGFBPs, and the presence of a large (150-kDa) circulating ternary IGF binding complex. This complex, composed of IGF-I (or -II), IGFBP-3 (or IGFBP-5), and an acid labile subunit (ALS), is the major circulatory reservoir for both IGFs. Normally, the majority of circulating IGF is bound to this saturated intravascular complex. However, with rapid IGF-I infusions, some IGF-I goes into the lower (50-kDa) unsaturated IGFBP fractions where transport into the extravascular space is possible. Partitioning of IGFs into various binding pools is critical to the biologic activity of both GH and IGF-I.

IGF-Binding Proteins (IGFBPs)

In the circulation and in tissues there are six IGFBPs. IGFBPs 1–6 belong to the same gene family and several features distinguish these known IGFBPs from one other (Rajaram *et al.*, 1997). Just as the IGFBPs serve important regulatory functions within the circulation, their role at the tissue level is also critical for the full biologic expression of IGFs. Binding of IGFBPs to IGFs normally blocks the interaction between IGFs and IGF-IR and consequently suppresses IGF actions (Kelley *et al.*, 1996; Collett-Solberg *et al.*, 1996). Nevertheless, binding of IGFBPs to IGFs can also protect IGFs from proteolytic degradation, and consequently enhances the IGF actions by augmenting their bioavailability in local tissues (Kelley *et al.*, 1996; Collett-Solberg *et al.*, 1996). Therefore, most of the IGFBPs function in a bipotential manner, and their impacts on IGFs depend to a large extent on the post-translational modification of IGFBPs by phosphorylation and proteolysis (Kelley *et al.*, 1996; Collett-Solberg *et al.*, 1996; Coverley *et al.*, 1997; Claussen *et al.*, 1997). Some IGFBPs also exhibit IGF-independent effects (Kelley *et al.*, 1996; Mohan *et al.*,1996).

The predominant binding protein in serum (and bone) is IGFBP-3, a 43-kDa glycosylated peptide. It is present in large concentrations in the serum and is easily measurable by radioimmunoassay (RIA). As noted earlier, IGFBP-3 is part of a larger saturated ternary complex including IGF-I (or -II) and an 80-kDa ALS. The association of these three proteins requires the presence of either IGF-I or IGF-II. In turn, this complex prolongs the half-life of the IGFs and provides a unique storage site.

The level of circulating IGFBP-3 is principally controlled by GH (Martin *et al.*, 1988). However, IGFBP-3 synthesis outside of the liver is regulated by other endocrine and paracrine factors. At a cellular level, IGFBP-3 has stimulatory or inhibitory effects on IGF-I depending on both the cell type and the physiologic milieu. IGFBP-3 action at the cell is characterized by its interaction with IGF-I or -II. *In vitro,* coincubation of IGFBP-3 with IGF-I can block IGF access to the IGF-IR (Rosen *et al.*, 1994; Conover, 1991). Conversely, preincubation of IGFBP-3 in certain cell systems facilitates receptor binding of the ligand by attaching to the cell membrane at a site remote from the receptor. In addition, very recent data suggest that IGFBP-3 may have IGF-independent actions on cell action. Although a putative IGFBP-3 receptor has not been cloned, IGFBP-3 has been shown to downregulate cell proliferation in certain cell lines and to enhance p53 production.

Further regulation of IGF-I by IGFBP-3 can occur in the extracellular space if IGFBP-3 undergoes proteolysis. Enzymatic degradation of IGFBP-3 produces low-molecular-weight IGFBP-3 fragments that differ in their affinities for the IGFs (Cohen *et al.*, 1992; Mohan, 1993). There are numerous IGFBP-3 proteases, which are produced by various cell types, that can be found in the intra- and extravascular space, and are regulated by both endocrine and paracrine factors. Prostate-specific antigen (PSA) is a serine protease that cleaves IGFBP-3 and may be important in defining skeletal metastases with prostate cancer (Nunn *et al.*, 1997).

IGFBP-1, -2, -4, and -5 are also important systemic and local regulators of IGF bioactivity. In contrast to IGFBP-3, these IGFBPs are not fully saturated and they easily translocate from the circulation into the extracellular space. IGFBP-1 is a 30-kDa peptide produced primarily in the liver. Serum IGFBP-1 levels correlate inversely with circulating insulin and, in poorly controlled insulin-dependent diabetes mellitus (IDDM), serum IGFBP-1 levels are quite high (Brismar *et al.*, 1988). Hepatic IGFBP-1 production is tightly regulated by insulin and substrate availability. However, unsaturated IGFBP-1 could also serve as a reservoir of binding activity for unbound IGF or as the initial binding site for cell-secreted IGF, prior to transfer to the more stable, GH-dependent 150-kDa complex. Shifts in the levels of IGFBP-1 may alter the distribution of the IGFs among the other IGFBPs and thus affect the relative distribution of the IGFs between the intra- and extravascular space. This mechanism could be critical in controlling metabolic and mitogenic activities of the IGFs (Arany *et al.*, 1994). In relation to the skeleton there is some *in vitro* suggestion that IGFBP-1 is synthesized by osteoblast and could inhibit IGF actions in bone during states of high IGFBP-1 production, such as starvation, and IDDM.

Human IGFBP-2 is a 31-kDa protein that preferentially binds to IGF-II (Christiansen *et al.*, 1991). It is the major IGFBP in cerebrospinal fluid and is likely produced by neural cells. Insulin and dexamethasone have been shown to decrease production of IGFBP-2 in rat osteoblasts (Schmid *et al.*, 1992). Excess rhIGFBP-2 inhibits IGF-I stimulated bone cell proliferation, bone collagen synthesis, and bone formation. Skeletal concentrations of IGFBP-2 are not nearly as high as IGFBP-3, -4, or -5 levels. Interestingly, IGFBP-2 concentrations increase with GHD and malnutrition as well as during aging. These changes are likely associated with its inhibitory properties. Yet, in puberty, there is a spike in IGFBP-2 production, predominantly from the liver. Khosla *et al.* (1998) reported that pro-IGF-II coupled to IGFBP-2 is present in the circulation of patients with osteosclerosis due to hepatitis C infections, suggesting that IGFBP-2 may have a permissive role in enhancing skeletal turnover, and in binding, through its heparin binding domain, to extracellular matrices. In addition, animal studies using IGF-II and IGFBP-2 in a complex have demonstrated that this combination can prevent disuse- and ovariectomy-induced bone loss (Conover *et al.*, 2002). Recently, IGFBP-2 null mice have been shown to have low bone mass as well.

IGFBP-4 is a glycosylated 24-kDa binding protein. It is one IGFBP that is consistently inhibitory for the IGFs in numerous cell systems. It was originally isolated from skeletal tissue and was found to inhibit IGF-mediated bone cell proliferation (Mohan *et al.*, 1989; Scharla *et al.*, 1993). The expression of IGFBP-4 in bone cells is regulated by cyclic adenosine monophosphate (AMP), parathyroid hormone (PTH), and 1,25-dihydroxyvitamin D. In addition, IGF-I stimulates IGFBP-4 proteolysis through the target enzyme, pregnancy-associated plasma protein-A (PAPP-A), thereby providing an autocrine/paracrine loop between the ligand and its binding protein (Durham *et al.*, 1994). PAPP-A deficient mice have been shown to have a growth phenotype with a reduced body size and a low bone mass, suggesting a complex role for this autocrine-paracrine loop, particularly in the skeleton.

Preliminary evidence suggests that circulating levels of IGFBP-4 may reflect local bone cell regulation. Rosen *et al.* (1992) have shown high serum levels of a 24-kDa IGFBP (likely to be IGFBP-4) in elderly women with hip and spine fractures. The relative ligand binding of IGFBP-4 in serum from osteoporotic women is closely correlated with circulating concentrations of PTH, suggesting that serum changes mirrored local skeletal activity (Rosen *et al.*, 1992). More recent data from Mohan *et al.*, utilizing a specific RIA for IGFBP-4, have shown an age-related increase in this binding protein and a relatively strong correlation between PTH and IGFBP-4 (Honda *et al.*, 1996). The age-related changes in IGFBP-4 have been confirmed in a larger study from the Framingham cohort, although those investigators did not find a relationship between PTH and IGFBP-4 (Karasik *et al.*, 2003). More recently, it has been reported that IGFBP-4 null mice are smaller and have a reduced BMD, suggesting IGFBP-4 may serve as a reservoir for IGF-I and that the relative proportions of IGF-I and IGFBP-4 are critical to define its function.

IGFBP-5 is a nonglycosylated 31-kDa IGFBP produced by osteoblasts and numerous other cell types. It is found in relatively high concentrations, both in bone and in serum, where it can be measured by RIA (Nicolas *et al.*, 1994). IGFBP-5 is the most abundant IGFBP stored in the bone, where it is bound to hydroxyapatite and extracellular matrix proteins (Nicolas *et al.*, 1995; Mohan *et al.*, 1995). IGFBP-5 is the only IGFBP that has been shown to consistently stimulate osteoblast cell proliferation *in vitro*, thus increasing the number of osteoblasts. Recent studies suggest that the mitogenic effects of IGFBP-5 may in part be independent of IGFs and mediated through rhIGFBP-5's own signal transduction pathway (Mohan *et al.*, 1995; Andress *et al.*, 1991; Schmid *et al.*, 1996; Slootweg *et al.*, 1996; Richman *et al.*, 1999). IGFBP-5 has the unique capacity to bind extracellular matrices, particularly hydroxyapatite. IGFBP-5 can also bind the ALS and IGF-I/IGF-II in the circulation, providing an alternative ternary complex. *In vitro,* IGFBP-5 enhances IGF bioactivity, especially in bone. Yet, *in vivo*, IGFBP-5 action can be either inhibitory or stimulatory depending on the relative concentration of IGF-I and its mode of administration (Salih *et al.*, 2005). Its synthesis is increased by PTH and other cyclic AMP analogs (Conover *et al.*, 1993). Intact IGFBP-5 can be found circulating in the extracellular space, attached to extracellular matrices, or cleaved into lower molecular weight protein fragments. Intact IGFBP-5's major role in the skeleton may be as a storage component for the IGFs because both IGF-I and IGF-II have very low binding affinity for hydroxyapatite, but bind avidly to IGFBP-5 (Rosen *et al.*, 1994). During remodeling, resorption enhances proteolytic cleavage of IGFBP-5. In addition, during formation and mineralization, synthesis and release of IGFBP-5 by bone cells facilitates attachment of IGFs to the newly mineralized matrix (Koutsilieris *et al.*, 1992).

IGFBPs are composed of three domains of approximately equal sizes (Baxter, 2000). The amino- and carboxyl-terminal domains are each internally disulfide-linked and share a high degree of sequence homology across the family (Neumann *et al.*, 1999). However, little homology is exhibited among their central L-domains. The disulfide linkages of the amino-domain of IGFBP-6 differ from those of other IGFBPs, whereas the carboxyl-domain disulfides are the same for all IGFBPs so far studied (Neumann *et al.*, 1999; Forbes *et al.*, 1998; Chelius *et al.*, 2001). IGFBP-6 differs functionally from other IGFBPs in binding IGF-II with 20- to 100-fold higher affinity than IGF-I, whereas IGFBPs 1–5 do not have a marked IGF binding preference (Headey *et al.*, 2004). Therefore, IGFBP-6 constitutes a relatively specific IGF-II inhibitor (Bach, 1999).

Proteases that Cleave IGFBPs

IGF bioactivity is regulated transcriptionally by hormones and paracrine factors. Tissue activity of the IGFs is also controlled by their respective binding proteins. Tissue specific proteases provide another form of regulation of IGFs at a post-translational level. Binding protein targeted proteases have been identified in serum and in various tissues including bone. These proteases alter the binding capacity of IGFs for the IGFBPs, thereby freeing the IGFs to bind to their respective IGFRs (Rosen, C. J. 1994). PSA and PAPP-A represent two of the most important circulating serine proteases.

Bone is a rich source of binding protein proteases. Proteases that cleave IGFBP play a pivotal role in determining the regulatory effects of IGFBPs on IGF actions. There are three categories of known proteases that proteolyze IGFBPs: 1) kallikreins; 2) neutral and acid-activated cathepsins; and 3) matrix metalloproteinases (MMPs) (Cohen et al., 1992; Cohen *et al.,* 1994; Conover *et al.,* 1995; Fowlkes *et al.,* 1994a). PSA is the first serine kallikrein IGFBP protease found to proteolyze both IGFBP-3 and IGFBP-5 into several lower molecular weight fragments and is regulated at least to some extent by testosterone and other androgens (Collett-Solberg *et al.*, 1996; Cohen *et al.*, 1992; Koutsilieris *et al.*, 1992). Its role in mediating the enhanced bone formation found in the lumbar spine of metastatic prostate cancer patients remains to be determined. However, it is likely to be one of several mechanisms whereby IGFs can stimulate mitogenic activity of both cancer and bone cells.

The concept of 'soil and seed' implies that the inherent bioactivity of the IGFs, whether bound or free, could stimulate growth of neoplastic tissue after there is homing of cancer cells to bone. Further studies are underway to define the nature of skeletal IGFs, the IGFBPs, and the proteases in this process, and the relationship of these to underlying morbidity associated with metastatic bone disease. A kallikrein-like IGFBP protease, 7S γ-subunit nerve growth factor, is found to cleave both IGFBP-4 and IGFBP-6, Cathepsin D is an acid-activated lysosomal protease, which is able to cleave all the six IGFBPs. MMPs are able to proteolyze a spectrum of IGFBPs, including IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-5 (Collett-Solberg *et al.*, 1996; Fowlkes *et al.*, 1994b; Rajah *et al.*, 1996; Claussen *et al.*, 1997; Marinaro *et al.*, 1999; Braulke *et al.*, 1995; Manes *et al.*, 1999). PAPP-A, which is not specific for IGFBP-4, is activated by IGFs and is found within skeletal tissue as well as in other organs. Particularly for PAPP-A, it is clear that the IGFs can regulate tissue-specific proteases, thereby establishing a complex regulatory loop in which the ligand (i.e., IGF) controls its own bioavailability through transcriptional and nontranscriptional means (Arany *et al.*, 1994). Global deletions of PAPP-A result in smaller mice with reduced bone mass, while targeted transgenic overexpression of PAPP-A in muscle causes hypertrophy and increased body weight (BW). IGF-I or -II can regulate PAPP-A, but GH does not regulate PAPP-A or other proteases (Angelloz-Nicoud *et al.*, 1995; Skjaerbaek *et al.*, 1998). Insulin and estrogen may also affect IGFBP protease activities (Bereket *et al.*, 1995; Bang *et al.*, 1998; Kudo *et al.*, 1996).

The IGF System Relative to Lineage Determination

Bone formation and remodeling requires integration of multiple signaling pathways, which in turn regulate the activities of osteoblast lineage-specific master genes as well as their cellular substrates. Despite the importance of the IGFs in cell growth and maintenance (i.e., cell cycling and apoptosis), their precise role in osteoblast and osteoclast differentiation is still a matter of debate. In part this relates to the complex yet developmentally sensitive and finely orchestrated process that drives mesenchymal stem cells into the bone lineage. Not unexpectedly, this pathway involves multiple transcription factors and cytokines, as well as the IGFs. The most notable transcription factors that control osteoblast fate are runt-related transcription factor 2 (Runx2) and osterix (Osx). Runx2, core binding factor 1 (Cbfa1)/ polyoma enhancer binding protien 2a (Pebp2A) a transcription factor that belongs to the Runx family is the α subunit of a heterodimeric transcription factor, PEBP2/CBF, which is composed of α and β subunits (Ito, 1999). Runx2 is involved in osteoblast differentiation and bone formation. In particular, Runx2 is required for early commitment of mesenchymal stem cells into osteoprogenitors, and also functions later in osteoblast differentiation to regulate the formation of the extracellular matrix (Ducy *et al.*, 1999). IGF-I can be shown to influence Runx2 through IGF-1 activation of PI3K, Pak1, and ERK (Xiao *et al.*, 2000; Qiao *et al.*, 2004).

Schnurri-3 (Shn3), a large zinc finger protein that functions as an adapter protein in the immune system (Oukka *et al.*, 2002), has been shown to control the protein expression level of Runx2 through promoting Runx2 degradation by recruitment of the WW domain containing E3 ubiquitin ligase1 (WWP1) to Runx2. It is a novel regulator of postnatal bone mass. Mice lacking Shn3 display adult-onset osteosclerosis with increased bone mass due to an augmented osteoblast activity (Jones *et al.*, 2006). Further studies are needed to elucidate whether IGF-I could influence Shn3 expression, or, could influence the Shn3 control of Runx2 degradation.

Osx is a master zinc-finger-containing transcription factor of osteoblast lineage progression that is highly specific to osteoblasts *in vivo*, which acts downstream of bone morphogenetic protein (BMP)-2 Smad signaling (Xiao *et al.*, 2000; Qiao *et al.*, 2004; Nakashima *et al.*, 2002). The Osx amino acid sequence predicts three C2H2-type zinc fingers that have a high degree of identity to similar DNA-binding domains in the transcription factors Sp1, Sp3, and Sp4. The expression of Osx is more specific to osteoblasts than Runx2 (Nakashima *et al.*, 2003). Because no Osx transcripts are detected in skeletal elements of Runx2-null mice, Osx must be downstream of Runx2 in the pathway of osteoblast differentiation (Nakashima *et al.*, 2002). Both IGF-I and BMP-2 are shown to upregulate Osx expression during early osteoblast differentiation (Celil *et al.*, 2005). In mesenchymal stem cells, it appears that both MAPK and PKD signaling pathways serve as points of convergence for mediating the IGF-I- and BMP-2-induced effects on Osx expression (Celil *et al.*, 2005). IGF-I-mediated Osx expression required all three MAPK components (Erk, p38, and JNK), whereas BMP-2 required p38 and JNK signaling, and the synergistic interactions of BMP-2 and IGF-I were also disrupted by PKD inhibition (Celil *et al.*, 2005).

It should be noted that Runx2-independent pathways of ossification may exist, including 1) the Wnt signaling pathway; 2) Msx2-dependent vascular ossification pathway; and 3) Osx induction via Dlx5, a homeobox transcription factor, which acts downstream of BMP-2 (Kato *et al.*, 2002; Cheng *et al.*, 2003; Lee *et al.*, 2003). These studies suggest additional pathways may act in parallel to, or independent of, Runx2 to regulate Osx expression during osteogenic lineage progression. Indeed, in the canonical Wnt signaling pathway, it is becoming apparent that there is some crosstalk with the IGF pathway. For example, β-catenin binds to insulin receptor substrate-1 (IRS-1), as well as other factors, to enhance its transport from the cytoplasm into the nucleus where it can affect a whole series of downstream target genes.

In sum, IGFs can serve to enhance differentiation of mesenchymal stem cells. Their mitogenic role in the skeleton is relatively limited. However, IGF-I may also be important in lineage allocation or in promoting adipogenesis within the marrow niche independent of key transcription factors. Moreover, IGF-I is essential for adipogenic differentiation and likely is downstream of peroxisome proliferator-activated receptor gamma (PPARG) activation. Because mesenchymal stem cells can enter several distinct lineages, the precise timing of IGF-I action and other factors, some undefined, that are active in the marrow niche, ultimately determine cell fate (Fig. 2). The details of this coordination have still not been defined.

PHYSIOLOGY OF THE GH/IGF/IGFBP SYSTEM

GHRH

In order to appreciate the complexity and redundancy inherent in the IGF regulatory system in bone, an overview of the physiologic aspects of IGF-I regulation is required. Although IGF-I is regulated by genetic, nutritional, hormonal (insulin and thyroxine), and environmental factors, growth hormone is the major controller of IGF-I expression in tissues and in the circulation. That process begins in the hypothalamus and ends at the tissue level (Fig. 3).

The regulation of GH secretion from the pituitary is complex and involves elaboration of discrete neurosecretory peptides from the hypothalamus. Hypothalamic-releasing factors were postulated to exist for more than five decades, but the exact structure of GH-releasing hormone (GHRH) was not elucidated until extracts of pancreatic islet cell tumors from two patients with ectopic acromegaly were characterized. Subsequently, two different GHRH peptides, one of 40 amino acids and the other of 44, were isolated from the hypothalamus.

GHRH is a hypothalamic hormone that is essential for normal expansion of the somatotrope lineage during pituitary development (Frohman *et al.*, 2002). GHRH, via binding to GHRH receptor (GHRHR), acts on somatotropes to

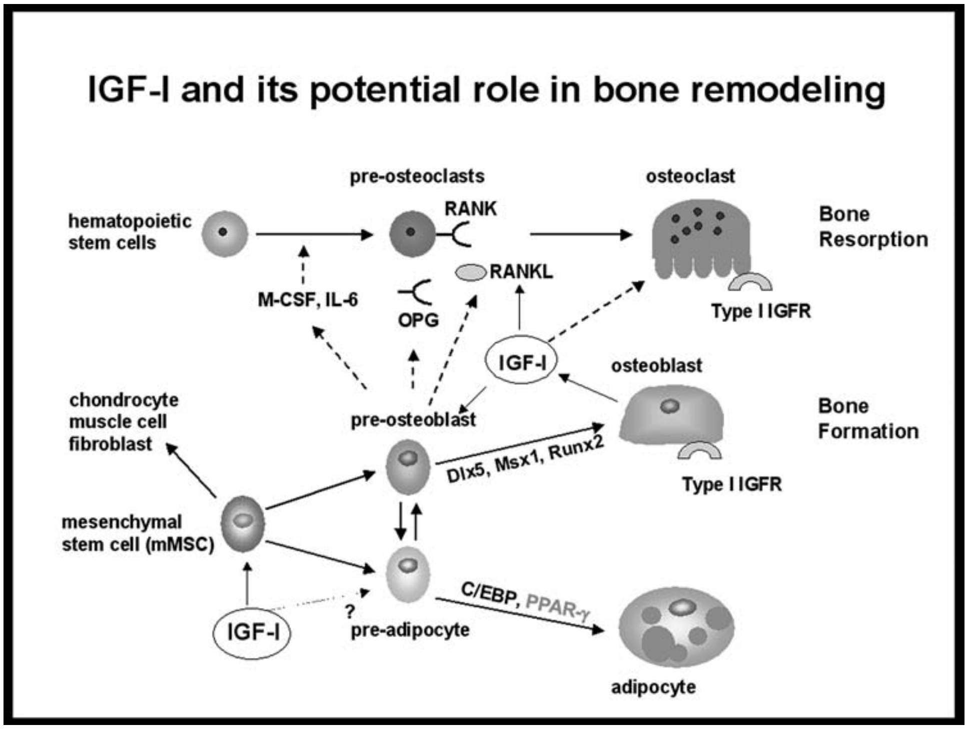
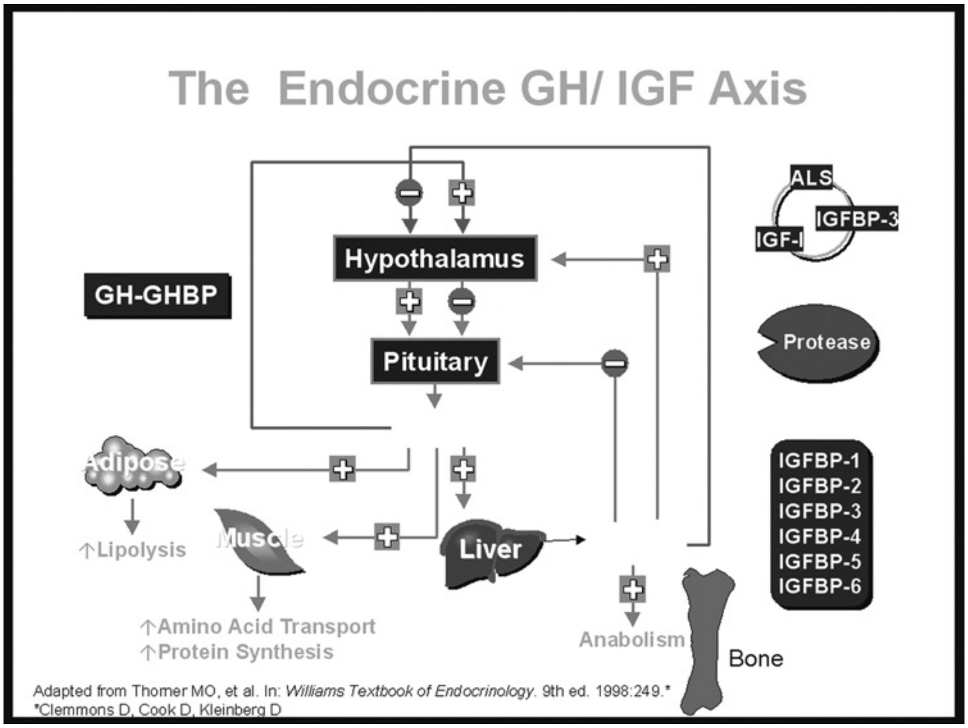

IGF-I and its potential role in bone remodeling
pre-osteoclasts
osteoclast
hematopoietic stem cells
RANK
Bone Resorption
RANKL
M-CSF, IL-6
OPG
Type I IGFR
IGF-I
osteoblast
chondrocyte
muscle cell
fibroblast
pre-osteoblast
Bone Formation
Dlx5, Msx1, Runx2
Type I IGFR
mesenchymal stem cell (mMSC)
?
IGF-I
pre-adipocyte
C/EBP, PPAR-γ
adipocyte

FIGURE 2 IGF-I serves as both a mitogen, albeit weak, and a differentiation factor for marrow stromal cells as they enter the osteoblast lineage. In addition, IGF-I can enhance osteoclast differentiation by stimulating RANKL production from stromal cells. There may be a direct effect of IGF-I on osteoclasts, because these cells express the type IGF-1 receptor and signal through IRS-2. IGF-I can also enhance adipocytes differentiation, and its role in promoting marrow adipogenesis is an area of recent investigations. Overall, IGF-I represents a coupling factor for bone remodeling.


The Endocrine GH/ IGF Axis
−
+
Hypothalamus
+
+
−
Pituitary
−
GH-GHBP
ALS
IGFBP-3
IGF-I
Protease
Adipose
+
+
↑Lipolysis
Muscle
+
Liver
↑Amino Acid Transport
↑Protein Synthesis
+
Anabolism
Bone
IGFBP-1
IGFBP-2
IGFBP-3
IGFBP-4
IGFBP-5
IGFBP-6

Adapted from Thorner MO, et al. In: *Williams Textbook of Endocrinology*. 9th ed. 1998:249.*
*Clemmons D, Cook D, Kleinberg D

FIGURE 3 IGF-I is an endocrine and paracrine/autocrine growth factor. It is regulated by GH via hypothalamic GHRH. The primary source of circulating IGF-I is liver, although bone is a very rich source of IGF-I and IGF-II. Target tissues for IGF endocrine action include fat, muscle, and bone. IGF binding proteins (IGFBPs) circulate in excess of IGFs, but there is a ternary circulating IGF complex that includes the acid labile subunit (ALS) and IGFBP-3. GHBP is growth hormone binding protein, a circulating GH receptor. The balance between circulating and skeletal IGF-I during growth and maintenance determines the overall effect of this peptide on bone.

increase GH biosynthesis and secretion, and is thought to cause somatotroph proliferation (Petersenn *et al.*, 2000). Release of GHRH is episodic, which accounts for the pulsatile release of GH from the pituitary. A decrease in GHRH is associated with somatotrope hypoplasia, whereas an increase in GHRH is associated with somatotrope hyperplasia (Frohman *et al.*, 2000).

GHRH is a potent stimulus for GH release, and synthetic analogs are now undergoing clinical trials for both diagnostic and therapeutic purposes in patients with hypothalamic-pituitary disorders. Preliminary investigations examining the utility of these GH-releasing peptides in elders have also recently been initiated.

GHRHR

GHRHR is a G-protein-coupled receptor with seven hydrophobic transmembrane domains. The human GHRHR gene spans 15 kb and is composed of 13 exons. The open reading frame was shown to extend 1269 bp and encodes a protein of 423 amino acids with a predicted molecular weight of 47-kDa. After release from the hypothalamus, GHRH binds to the GHRHR predominantly located on the pituitary somatotrope (Lin-Su *et al.*, 2002). GHRHR activation leads to the opening of a sodium channel in the somatotrope, which causes its depolarization. The resultant change in the intracellular voltage in turn opens a voltage-gated calcium channel, allowing for calcium influx, which directly causes the release of premade GH stored in secretory granules (Petersenn *et al.*, 2000). The cAMP elevation stimulates protein kinase A, which phosphorylates and activates the transcription factor cAMP response element binding protein (CREB), which then stimulates de novo GH production (Petersenn *et al.*, 2000; Muller *et al.*, 1999; Mayo *et al.*, 1995). Autosomal recessive mutations in the GHRHR can result in near total absence of GH, and lead to short stature in humans, and the *little* phenotype in mice.

Somatostatin

Somatostatin (SMS) is a small (14-amino-acid) but ubiquitous polypeptide that inhibits GH synthesis and release. In concert with GHRH, SMS regulates GH secretion through a dual control system; one stimulatory, the other inhibitory. Several molecular forms of somatostatin, distinct from the native 14-amino-acid peptide, have been isolated. In addition to inhibition of GH release, SMS also inhibits secretion of thyrotropin as well as several pancreatic hormones, including glucagon and insulin. The SMS receptor has been localized to various cell types, especially those of neuroendocrine origin. Localization of this receptor suggests that SMS acts as both an endocrine and a paracrine regulator in diverse tissues. A highly potent synthetic analog of SMS, octreotide, has been used therapeutically in acromegaly and diagnostically (in a radiolabeled form) for scintigraphic visualization of neuroendocrine tumors.

Secretion of GH from the Pituitary

Mechanism of GH Secretion

GH is an anabolic polypeptide hormone produced by the somatotropes of the anterior pituitary gland. GH is also lipolytic, which activates lipase and leads to mobilization of fat from the adipose tissue, and plays an important role in carbohydrate metabolism (Liu *et al.*, 2004; Heffernan *et al.*, 2001). Deletion of the GHR gene results in a small mouse with low IGF-I but enhanced insulin sensitivity (Liu *et al.*, 2004). GH and its receptor belong to the cytokine superfamily of receptors and ligands. GH is secreted under the influence of three hypothalamic hormones: 1) GHRH, which acts via the GHRHR 2) GHS (also known as Ghrelin), which acts through the GHS receptor; and 3) SMS, which acts on the pituitary to suppress basal and stimulated GH secretion, but is not believed to affect GH synthesis (Petersenn *et al.*, 2000; Lin-Su *et al.*, 2002).

GH secretion is pulsatile (due to the episodic release of GHRH) and circadian with the highest pulse amplitude occurring between 02:00 and 06:00 (Ho *et al.*, 1990). Puberty has a dramatic effect on the amplitude of GH pulses, due to changes in the hypothalamic milieu as a result of rising sex steroid concentrations (Jansson *et al.*, 1985). Through binding directly to GHR, GH has profound effects not only on linear bone growth, but also bone metabolism and bone mass. The GHR is found on the cell surface of osteoblasts and osteoclasts, but not on mature osteocytes (Andreassen *et al.*, 2001). Although GH can act on cells directly through specific receptors, most of its anabolic actions are mediated through IGF-I (Mathews *et al.*, 1988). GH stimulates the secretion of IGF-I, largely from the liver, which then acts in an endocrine fashion (Daughaday, 1989). GH also stimulates IGF-I locally in target tissues such as bone, where it may act in a paracrine or autocrine fashion. Perturbations of the GH-IGF-I axis may predispose to the development of osteoporosis.

Effects of Gonadal Status on GH-IGF-I Axis

Not surprisingly, the pattern of GH secretion in animals and humans depends highly on age and sex (Ho *et al.*, 1990; Jansson *et al.*, 1985). Both factors strongly influence the frequency and amplitude of GH pulses, which in turn determines GH basal secretory rates, and the levels of serum IGF-I. Characteristic changes during puberty in rats parallel pubertal changes in humans (Jansson *et al.*, 1985). GH secretion in male and female rats is identical after birth but at puberty, a sexually differentiated pattern of secretion appears with male rats displaying high-amplitude, low-frequency pulses, and with female rats displaying pulses of high frequency but low amplitude (Jansson *et al.*, 1985). This sexual dimorphism can be altered by manipulating the gonadal steroid environment, suggesting that sex steroids are important modulators of GH secretion.

In humans, sexual differences in GH secretion during puberty are less pronounced, even though administration of gonadal steroids to prepubertal children increases GH pulses and mimics the pubertal milieu of the hypothalamus. Various sampling techniques (profiles vs. stimulatory tests) and assays with different sensitivities have produced disparate findings. However, spontaneous and stimulated GH peaks in humans are enhanced during puberty. Matched for age and body mass index, young girls were found to have higher integrated GH concentrations (IGHC) than boys (Ho *et al.*, 1990). Other secretory characteristics, including pulse amplitude, frequency, and the fraction of GH secreted as pulses (FGHP), were similar in both sexes of the same age. In a preliminary study, black adolescents (males and females) had higher GH secretory rates than age-matched white adolescents (Wright *et al.*, 1995). Higher GH secretion rates in adolescent blacks could lead to a greater acquisition of bone mass.

Gonadal steroids affect GH secretion in perimenopausal women. IGHC mean pulse amplitude of GH and FGHP are lower in older women than premenopausal women. GH secretory indices in postmenopausal women correlate with serum estradiol, but not with total serum androgen levels. During menopause, GH secretion is reduced (Ho *et al.*, 1990). However, oral administration of estradiol (or conjugated equine estrogens) increases GH secretion as a result of reduced hepatic generation of IGF-I (Ho *et al.*, 1990; Dawson-Hughes *et al.*, 1986). On the other hand, transdermal administration of 17-β estradiol increases serum IGF-I concentration, suggesting that suppression of IGF-I by oral estrogens is due to a "first-pass" hepatic effect. Impaired IGF-I generation in the liver removes a key component of negative feedback on the hypothalamus, resulting in increased GH release.

Effects of Age on the GH/IGF-I/IGFBP System

The GH-IGF-I axis undergoes changes over a life span so that elders have lower spontaneous GH secretion rates and serum IGF-I levels than younger people (Kelijman, 1991; Rudman *et al.*, 1981; Donahue *et al.*, 1990). Most of these age-related differences are a function of an altered hypothalamic-pituitary set point due in part to changes in lifestyle and nutrition. The GH secretory response to common stimuli such as GHRH, clonidine, L-dopa, physostigmine, pyridostigmine, hypoglycemia, and met-enkephalin, but not arginine, is reduced by aging. Somatotrope responsiveness to GHRH and arginine does not vary with age, implying that the maximal secretory capacity of somatotropic cells is preserved in elderly people (Corpas *et al.*, 1993).

In sum, circulating IGF-I concentrations decline with advancing age as a result of reduced GH secretion, lower energy intake, altered gonadal status, and other factors. These changes have been causally linked to the frailty of aging in respect to musculoskeletal function. However, large cross-sectional studies have demonstrated only a weak association (i.e., correlation coefficient of $r = 0.2–0.4$) between diminished serum IGF-I and age-related bone loss, or between serum IGF-I and BMD (Rudman *et al.*, 1981; Nicolas *et al.*, 1994; Donahue *et al.*, 1990; Corpas *et al.*, 1993; Langlois *et al.*, 1998). In one large cohort study, the lowest serum IGF-I quartile was associated with a significantly greater risk of hip fractures (Garnero *et al.*, 2000). On the other hand, skeletal concentrations of IGF-I, IGF-II, and IGFBP-5 in femoral cortical and trabecular bone decline significantly with age and these have been associated with low BMD (Rudman *et al.*, 1981; Nicolas *et al.*, 1994; Donahue *et al.*, 1990; Corpas *et al.*, 1993; Langlois *et al.*, 1998). In contrast to the multitude of studies linking serum IGF-I to age-related frailty and muscle performance, differences in GH secretion are difficult to determine due to its normal pulsatility. There is only one study in older postmenopausal women relating changes in 24-hour GH levels with BMD (Dennison *et al.*, 2003).

GH exerts a multitude of biological effects on various tissues through the GHR. Regulation of GH bioactivity occurs at several pre- and postreceptor levels. GHBP is a plasma binding protein identical to the extracellular domain of the tissue GHR. GHBP binds exclusively to GH and most, if not all serum GH, is bound to this carrier protein. Measurements of GHBP in serum are relatively stable and reflect the endogenous status of the GH receptor in responsive tissues (Baumann *et al.*, 1989). With advanced age, GHBP concentrations increase substantially.

GH/IGF ACTIONS ON THE SKELETON

GH-IGF-I Effects on Longitudinal Growth

GH has distinct effects on the skeleton in terms of both linear growth and bone remodeling. However, it has been extremely difficult to ascertain a role for IGF-I independent of GH. The interaction of GH and IGF-I in bone during growth is complex and has been labeled a "dual-effector" process. Indeed, in double GH/IGF-I knockout mice there is significant growth retardation suggesting that each of these two growth factors contribute equally but distinctly to longitudinal growth (Lupu *et al.*, 2001). Interestingly, in those studies, only 17% of the growth in these mice could be attributed to non-GH and non IGF-I determinants (Lupu *et al.*, 2001).

Longitudinal growth results from the activity of GH on the skeleton, particularly at the cartilaginous growth plate. In human bone, proliferating chondrocytes express IGF-IRs and are responsive to paracrine IGFs secreted by differentiated cartilage cells (Isaksson *et al.*, 1987). The target for GH in the growth plate is the differentiated chondrocyte that synthesizes IGF-I in response to GH. Proliferating chondrocytes respond to locally produced IGF-I by differentiation, which in turn leads to cartilage expansion and linear growth. Thus, GH's stimulatory properties on the endochondral growth plate are mediated by induction of IGF-I.

GH may have its own effect on linear growth, independent of IGF-I. For example, GH stimulates longitudinal bone growth in normal rats but rhIGF-I does not (Schmid *et al*., 1991). Similarly, transgenic mice that overexpress GH grow to twice their normal size, even though administration of IGF-I to normal mice does not provoke a similar growth response. These effects are almost certainly a result of distinct GHRs on osteoblasts and marrow stromal cells. The presence of both GHR and IGFRs on bone cells complicate interpretation of GH's action but recent Cre/loxP technology for targeted *in vivo* and *in vitro* deletion or overexpression has allowed investigators to clarify the anabolic activity of GH and IGF-I in bone. *In vitro,* GH stimulates osteoblastic proliferation, differentiation, and matrix mineralization (Ernst M *et al*., 1990; Maor *et al*., 1989). GH also induces the synthesis and release of IGFBP-3, -4, and -5 in rodent calvarial cells. The response to GH in human bone cells (hOB) is very dependent on specific cell culture conditions. For subconfluent cultures, GH stimulates cell proliferation, while in confluent hOB cultures, GH induces cell differentiation (as measured by cell alkaline phosphatase and procollagen type I propeptide synthesis) (Kassem *et al*., 1993).

GH and IGF-I Actions on the Skeleton: Local vs. Systemic

Local Skeletal Actions of the GH/IGF-I Axis

The effects of GH on bone remodeling are complex, in part because there are both local and circulating IGF-I complexes directly induced by GH. Several *in vivo* studies have shown that skeletal IGFs are critical in mediating the GH skeletal response. Remodeling is the sum of several distinct events beginning with activation of lining cells, followed by osteoclast recruitment, osteoblast differentiation, and osteocyte formation. Induction of IGF-I synthesis by GH in osteocytes, mature osteoblasts, or lining cells is one potential mechanism for activation of remodeling. Several lines of evidence showing that IGFs are critical to the remodeling sequence (and not just osteoblastic activity) are derived from clinical and basic studies. First, large quantities of growth factors [e.g., IGFs, transforming growth factor (TGF)-beta, fibroblast growth factors (FGFs)] are stored in bone and released during active resorption, suggesting that local growth factors could couple formation to resorption (Mohan *et al*., 1991). Second, IGFs stimulate the differentiation and activation of osteoclasts, possibly in concert with cytokines such as receptor activator of nuclear factor Kappa β ligand (RANKL), and macrophage colony-stimulating factor (M-CSF) (Slootweg *et al*., 1992; Mochizuki *et al*., 1992). Third, administration of IGF-I enhances bone formation and bone resorption to a similar degree (Ebeling *et al*., 1993). Fourth, bone marrow stromal cells that produce osteoclast-activating cytokines are also rich sources of IGFs and IGFBPs. Fifth, several proteases including PAPP-A and MMP-9 are physiologically active at low pH (Conover *et al*., 1994). The acidic pH necessary for protease activation is approximately the same pH present within the microenvironment of the osteoclast during its active proton secretion phase. This raises the possibility that bone resorption activates proteolytic cleavage, which permits the IGFs to become free of the IGFBPs, and allows for further recruitment of both osteoblasts and osteoclasts.

In vitro, GH, IGF-I, and IGF-II all have modest mitogenic effects on bone cell growth (Canalis *et al*., 1989; Mohan *et al*., 1988). This suggests that GH could act through the IGFs to activate skeletal remodeling. Indeed, GH-induced cell proliferation can be blocked by simultaneous addition of a specific monoclonal antibody to IGF-I (Mohan *et al*., 1991). IGF-II, on the other hand, stimulates mitogenesis independent of GH and even if high doses of IGF-I are coadministered. This implies that IGF-II could regulate osteoblastic proliferation via the IGF-IIR (Mohan, *et al*., 1989a). *In vitro*, both IGF-I and IGF-II are mitogenic to rodent preosteoblasts, and both rapidly increase mRNA expression of the proto-oncogene, c-*fos,* 20- to 40-fold in less than 30 minutes (Merriman *et al*., 1990). The IGFs also stimulate type I collagen synthesis, alkaline phosphatase activity, and osteocalcin in more differentiated human osteoblast-like cells (Mohan *et al*., 1989; Schmid *et al*., 1984). Taken together, the IGFs are important for osteoblast activity but the effects are almost certainly dose and time dependent.

Zhang *et al*. (2002) demonstrated the critical importance of IGF-I in the process of mineral apposition, which is a late osteoblast function. These investigators used a Cre/loxP system to delete the IGF-IR in mature osteoblasts and found that those mice with the deletion had a significant impairment in mineralization lag time. Similarly, targeted overexpression of IGF-I in osteoblasts resulted in mice with increased osteoblast function, but no change in the number of osteoblast precursors. These findings are reinforced by *in vitro* studies showing that IGF-I can increase type I collagen synthesis, alkaline phosphatase activity, and osteocalcin production in osteoblasts (Schmid *et al*., 1992). IGF-I also acts as a potent anti-apoptotic factor, particularly for differentiated osteoblasts and osteocytes, probably via the PI3's kinase signaling pathway. On the other hand, Rowe *et al.* used timed micro array technology with a green fluorescent protein (GFP) Col3.6 promoter to show that during marrow stromal cell recruitment, IGF-I expression declines, particularly as these cells finally enter the osteoblast lineage.

In addition to regulating osteoblast function, several groups have shown that skeletal IGF-I can stimulate osteoclast recruitment and differentiation either directly through the IGF-IR or via RANKL expression (Mochizuki *et al.*, 1992; Rubin *et al.*, 2002). This would place IGF-I in the category of a "coupler" for bone remodeling. Indeed, IGF-I null mice are growth retarded, and most die after birth. Those that survive are very small, have developmental defects in brain, muscle, bone, and lung, and are infertile. However, their skeletal phenotype is particularly striking and is characterized by very little cortical bone but an increased trabecular bone volume fraction (Bikle *et al.*, 2001; Bikle *et al.*, 2006). Recently, it has been demonstrated both *in vivo* and *in vitro* that the absence of IGF-I impairs osteoclast recruitment and activity, although the exact mechanism is unknown (Wang *et al.*, 2006).

Effects of Systemic GH-IGF-I Axis on the Skeleton

Although skeletal IGF-I is essential for bone growth, it is also apparent that the circulating IGF-I may play an important role in modulating skeletal development and acquisition. Liu *et al.* (1993) were the first to report that newborn mice homozygous for a targeted disruption of *Igf1* exhibit a growth deficiency similar in severity to that previously observed in viable *Igf2* null mutants (i.e., 60% of normal birth weight). Depending on the genetic background, Liu found that some of the *Igf1*(-/-) dwarfs died shortly after birth, whereas others survived and reached adulthood (Liu *et al.*, 1993). On the contrary, null mutants for the *Igf1R* gene die invariably at birth, of respiratory failure and exhibit a more severe growth deficiency (45% normal size) compared to wild-type animals. In addition to generalized organ hypoplasia in *IgfR*(-/-) embryos, including the muscles and developmental delays in ossification, deviations from normalcy were found in the central nervous system as well as epidermis. *Igf1* -/- /*Igf1R*(-/-) double mutants did not differ in phenotype from *Igf1R*(-/-) single mutants, while in *Igf2(-)/Igf1R*(-/-) and *Igf1(-/-)/Igf2*(-) double mutants, which are phenotypically identical, dwarfism was further exacerbated (i.e., 30% normal size).

To investigate the role of IGF-I in normal development, Powell-Braxton *et al.* generated mice with an inactive *Igf1* gene by homologous recombination in embryonic stem cells. Heterozygous *Igf1*(+/-) mice appear healthy and fertile; however, they are 10–20% smaller than wild-type littermates and have lower than normal levels of IGF-I (Powell-Braxton *et al.*, 1993). The size reduction is attributable to a decrease in organs and BMD. This was recently confirmed by studies from the Kream laboratory (He *et al.*, 2006). These investigators showed that heterozygous IGF-I null mice had reduced OB function, as well as impaired osteoclast activity *in vitro*.

As previously noted, at birth homozygous mutant *Igf1* (-/-) mice are less than 60% BW of wild-type and greater than 80% of the pups die perinatally. The survivors are sometimes compromised in terms of several homeostatic processes but the compensatory mechanisms in the survivors are interesting. For example, Bikle *et al.* (2001) analyzed the structural properties of bone from mice rendered IGF-I deficient by homologous recombination using histomorphometry, peripheral quantitative computerized tomography (PQCT), and micro-computerized tomography. The knockout mice were 24% the size of their wild-type littermates at the time of study (four months). The knockout tibias were 28% and L_1 vertebrae were 26% the size of wild-type bones. Bone formation rates (BFR) of knockout tibias were 27% that of the wild-type littermates. They have shown that the bone formation rate was reduced by 77% in the tibia of *Igf1-/-* mice compared with corresponding littermates (Bikle *et al.*, 2001). The bones of knockout mice responded normally to GH (1.7-fold increase) and supranormally to IGF-I (5.2-fold increase) with respect to BFR. Cortical thickness of the proximal tibia was reduced 17% in the knockout mouse. However, trabecular bone volume (bone volume/total volume [BV/TV]) in knockout mice compared with wild-type controls was increased 23% in male mice and 88% in female mice as a result of increased connectivity, increased number, and decreased spacing of the trabeculae. Thus, absence of IGF-I leads to the development of a smaller bone that may be more compact, almost certainly due to reduced osteoclastic activity as noted earlier. The structural consequences of these bones in respect to fracture have not been studied.

To further separate the role of endocrine and paracrine IGF-I in skeletal development, Yakar *et al.* (1999) used the Cre/loxP recombination system whereby mice with loxP-flanked *Igf1* gene were mated with albumin-Cre transgenic mice expressing the Cre recombinase exclusively in the liver. Liver-specific *Igf1* gene-null mice were macroscopically normal, suggesting that autocrine/paracrine IGF-I could support normal postnatal growth and development (Yakar *et al.*, 1999). Nevertheless, more extensive developmental phenotyping of the liver-specific *Igf1* gene-null mouse revealed a marked reduction in bone volume, periosteal circumference, and medial lateral width, consistent with the hypothesis that circulating IGF-I had an important role in bone modeling (Yakar *et al.*, 2002). Moreover, recent studies from Yakar *et al.* have shown that ALS knockout mice also have reduced cortical thickness and enhanced trabecular bone, consistent with an endocrine effect of the circulating IGF complex on skeletal acquisition. Finally, the double ALS and IGF-I knockout mice have a major growth phenotype as well as markedly reduced BMD, despite normal expression of skeletal IGF-I (Yakar *et al.*, 2006). Thus, it seems likely that both local and circulating IGF-I concentrations are essential for peak bone acquisition and maintenance.

ROLE OF GH/IGF/IGFBPs IN OSTEOPOROSIS

Effects of GH-Deficiency on Bone Metabolism

GHD in childhood is associated with growth failure and short stature. However, the effects of GHD on BMD in prepubertal children have been more difficult to quantify, in part because of the mixed hypopituitary syndromes that often accompany GHD. Thus there are a paucity of studies examining BMD status in GH-deficient children. By single photon absorptiometry (SPA) of the wrist, children with GHD have been found to have a low BMD (Wuster *et al.*, 1992). Serum concentrations of osteocalcin are also reduced in children with GHD, but the response of osteocalcin to GH administration does not correlate with linear growth (Delmas *et al.*, 1986). In several cross-sectional studies of adults with GHD, lumbar spine BMD is reduced compared to that in age-matched controls (Wuster *et al.*, 1991; Johansson *et al.*, 1992; Hyer *et al.*, 1992; Rosen *et al.*, 1993; Bing-You *et al.*, 1993; DeBoer *et al.*, 1994). In one group of adult GHD patients, the lowest spinal BMD was found in people who were previously treated with rhGH during childhood (Wuster *et al.*, 1991; Wuster *et al.*, 1993). This degree of osteopenia was not due to cortisone or thyroxine substitution because the BMDs of patients on hormonal substitution did not differ from those without hormone replacement (Wuster *et al.*, 1993). In that same study, Wuster *et al.* (1991) showed an increased prevalence of vertebral osteoporotic fractures among GH-deficient adults. Kaufman *et al.* (1992) confirmed low BMD in GHD adults with or without hormonal deficiencies. However, Kann *et al.* (1993) found no difference in the apparent phalangeal ultrasound transmission velocity of GHD patients compared to age- and sex-matched controls. De Boer *et al.* (1994) noted that low BMD was partly explained by reduced body height, but with correction for body mass index, BMD was still significantly reduced compared to age- and sex-matched controls (Wuster *et al.*, 1992).

The cause of low BMD in adult GHD has been thought to be due to insufficient bone acquisition during the adolescent years (DeBoer *et al.*, 1994). This hypothesis is supported in one study by bone histomorphometry. In 36 men with GHD (primarily of juvenile onset), there were increased eroded surfaces, increased osteoid thickness and increased mineralization lag time, all indicative of delayed mineralization probably due to changes in the timing of puberty (DeBoer *et al.*, 1994; Bravenboer *et al.*, 1994). In support of those histomorphometric changes, low serum levels of osteocalcin have been detected in some adult GHD patients (Delmas *et al.*, 1986). This is in sharp contrast to patients with normal GH secretion but multiple pituitary hormone deficiencies, where serum osteocalcin levels are normal but there is markedly increased urinary pyridinoline excretion (de la Piedra *et al.*, 1988).

Although an inadequate acquisition of BMD during childhood may be one explanation for the osteopenia of GHD, the role of gonadal steroids in this process has not been completely clarified. Furthermore, there are no data on hip fractures in GHD adults, and evidence that spinal fractures are more prevalent in GHD is still preliminary. Only longitudinal studies of GHD patients will be able to determine the precise cause of osteopenia in the acquired GHD syndrome.

Effects of GH Excess on Bone Mass and Bone Turnover

Chronic GH excess in adults (i.e., acromegaly) has been a surrogate model for studying the effects of GH on the skeleton. However, this disease is complicated by changes in vitamin D metabolism and gonadotropin secretion (Bouillon, 1991). Increased bone turnover has been reported in acromegaly by biochemical markers and histomorphometric studies (de la Piedra *et al.*, 1988; Ezzat *et al.*, 1993). However, bone mass determinations in acromegaly vary according to the site of measurement. Cortical BMD is increased compared to age-matched controls and is directly related to the degree of GH excess (Diamond *et al.*, 1989). Trabecular BMD, however, can be high, normal, or low (Ezzat *et al.*, 1993; Diamond *et al.*, 1989; Seeman *et al.*, 1982). In one study, computed tomography (CT) measurements of the lumbar spine revealed that trabecular BMD was elevated in only 1 of 14 patients with active acromegaly (Ezzat *et al.*, 1993). This may have been due to hypogonadism in the acromegalics. Wuster *et al.* (1993) recently studied five patients with active acromegaly treated with octreotide for five years. All had achieved normal IGF-I levels during therapy. Spinal BMD was initially decreased in all five patients but normalized in three of them with octreotide. All patients remained eugonadal throughout follow-up.

As noted earlier, biochemical markers of bone turnover are altered in acromegaly. Many of these changes can be related to alterations in gonadal status during the disease and its treatment. However, changes in bone turnover with acromegaly reflect persistent coupling of the remodeling cycle with increased resorption and formation. Serum osteocalcin and skeletal alkaline phosphatase are increased in acromegaly as are urinary calcium and hydroxyproline excretion (de la Piedra *et al.*, 1988; Ezzat *et al.*, 1993). Although serum calcium, total alkaline phosphatase, and phosphorus are usually normal, there may also be increased synthesis of 1,25-dihydroxyvitamin D. This results from significant intracellular phosphate shifts due in part to an increased circulating IGF-I. The consequence of this change, however, is not entirely clear.

Changes in the GH-IGF-1 Axis in Patients with Osteoporosis

For several years, attempts have been made to link GH secretory status with a low BMD and osteoporosis. As noted previously, efforts to find a relationship between GH secretion and age-related bone loss have been conflicted at best. However, other investigators have examined the relationship of GH to BMD in the immediate menopausal period. These efforts gained prominence in the 1980s when it was reported that GH secretion in patients with osteoporosis was reduced even after stimulation with L-arginine. Low serum IGF-I, IGF-II, and IGFBP-3 levels (by RIA) were noted in 98 females with postmenopausal osteoporosis compared to 59 normal controls and 91 patients with osteoarthritis or degenerative bone disease (Wuster *et al.*, 1993). In a cross-sectional study of a large cohort of older postmenopausal women from Framingham, Langlois *et al.* (1998) reported very strong correlations between the lowest quintile of IGF-I and BMD at the spine hip and radius. Bauer *et al.* reported that in the Study of Osteoporotic Fractures (SOF), women in the lowest quartile for serum IGF-I had a 60% greater likelihood of hip or spine fractures, even when controlling for BMD (Bauer *et al.*, 1997). Gamero *et al.* (2000) noted that low serum levels of IGF-I were associated with a significantly greater risk of hip fractures among a large cohort of older postmenopausal women in France. In a study of 61 community-dwelling men over the age of 27, who were randomly selected from the Calgary cohort of 1,000 subjects in the Canadian Multicentre Osteoporosis Study, IGF-I was found to be a significant predictor of BMD at the total hip, femoral neck, and femoral trochanter neck ($p \leq 0.001$). Szulc *et al.* (2004) evaluated the correlation of BMD with serum IGF-I in a large cohort of 721 men aged 19–85 taking into account age, BW, 17 β-estradiol, free testosterone, and PTH. Serum IGF-I decreased with age ($r = -0.44$, $p = 0.0001$). IGF-I correlated positively with BMD at the whole body and at the third lumbar vertebra. BMD of the total hip was 6% higher in men in the highest quartile of IGF-I than in men in the lowest quartile. However, others have not found a relationship between serum IGF-I and BMD in patients with fractures, in postmenopausal osteoporosis or in otherwise healthy subjects (Kassem *et al.*, 1994; Lloyd *et al.*, 1996; Rudman *et al.*, 1994).

In male osteoporotics, serum IGF-I as well as IGFBP-3 concentrations were reported to be low and correlated with lumbar BMD (Ljunghall *et al.*, 1992). Comparable results have been noted for IGF-I by at least two other groups (Nakamura *et al.*, 1992). Johannsen *et al.* (1994) reported that among healthy males, IGFBP-3 was the best predictor of femoral BMD. Kurland, E.S. *et al.* (1997) reported that younger males with idiopathic osteoporosis had low serum levels of IGF-I in relation to age-matched controls. Moreover, these men also had low rates of bone turnover by histomorphometry but normal GH dynamics (Kurland *et al.*, 1998). Of potential pathophysiological importance is the observation that patients with osteoarthritis have higher concentrations of IGF-II than normal controls (Wuster *et al.*, 1993; Mohan *et al.*, 1991). Other studies have related serum IGFBP-4 and IGFBP-5 due to aging and to a low bone mass, although causality was not established (Karasik *et al.*, 2002). Also, Dennison and colleagues reported that low 24-hour GH profiles were associated with low BMD of the lumbar spine in older British women (Dennison *et al.*, 2003). Further longitudinal studies will be required to determine the precise relationship among the IGFs, GH, and osteoporosis.

GH AND IGF-I AS TREATMENTS FOR BONE DISORDERS

Anti-resorptive agents have been considered as cornerstone therapeutics for osteoporosis (Agnusdei *et al.*, 2005). Anabolic agents can directly stimulate bone formation, which might have greater potential than the anti-resorptives to increase bone mass and to decrease fractures. Therefore, there is an emerging interest in developing anabolic agents including PTH, GH, and IGF-I to treat osteoporosis (Rubin *et al.*, 2002).

GH Treatment for Skeletal Disorders

GH has direct and indirect effects on bone, depending on age and skeletal maturity. Indirectly, GH can enhance bone mass through its effects on muscle mass and calcium transport in the gut, as well as suppressing adipocyte differentiation (Fleet *et al.*, 1994). In addition, GH can directly stimulate bone remodeling and increase endochondral growth through its actions on the osteoblast. Overall, GH is considered essential for both the growth and maintenance of skeletal mass. Moreover, it is established that for virtually all cohorts of GHD subjects, whether onset is in childhood or adulthood, male or female, there is reduced areal BMD. In some but not all studies, volumetric BMD, measured either by computed tomography (CT) or areal-adjusted algorithms, was reduced in children with GHD (Baroncelli *et al.*, 1998). In the largest observational trial of GHD subjects to date, i.e., KIMS, GHD was associated with a marked increase in fracture risk, particularly when compared to age-matched normals (Rosen *et al.*, 1997; Wuster *et al.*, 2001). Hence, there is a strong rationale to treat GHD in children.

Substantial differences between the direct and indirect (i.e., via IGF-I) effects of GH on the osteoblast, the marrow stromal cell precursor, and the osteocyte, may partially explain changes in skeletal responsiveness to GH and IGF-I. For example, exogenous GH stimulates longitudinal growth in normal rats, but rhIGF-I does not (Schmid *et al.*, 1991).

Similarly, transgenic mice who overexpress GH grow to twice their normal sizes whereas exogenous administration of IGF-I is far less efficient in stimulating long bone growth. Thus, despite the fact that GH induces IGF-I production in the skeleton and elsewhere, treatments with GH and with IGF-I are not equivalent. In general, skeletal responsiveness to GH and IGF-I depend on the species, the GH status of the animal, and the mode of administration. Even the systemic side effects of rhGH and rhIGF-I therapy may differ substantially.

GH Treatment for Children with Growth Impairment

Early clinical experiences with rhGH in GHD children provided investigators with a model for studying skeletal responsiveness to somatotropin. Intermittent (daily or three times weekly) injections of rhGH resulted in a prolonged and sustained GH profile with resultant catch-up growth evident during the first year of treatment (Rappaport *et al.*, 1993). This increase in skeletal growth was accompanied by a rise in serum levels of type I procollagen peptide. Although dosage schemes varied between the United States and Europe (0.1 mg/kg/tiw[U.S.] to 0.7 U/kg/week[Europe]), there was a strong dose-related growth response to rhGH (de Muinck Keizer-Schrama *et al.*, 1992). Indeed, most studies of pre-adolescent GHD children have shown significant improvements in areal BMD with GH replacement. However, the skeletal response to GH depends on several factors including: 1) GH secretory status; 2) pretreatment IGF-I levels; 3) pretreatment height velocity; and 4) GH dosage (de Muinck Keizer-Schrama *et al.*, 1992). The rate of change in serum IGF-I (rather than the absolute level of IGF-I attained by GH treatment) is a relatively nonspecific predictor of growth as are procollagen I and osteocalcin concentrations (Delmas *et al.*, 1986; de Muinck Keizer-Schrama *et al.*, 1992). Serum procollagen III levels correlate with growth rates during GH treatment (Delmas *et al.*, 1986).

Linear growth is a measurable response to exogenous GH, but changes in BMD in children are more difficult to quantify. In some studies, bone mineral content (BMC) is increased during GH treatment to a greater extent than expected for change in bone size (Inzucchi *et al.*, 1994). In one of the longest intervention trials to date, 26 GHD children were given rhGH (0.6 IU/kg per week) for 12 months (Saggese *et al.*, 1993). Baseline radial BMC *Z*-scores (corrected for their chronological, statural, and bone ages) were significantly reduced as were serum osteocalcin and procollagen peptide levels. Treatment with rhGH six times per week increased BMC and normalized *Z* scores of the radius in nearly 50% of the subjects. Serum levels of procollagen peptide during the first week of treatment were positively related to growth velocity at 6 and 12 months and radial BMC at 12 months. In another nonrandomized trial 32 children with GHD aged 7–16 were treated for nearly 1 year with rhGH and found to have significant improvements in areal BMD and final adult height (Saggese *et al.*, 1996). In adolescent GHD subjects, GH replacement has variable effects on peak bone acquisition. Even with higher rhGH doses, significant changes in volumetric BMD in these subjects have not been found, nor has acceleration in skeletal maturation (Baroncelli *et al.*, 2004; Mauras *et al.*, 2000; Kamp *et al.*, 2002). Controversy continues as to whether GH treatment impacts BMD in children with idiopathic short stature or children born small for gestational age (SGA). Arends *et al.* (2003) demonstrated that prepubertal SGA children given 33 ug/kg/d of rhGH for three years had significant increases in height, areal BMD, and areal adjusted spine BMD. However, rhGH to children with idiopathic short stature who had low volumetric BMD did not result in further increases in BMD, despite significant changes in lean body mass and bone turnover indices (Hogler *et al.*, 2005). In cerebral palsy children (aged 4–15 years), 18 months of rhGH (50 μg/day) in a randomized placebo-controlled trial RPCT, increased areal BMD significantly, as did height, IGF-I, IGFBP-3, and osteocalcin (Ali *et al.*, 2007). In sum, rhGH improves adult height, and areal BMD in prepubertal GHD children treated for at least one year. These changes are accompanied by favorable effects on body composition, muscle strength, and overall quality of life. It is still not certain, however, how beneficial these GH-induced effects are in late adolescent GHD subjects in non-GHD states, or whether true volumetric BMD is significantly improved by long-term GH therapy.

GH Administration for Healthy Adults

Although there were striking differences between longitudinal growth in children and remodeling in adults, criteria that determine rhGH responsiveness in children may be relevant for older individuals. It has already been established that biochemical and histomorphometric responses to rhGH in children may differ according to their GH secretory status. The same principle probably holds for adults treated with GH. Three adult populations have been studied before and after GH treatment in order to examine predictors of skeletal responsiveness: 1) healthy adults, 2) GHD adults, and 3) elderly men and women with/without osteoporosis.

Initial studies with rhGH in adults focused primarily on changes in body composition. Short-term treatment with rhGH leads to a decrease in adiposity and an increase in lean body mass (Crist *et al.*, 1988). There is also a marked shift in extracellular water (Holloway *et al.*, 1994). Detailed analysis of skeletal markers during GH treatment was first reported by Brixen *et al.* (1990). Twenty male volunteers (ages 22–31) were given a relatively large dose (0.1 IU/kg) of rhGH twice daily for seven days. Serum osteocalcin increased after two days of treatment and remained elevated for six months. Bone alkaline phosphatase decreased initially (during the seven days of GH treatment) but then increased slightly over six months (Brixen *et al.*, 1990). Serum calcium and phosphate increased but only during the seven-day treatment phase. Like bone formation indices, urinary markers of bone resorption (urinary Ca/Cr and hydroxyproline/creatinine) rose during treatment and remained elevated for up to four weeks after discontinuation of therapy.

Treatment with rhGH stimulates bone remodeling. More importantly, the anabolic effect on bone may persist well beyond discontinuation of GH. Early (two-day) and late (two-week) osteocalcin responses imply that GH can stimulate existing osteoblasts and enhance recruitment of new osteoblasts. Still, it is uncertain if those effects are mediated through IGF-I. For example, Brixen *et al.* (1990) were unable to find a significant correlation between the rise in serum IGF-I and an increase in osteocalcin or bone alkaline phosphatase. The absence of a significant correlation between bone formation markers and serum IGF-I, however, may be due to the low skeletal specificity of serum IGF-I.

Skeletal resistance to GH has been considered a possible cause for postmenopausal osteoporosis. However, on the basis of one double-blinded rhGH trial in postmenopausal women, this is unlikely. Kassem *et al.* (1994) noted that administration of rhGH (0.2 IU/kg/day) for three days increased serum IGF-I, osteocalcin, and procollagen type I C-terminal propeptide (PICP) to the same extent in 15 women with severe postmenopausal osteoporosis as in 15 age-matched control women. Serum and urinary markers of bone resorption also did not differ between the two groups. *In vitro* studies of marrow stromal cells from osteoporotic women demonstrate full GH responsiveness. Therefore, it is unlikely that the osteoporotic skeleton is resistant to rhGH therapy.

GH Treatment for GHD Adults

GHD can be documented by provocative stimuli (GHRH, insulin, glucagon) and serial GH measurements. The majority of adult patients treated with rhGH have either idiopathic GHD or a history of previous central nervous system (CNS)/pituitary-hypothalamic tumors. Early trials with rhGH replacement therapy examined changes in muscle mass, muscle strength, and body fat. Daily administration of subcutaneous rhGH to GHD patients produced a marked rise in serum IGF-I and an increase in muscle mass and basal metabolic rate (Jorgensen *et al.*, 1991). Some of those anabolic changes were noted soon after the initiation of rhGH. For example, mean nitrogen retention during the first 15 days of rhGH treatment was as much as 2.8 g per day (approximately 20 g of muscle mass) (Valk *et al.*, 1994). GH treatment can also increase the total cross-sectional area of thigh muscles and quadriceps as well as improve hip flexors and limb girdle strength (Jorgensen *et al.*, 1991; Valk *et al.*, 1994). At least one group has suggested that rhGH can increase the number of type II muscle fibers. Total fat mass, however, consistently decreases during rhGH treatment (Jorgensen *et al.*, 1989; Jorgensen *et al.*, 1991). Based on these and other studies, the U.S. FDA approved the use of rhGH in patients with established GH.

Several biochemical parameters reflect the pharmacologic action of GH on the skeleton. Serum calcium, osteocalcin, and urinary hydroxyproline all increase, while PTH declines slightly during rhGH treatment. Newer and more sensitive markers of bone turnover also reflect changes during rhGH treatment. Urinary deoxypyridinoline increases threefold and the amino-terminal propeptide of type III procollagen doubles during four months of daily rhGH (Christiansen *et al.*, 1991; Johansen *et al.*, 1990). After cessation of rhGH treatment, deoxypyridinoline excretion decreases but type III procollagen levels remain higher than controls for several months(Johansen *et al.*, 1990). Serum osteocalcin and procollagen 1 N-terminal propeptide (P1NP) markers of bone formation also increase significantly with rhGH therapy in adults, not unlike the change noted in children.

If prolonged, GHD in adults results in profound changes in the musculoskeletal system, then GH replacement would be expected to enhance muscle performance and subsequently BMD. The first waves of studies with rhGH for adults were uncontrolled observational trials. Fourteen GHD adults given a nightly dose of rhGH (0.5 IU/kg/week) showed increases in exercise capacity, maximum oxygen consumption, and alkaline phosphatase even though quadriceps strength and spinal BMD did not change over one year (Whitehead *et al.*, 1992). When 0.25 IU/kg/week of rhGH was administered to 12 GHD adults for one year, there was a marked increase in trabecular BMD (measured by single and dual energy QCT of the spine) at 6 and 12 months. At 12 months proximal and distal forearm BMC increased, mid-thigh muscle area was greater, and fat cross-sectional area decreased. Because the rise in spine BMD was noted with both single and dual energy CT measurements of the spine, it is possible that the enhancement in BMD was significant and not related to the reduction in marrow fat. However, more studies are needed to define the response of the bone marrow, and particularly the marrow adipocytes, to rhGH treatment. New BMD studies using magnetic resonance imaging (MRI) to quantify changes in the skeleton are likely to generate data on the response of marrow fat to systemic rhGH use.

Several groups have performed longer, although not randomized, trials with rhGH in the GHD syndrome. Changes in BMD were not significant at 12 months, however by 24 and 36 months BMD has been reported to increase by as much as 5–8% in the spine (Janssen *et al.*, 1998; Baum *et al.*, 1996; Papadakis *et al.*, 1996).

In addition, other investigators have reported a concomitant increase in muscle strength after two years of rhGH treatment. It appears from those studies that individuals with earlier onset of GHD, as well as those with the lowest BMD, had the greatest likelihood of showing significant changes in BMD with rhGH. More recently, four RPCTs have been conducted in adult GHD subjects treated with rhGH for at least 18 months. In one of the studies of men only, BMD increased in the lumbar spine by 5.1% and in the femoral neck by 2.4% (Baum *et al.*, 1996). In another study of both men and women, there were no significant differences in BMD after 18 months between the group treated with rhGH and those taking placebo (Sneppen *et al.*, 2002). In the third trial, men but not women showed increases in spine BMD after 24 months of rhGH compared to no therapy. The most definitive and most recent study was a true randomized placebo-controlled trial using physiologic rather than pharmacologic doses of rhGH in GHD patients. In contrast to the three previous studies, changes in serum IGF-I were titrated within the normal range, rather than to the superphysiologic levels achieved with higher doses of rhGH. Interestingly, in that study of 67 men and women, spine BMD increased after 18 months by nearly 4% in men, which was statistically different than the placebo control, but increased much less in women, and those changes were not statistically different than placebo (Snyder *et al.*, 2007). Neither gender showed a significant change in hip BMD in response to rhGH, in doses that were up to 12 μg/kg/day. In sum, there are clear gender- and dose-dependent effects of rhGH on BMD in GHD subjects treated for at least 18 months. There are no studies showing fracture risk reduction with rhGH treatment in GHD subjects.

GH Administration to Elderly Men and Women

As previously noted, elderly people have lower GH secretory amplitudes and reduced serum levels of IGF-I and IGFBP-3 compared to younger adults (Kelijman, 1991; Rudman *et al.*, 1981; Donahue *et al.*, 1990). Moreover, the pulse frequency for GH is less in the elderly population. Based on these data, it was assumed that skeletal responsiveness to GH in elders would be identical to that seen in GHD patients. In elderly men, one group reported a blunted serum IGF-I response to 0.1 mg/kg GH (36% lower) compared to that in younger men or adults with GHD (Lieberman *et al.*, 1994). However, Rosen *et al.* (1999) and others have noted that generation of IGF-I after various doses of rhGH to frail elders was not associated with significant GH resistance. Based on some recent data it appears that GH replacement for adult GHD or for pharmacologic treatment results in similar IGF-I responses independent of age. Side effects such as fluid retention, gynecomastia, and carpal tunnel appear to be more common in the elderly given rhGH compared to young adults with GHD.

The most widely publicized GH RPCT in elders involved 21 men over age 65, randomized to receive 0.03 mg/kg of rhGH three times per week (as a subcutaneous injection), or placebo. Twelve men received rhGH while nine men served as observational controls. The men were selected on the basis of a low serum IGF-I (<350 IU/liter) concentration (Rudman *et al.*, 1990). The rhGH produced a threefold rise in circulating IGF-I, an increase in lean body mass (as measured by ^{40}K analysis), and a decline in total adipose mass. BMD of the lumbar vertebrae (L_1–L_4) as measured by dual-energy x-ray absorptiometry (DXA) increased 1.6% after six months in the treatment group while no change was noted in controls. Biochemical markers of bone turnover were not examined and no changes in BMD were detected in the mid- or distal radius or three areas of the hip. Furthermore, the spinal BMD changes at six months were not sustained at one year.

Marcus *et al.* studied the effects of rhGH in 16 men and women over age 60 (Marcus *et al.*, 1990). Daily doses of rhGH (0.03, 0.06, or 0.12 mg/kg BW/day) were randomly assigned to each subject and administered once daily for seven days. Serum IGF-I, osteocalcin, PTH, and calcitriol concentrations all increased during treatment. In this short-term study, there was also a significant rise in urinary hydroxyproline and urinary calcium excretion with a concomitant decline in urinary sodium excretion.

Holloway *et al.* conducted a longer double-blind RPCT of daily rhGH for one year in 27 healthy elderly women, 8 of whom took a stable dose of estrogen throughout the study (Holloway *et al.*, 1994). Thirteen women completed six months of treatment and 14 women completed six months in the placebo group. Side effects prompted a 50% reduction in the original dose of rhGH (from 0.043 mg/kg BW or approximately 0.3 mg rhGH/kg/week to 0.02 mg/kg/day) and led to several dropouts in the treatment group. Fat mass and percentage body fat declined in the treatment group but there were no changes in BMD at the spine or hip at 6 or 12 months in other groups (Holloway *et al.*, 1994). Although BMD did not change, there were changes in some biochemical parameters. In particular, urinary markers of bone resorption (e.g., hydroxyproline and pyridinoline) increased after six months of rhGH treatment. The response of bone formation markers was more variable. Osteocalcin increased but type I procollagen peptide levels did not change. For women taking estrogen replacement therapy, indices of bone turnover (both formation and resorption) were blunted.

Rosen *et al.* (1999) reported that there was a dose-dependent decrease in total body BMD after one year of rhGH in frail elderly men and women. This occurred despite striking increases in osteocalcin and serum IGF-I with the highest doses of rhGH (0.01 mg/kg/day). In part, the absence of a GH effect on BMD is not surprising because resorption is coupled to formation and GH activates the entire remodeling sequence. Moreover, the skeletal response was only measured after one year of treatment; this was probably inadequate to determine the true effect of rhGH on BMD as noted from earlier studies in GHD. Not surprisingly, in the same trial of 132 frail elderly subjects by Rosen and colleagues, urinary N-telopeptide and osteocalcin both rose to the same extent suggesting that total bone turnover, not just bone formation, was increased by rhGH therapy. The relatively high incidence of acute side effects (weight gain, carpal tunnel syndrome, edema, glucose intolerance) in GH trials, especially in the frail elderly, has remained particularly troublesome even with titrating doses. Moreover, high serum IGF-I levels for long periods of time may predispose individuals to certain malignancies. Thus, there is limited enthusiasm for rhGH or rhGHRH treatments in the frail elderly.

GH Treatment for Osteoporotic Patients

Short nonrandomized clinical trials with GH in osteoporosis were attempted well before GH replacement therapy was considered. As early as 1975, two patients with osteogenesis imperfecta and one patient with involutional osteoporosis were treated with GH. Histomorphometric parameters of increased bone formation and resorption were noted. Subsequent studies employed GH with and without anti-resorptive agents. Aloia *et al. (*1977) administered between 2 and 6 U/day of GH for 12 months to 8 patients with postmenopausal osteoporosis (the first 6 months of treatment featured low-dose GH; the last 6 months consisted of high-dose GH (6 U/day). Radial BMC dropped slightly and histomorphometric parameters did not change during treatment. However, severity of back pain decreased considerably in several people. Daily GH injections (4 U/day) combined with alternating doses of calcitonin produced an increase in total body calcium (measured by neutron activation analysis) but a decline in radial bone mass after 16 months. In a separate trial, 14 postmenopausal women were given two months of GH then three months of calcitonin in a modified form of coherence therapy. Total body calcium increased 2.3%/year and there were few side effects, but there were no changes in BMD or histomorphometric indices. One study administered 16 U of rhGH every other day along with daily sodium fluoride to six women with postmenopausal osteoporosis. On histomorphometric analysis, there was a significant increase in the number of osteoblasts and osteoclasts but BMD was unchanged.

Johansson et *al*. (1994) conducted a crossover double-blinded RPCT of rhGH and with idiopathic osteoporosis. In this seven-day trial with rhGH 2 IU/m^2, procollagen peptide and osteocalcin levels increased after treatment as did urinary markers of bone resorption. The changes in osteocalcin were relatively small and were not sustained after discontinuation of GH treatment.

There are no GH trials (past or present) that have examined spinal fractures as a therapeutic end point. Therefore it is difficult to judge the potential efficacy of GH in the treatment of osteoporosis. However, GH stimulates bone remodeling activity, thereby leaving open the possibility that GH can be coupled to anti-resorptive agents. This thesis was tested in a two-year randomized trial by Holloway and colleagues (Holloway *et al*., 1997). In that study, rhGH and nasal calcitonin increased spine BMD by approximately 2%. This, however, was not much different than the use of CT alone, and certainly less than what has been seen in very large randomized trials with anti-resorptive agents. Once again, there were several side effects that produced limited enthusiasm for rhGH as a primary treatment for osteoporosis. In another combination trial that was larger and longer, 80 osteoporotic postmenopausal women on hormone replacement therapy (HRT) (estrogen with or without progestin) were administered rhGH ten 2.5 U/day or placebo for 18 months, and then open label rhGH for another 18 months. These women were then followed for an additional 24 months. Women given GH and HRT had a marked increase in total body and spine BMD compared to placebo, which was maintained to year 3, but disappeared by year 5 (Landin-Wilhelmsen *et al*., 2003). This trial suggested that combination therapy of an anabolic and an anti-resorptive could be used in postmenopausal osteoporosis. A similar result was noted in a seven-year follow-up of 30 men and women who received rhGH for four years and then were treated with alendronate for an additional three years. BMD increased significantly versus a control group after three years, especially in the males, and the addition of alendronate further enhanced spine BMD at year 7 (Biermasz *et al*., 2004). Therefore, it is likely that GH may induce small but significant changes in BMD that over an extended period could translate into fewer spine fractures. In the meantime, several very small trials have looked at the effects of GH-releasing analogs on bone turnover and BMD. Not unlike rhGH, however, these studies have been small, and the results somewhat conflicting. However, in contrast to rhGH, GH-releasing analogs are not associated with the significant side effects of weight gain and carpal tunnel syndrome. Hence, further trials may continue with these analogs.

IGF-I for the Treatment of Osteoporosis

Overview

In the late 1980s, clinical trials with rhIGF-I for diabetes mellitus were begun. The availability of this recombinant peptide and the absence of other treatments to stimulate bone formation accelerated animal and human studies of rhIGF-I in metabolic bone diseases. Theoretically, there are potential benefits for rhIGF-I compared to rhGH. These include: 1) more direct stimulation of bone formation; 2) bypass of skeletal GH resistance; and 3) reduction in GH-induced side effects such as carpal tunnel and diabetes mellitus. There are, however, considerably fewer animal and human studies using rhIGF-I than rhGH. Therefore, these advantages have either yet to be fully realized or have not been validated.

IGF-I is not a potent mitogen in most tissues and bone is no exception (see Chapter 8 by Canalis). There are high-affinity receptors for IGF-I expressed on osteoblasts and IGF-I can stimulate preosteoblast replication and provoke resting cells to proceed through their growth cycles. IGF-I maintains the differentiated osteoblast phenotype, stimulates collagen synthesis, and prevents collagen degradation. Theoretically, therefore, despite its relatively weak mitogenic properties, IGF-I could have significant anabolic activity on the skeleton.

Animal Studies with IGF-I

In hypophysectomized rats, growth can be fully restored by administration of either GH or IGF-I but not IGF-II (Schoenle *et al.*, 1985). A similar growth response occurs after rhIGF-I in streptozotocin-diabetic rats but not in sex-linked dwarf-mutant chickens (Schoenle *et al.*, 1985; Tixier-Boichard *et al.*, 1992). In normal rats, rhIGF-I administrated either systemically or locally (hind limb infusions), does not stimulate longitudinal bone growth. In the spontaneously diabetic BioBreeding (BB) rat, rhIGF-I treatment does not result in changes in epiphyseal width, osteoblast surfaces, or osteocalcin concentration (Verhaeghe *et al.*, 1992).

The skeletal response to rhIGF-I is determined by the GH/IGF-I status of the animal. For example, IGF-I does not increase bone formation in normal rats, whereas it stimulates bone growth and normalizes type I procollagen mRNA levels in hypophysectomized rats (Schmid *et al.*, 1989; Spencer *et al.*, 1991; Tobias *et al.*, 1992). Similarly, in the spontaneous mouse mutant (*lit/lit*), the absence of GHRs results in very low levels of IGF-I and skeletal dwarfism. IGF-I treatment restores growth and increases total body water but does not enhance BMD in these mice (Donahue *et al.*, 1993). These findings are somewhat similar to the effects of GH on the skeleton in GHD animals. However, rhIGF-I and rhGH differ in their actions on the circulatory IGF regulatory system. GH stimulates hepatic production of both IGF-I and IGFBP-3 while rhIGF-I administration increases the total circulating pool of IGF-I but suppresses hepatic production of IGFBP-3, primarily through feedback inhibition of GH secretion. It is conceivable that variations in IGF-I biological activity (between direct IGF-I administration and endogenously produced IGF-I as a result of GH treatment) may be due to the relative proportion of IGF-I bound to IGFBP-3.

Several experimental paradigms have been employed to study the effects of IGF-I on bone turnover in animals. These include: 1) oophorectomy, 2) diabetes mellitus (spontaneous or induced), and 3) immobilization. In each situation, bone remodeling is markedly altered prior to IGF-I treatment in order to study growth factor actions on bone resorption and formation. These experimental models provide useful clinical information since IGF-I has been considered a potential therapeutic agent in conditions similar to those produced experimentally.

In oophorectomized rats, administration of rhIGF-I has variable effects on bone remodeling, BMD, and bone strength. Kalu reported partial restoration of trabecular bone volume after oophorectomy in adult rats treated with rhIGF-I. In older oophorectomized rats, rhIGF-I increased mid-shaft tibial BMD and enhanced periosteal bone apposition (Ammann *et al.*, 1996). Six weeks of rhIGF-I (delivery by mini-osmotic pump) to older rats caused a dose-dependent increase in BMD in the lumbar spine and proximal femur although bone strength and stiffness did not change. Muller reported that subcutaneous administration of rhIGF-I to adult oophorectomized rats stimulated bone formation as evidenced by increased osteoid surfaces, osteoblast surfaces, and mineral apposition rates. At high doses of rhIGF-I, osteoclast surface and osteoclast number also increased. In contrast, Tobias *et al.* (1992) found that rhIGF-I (200 mg/kg) administered for 17 days to 15-week-old rats increased longitudinal and periosteal growth but suppressed trabecular bone formation in both oophorectomized and control rats. Bone resorption was also slightly suppressed during rhIGF-I treatment, although not to the extent that bone formation was inhibited.

IDDM is associated with decreased cortical BMD. Although the pathophysiology of diabetic osteopenia remains unknown, it appears that the duration of diabetes, the extent of diabetic control, and the timing of disease onset are each associated with higher risks of low BMD (McNair, 1988). Serum markers of bone formation are reduced in type I diabetics, suggesting a possible defect in osteoblastic activity (Verhaeghe *et al.*, 1992). Serum IGF-I levels are either normal or low in IDDM, but often are reduced in patients with poor diabetic control. In these same people, serum IGFBP-1 levels are quite high. This has led investigators to believe that changes in the IGF regulatory system during poor metabolic control contribute to impaired growth.

Spontaneously diabetic BB rats exhibit osteopenia and therefore provide a useful model for studying the effects of IGF-I on bone remodeling. Even though bone formation is lower in BB than control rats (as measured by serum markers), administration of rhIGF-I does not increase bone epiphyseal width, osteoblast surfaces, or serum osteocalcin (Verhaeghe *et al.*, 1992). Thus, despite evidence that circulating levels of IGF-I are reduced in some patients with type I IDDM, preliminary animal studies have failed to show that IGF-I administration can correct any inherent defect in bone formation.

Chronic immobilization inhibits bone formation and leads to significant bone loss. The pathophysiology of immobilization caused by bedrest, hind quarter elevation, or space flight is unknown, but the bone remodeling unit is uncoupled due to a transient decrease in bone formation and a marked rise in bone resorption (Heaney, 1962). Some investigators have proposed that reduced bone formation during immobilization results from resistance to skeletal IGF-I. Immobilization in rats by the hind limb elevation method causes cessation of bone growth (Bikle *et al*., 1994). Paradoxically, mRNA levels for IGF-I and the IGF-IR are substantially increased in the proximal tibia and distal femur of hind limb elevated rats (Bikle *et al*., 1994). Infusion of rhIGF-I (200 mg/day) during hind limb elevation does not reverse the cessation in linear growth induced by immobilization, even though growth and bone formation resume relatively soon after immobilization is stopped (Bikle *et al*., 1994). This would suggest that there may be, at least transiently, resistance to IGF-I bioactivity.

Other investigators have reported contrasting results during hind limb elevation. Machwate *et al*. (1994) continuously infused rhIGF-I (1.3–2.0 mg/kg/day) for 14 days to 5-week-old hind limb elevated rats. The decline in BMD of the proximal femur with unloading was blunted by infusions of IGF-I. At the tibial metaphysis of IGF-infused animals, bone formation rate and trabecular number were markedly increased. Marrow stromal cells from unloaded rats exhibit decreased proliferative characteristics, but addition of IGF-I greatly increased alkaline phosphatase positive cell proliferation. The rhIGF-I also enhanced serum alkaline phosphatase activity and osteocalcin levels in immobilized rats.

Alternate ways of exploiting the anabolic properties of IGF-I in bone have been proposed. IGF-I has been administered by intra-arterial infusion or coupled to IGFBP-3. Infusion of rhIGF-I continuously into the arterial supply of the right hind limb of ambulatory rats for 14 days leads to a 22% increase in cortical and trabecular bone formation in the infused limb (Spencer *et al*., 1991). By histomorphometry the number of osteoblasts, but not osteoclasts, increases.

Using an alternative model, Bagi *et al*. (1994) administered rhIGF-I or a complex of IGF-I-IGFBP-3 to 16-week-old oophorectomized rats. The IGF-I-IGFBP-3 complex (7.5 mg/kg/day) increased bone formation more than did IGF-I alone, even though both treatments increased longitudinal bone growth. The highest doses of rhIGF-I and rhIGF-I-GFBP-3 enhanced trabecular thickness in the lumbar vertebrae and femoral epiphyses and increased bone resorption but only in the femoral metaphysis. A similar study contrasting IGF-I with IGF-I-GFBP-3 was performed in 22-week-old oophorectomized rats (Brommage *et al*., 1993). BMD increased in both groups but fewer than 10% of the rats treated with IGF-I-IGFBP-3 complex developed hypoglycemia, compared to nearly 50% with rhIGF-I alone.

Human Studies of IGF-I and BMD

There is one published study of bone markers that employed rhIGF-I to healthy young postmenopausal women. Doses of rhIGF-I from 30 to 180 mg/kg/day were administered daily by subcutaneous injection for six days to older postmenopausal women without fractures and normal BMD (Rajaram *et al*., 1997). Very significant dose-dependent increases in serum PICP, osteocalcin, and urinary deoxypyridinoline were reported. Although the rise in PICP was greater than the increase in collagen breakdown (measured by deoxypyridinoline), it is uncertain whether this meant that formation was stimulated more than resorption. For the two highest doses of rhIGF-I (120 and180 mg/kg/day), orthostasis, weight gain, edema, tachycardia, and parotid discomfort were noted. At lower doses (30 and 60 mg/kg/day) fewer side effects were reported, but less discrete changes in PICP were noted. As noted later, high and low doses of rhIGF-I were also administered for 28 days to elderly postmenopausal women and the results are somewhat different in that bone formation was stimulated by lower doses of rhIGF-I.

One indication for rhIGF-I, which has been approved in the United States, Sweden, and other countries, is the GH-resistant short stature syndrome (Laron dwarf). Patients with the Laron dwarf syndrome lack functional GHRs and thus do not respond to GH; their IGF-I levels are very low, growth is slow, and circulating GH levels are high (due to a lack of negative feedback on GH by IGF-I) (Bondy *et al*., 1994). Underwood treated one such boy (age 9) with two weeks of continuous intravenous rhIGF-I (Bondy *et al*., 1994). Urinary calcium excretion increased while urinary phosphate and sodium decreased. After a two-week continuous infusion of rhIGF-I, the patient was treated with twice daily sc rhIGF-I (120 mg/kg) for two years. Growth occurred at a rate of 10 cm/year, compared to 5 cm for the three years prior to treatment. Subsequently, Underwood and colleagues have treated eight patients in this manner without hypoglycemia while Laron and his group have treated five children (Bondy *et al*., 1994; Laron *et al*., 1992). More recently, a child with an IGF-I deletion mutation in exon 5 has been reported. This patient had very short stature, mental retardation, and other abnormalities along with very low levels of circulating IGF-I (Woods *et al*., 1996). The rhIGF-I treatment led to a marked increase in linear growth and a huge increase in spinal bone mass. However, when corrected for changes in size of the bone, the incremental changes in volumetric bone mass were much less impressive (Camacho-Hubner *et al*., 1999). Hypoglycemia was avoided in these cases by having children eat three to four hours after their IGF-I injection, although several children had selective growth of adenoidal tissue.

Two unique aspects about these IGF-I data challenge previous concepts about the role of GH in skeletal homeostasis. First, IGF-I can act as a classical endocrine hormone stimulating longitudinal growth independent of GH; second, GH may not be absolutely essential for statural growth; i.e., the stimulatory effect of GH on chondrocytes that permits skeletal responsiveness to IGF-I may not be as critical as once perceived. However, caution must be undertaken in examining the effects of rhIGF-I on BMD in children because most of the changes in the skeleton relate to linear growth and periosteal enhancement, both of which can contribute to two-dimensional changes in BMD as measured by DXA, but lesser changes when corrected for size (Bachrach *et al.*, 1998).

Idiopathic osteoporosis in men is an ill-defined syndrome of low bone mass and spinal fractures without associated hypogonadism. By histomorphometry, these men often have low bone turnover, suggesting a possible defect in bone formation. Several groups of investigators have suggested that this syndrome is related to low serum IGF-I levels (Ljunghall *et al.*, 1992; Kurland *et al.*, 1997; Kurland *et al.*, 1998). Because the therapeutic options in males with osteoporosis are somewhat limited and treatment for low bone turnover states, in general, is frustrating, the therapeutic potential for anabolic agents like IGF-I in this condition should be quite high. In one male with idiopathic osteoporosis and low serum IGF-I, Johansson *et al.* (1994) administered subcutaneous rhIGF-I (160 mg/kg/day) for seven days. Bone alkaline phosphatase, osteocalcin, and the PICP all increased more than 40% over baseline. However, urinary calcium/creatinine and hydroxyproline excretion rose during treatment. In a recent trial rhIGF-I (at a dose of 80 mg/kg/day) and rhGH (2 IU/m^2/day) in 12 men, serum osteocalcin, serum procollagen peptide, and urinary deoxypyridinoline excretion all increased following seven days of rhIGF-I treatment (Holloway *et al.*, 1997). Although there were slight differences in the response of certain biochemical markers to IGF-I and GH, both forms of therapy produced significant increases in bone resorption.

Anorexia nervosa is a condition that is characterized by amenorrhea, profoundly low BMD (due either to low peak bone mass or rapid bone loss), as well as reduced body weight, low circulating IGF-I, resistance to GH, and a marked propensity for fractures. Hence, rhIGF-I might be considered an ideal therapeutic option for this group of adolescents and young adults with severe bone disease, particularly because oral contraceptive pills (OCPs) have virtually no effect on BMD in these patients. Grinspoon *et al.* (2003) studied 60 anorexic women with low BMD in a RPCT of nine months' duration using rhIGF-I 30 ug/kg/d, with or without OCPs. The group of women receiving rhIGF-I and OCPs had the greatest increase in spine BMD (+1.8%); rhIGF-I alone also increased BMD (+1.1%) while bone loss occurred in the group receiving placebo or OCPs alone. Interestingly, there were virtually no side effects in the anorexic women, and serum IGFBP-2 was inversely correlated with the changes in hip BMD. Although the increase in BMD was relatively modest, considering the lack of other available therapies, these changes are encouraging and suggest further studies are needed.

Clinical trials provide evidence that IGF-I acts by increasing the birth rate of remodeling osteons, thereby promoting bone resorption and formation. This action might be ideal for older individuals, because one characteristic of age-related osteoporosis is suppressed bone formation. However, concerns about dosing and side effects have limited enthusiasm for this approach. Yet, it is conceivable that low doses of rhIGF-I (<30 mg/kg/day) could differentially stimulate bone formation. In one trial of 16 healthy elderly women, 60 ug/kg/day(high dose) and 15 mg/kg/day(low dose) of rhIGF-I were tested for 28 days. The high dose rhIGF-I increased markers of bone resorption and formation. However, low doses of rhIGF-I caused increases in serum osteocalcin and PICP, but had no effect on total pyridinoline excretion (Ghiron *et al.*, 1995). These data would support the thesis that low doses of rhIGF-I may directly increase osteoblastic function with only a minimal increase in bone resorption. However, further studies will be needed to assess the future therapeutic role of low doses of rhIGF-I in osteoporosis.

Recently, novel approaches to enhancing IGF-I action in bone have been proposed. One strategy is to administer a bone-specific agent that stimulates bone mass such as PTH. Intermittent hPTH increases trabecular bone by stimulating osteoblasts to synthesize IGF-I and other growth factors (Rosen and Donahue, 1998). Another strategy is to administer IGF-I along with an IGFBP. Bagi *et al.* (1994) previously reported that IGF-I/IGFBP-3 complex could enhance bone mass in the metaphysis and epiphysis of rats. One very small randomized trial utilized subcutaneous infusions of IGF-I/IGFBP-3 in 24 older women with hip fractures. Bone loss in the contralateral hip was reduced considerably after six months (i.e., from 6% to 1.5%) in those subjects who were given the complex versus those receiving saline (Geusens *et al.*, 1998; Boonen *et al.*, 2002). Accompanying that change in BMD was also an increase in grip strength in those that received the active agent, while no significant side effects were reported.

Limitations to the Clinical Use of Recombinant Human IGF-I

IGF-I treatment has its limitations (Zofkova, 2003). The impacts of serious sequelae of long-term administration of IGF-I remain to be evaluated. During IGF-1 treatment, undesirable metabolic manifestations may develop hypoglycemia (in particular after large intravenous doses) and hypophosphatemia with subsequent hypotension (Zofkova, 2003). A more frequent incidence of gynecomastia was also observed. The use of IGF-I/IGFBP-3 complex seems to be very useful and safe in women who are older and who have a recent hip fracture (Agnusdei *et al.*, 2005). Furthermore, the combination of an anabolic agent with an anti-resorptive drug (such as calcitonin or alendronate) could be more potent than either agent alone. However, therapeutic effects of such combined approach on BMD at different skeletal sites have been controversial (Agnusdei *et al.*, 2005).

SUMMARY

Several lines of evidence point to the importance of local IGF-I in skeletal turnover. Systemic IGF-I, regulated by GH, the IGFBPs, and several proteases, as well as the IGF receptors, also must be important for full linear growth and peak skeletal acquisition. Recent work suggests that recombinant growth factors may be anabolic for the skeletal remodeling unit. First, both GH and IGF-I stimulate osteoblastic differentiation and *in vivo* models using targeted deletion or overexpression of IGFs or the IGF-IR support a critical role for this regulatory circuit in peak bone acquisition. Second, in animal models, GH and IGF-I treatments stimulate longitudinal growth, bone formation, and BMD. Third, in GHD children, rhGH and rhIGF-I both enhance trabecular and cortical BMD. However, rhGH needs to be continued for several years in order to see a positive skeletal response in adults; the response is greater in men than women, and the potential long-term risks of elevating IGF-I as well as the availability of other less expensive therapies, precludes major development of these peptides. Therefore, unless more favorable responses in properly controlled clinical trials are seen with rhGH or rhIGF-I, these drugs are not recommended for the treatment of postmenopausal osteoporosis.

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Chapter 50

Platelet-Derived Growth Factor and the Skeleton

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INTRODUCTION

Platelet-derived growth factor (PDGF), a polypeptide with a molecular mass of 30-kilo Daltons (kDa), was originally isolated from human platelets. PDGF is composed of two polypeptide chains that form a homo- or heterodimer. The chains are the products of distinct but related genes. There are four members of the *pdgf* gene family: *pdgf a*, *pdgf b*, *pdgf c*, and *pdgf d* (Fredriksson *et al.*, 2004). In addition, vascular endothelial growth factor (VEGF) shares a high degree of sequence homology with members of the *pdgf* gene family, which is often referred as the PDGF/VEGF family (Ferrara and Davis-Smyth, 1997). The *pdgf a* gene is localized to chromosome 7 and the mature peptide shares 56% sequence homology with PDGF B (Betsholtz *et al.*, 1986). The *pdgf b* gene shares extensive sequence homology with *p28-sis*, the oncogene product of the simian sarcoma retrovirus (Doolittle *et al.*, 1983). *Pdgf* genes encode a highly conserved cytine knot motif, and *pdgf c* and *d* have a Clr/Cls, urchin endothelial growth factor, bone morphogenetic protein (BMP)-1 or CUB domain linked to the cystine knot core motif, by a hinge domain (Bergsten *et al.*, 2001; Gilbertson *et al.*, 2001; LaRochelle *et al.*, 2001; Reigstad *et al.*, 2005). The proteolytic release of the PDGF C and D core from the CUB domain is required for the activation of the growth factor, although activity for the full-length peptide and independent activity for the CUB domain have been reported. PDGFs must form homo- or heterodimers to exhibit activity, and they can form PDGF AA, BB, AB, CC, or DD dimers (Reigstad *et al.*, 2005).

Although PDGF was initially isolated from human platelets, its presence was subsequently found in selected tissues, including bone. In the circulation, PDGF is transported in platelet granules, where its composition is 70% PDGF AB heterodimer, 25% PDGF BB, and 5% PDGF AA homodimer (Hammacher *et al.*, 1988; Hart *et al.*, 1990). *Pdgf* genes are expressed in normal and malignant cells, where they act as local regulators of cell growth. The various *pdgf* genes are conspicuously expressed during development and in adult tissues. Transcripts for the *pdgf a*, *pdgf b,* and *pdgf c* genes can be detected in osteoblasts, but the basal level of PDGF expression is relatively low (Betsholtz *et al.*, 1986; Ding *et al.*, 2000; Reigstad *et al.*, 2005; Rydziel *et al.*, 1994). *Pdgf d* is expressed by myocardial and vascular cells, but not by the osteoblast (Ponten *et al.*, 2005).

SYNTHESIS OF PDGF BY SKELETAL CELLS

The major source of PDGF is the systemic circulation, and skeletal cells become exposed to PDGF following platelet aggregation. Skeletal cells express the *pdgf a*, *pdgf b,* and *pdgf c* genes, indicating that PDGF isoforms may act as autocrine regulators of skeletal cell function (Table I) (Ding *et al.*, 2000; Rydziel *et al.*, 1992; Rydziel *et al.*, 1994). The expression of the *pdgf a* gene in osteoblasts is regulated by other growth factors and TGF β as well as PDGF induce *pdgf a* expression, suggesting that an autoregulatory mechanism is in place to maintain local levels of the growth factor (Rydziel *et al.*, 1992; Rydziel *et al.*, 1994). This autoregulation of the *pdgf a* gene is not unique to the osteoblast, as it also occurs in nonskeletal fibroblasts and mesangial cells (Bhandari *et al.*, 1994). Furthermore, growth factor autoregulation is not specific to PDGF, and other growth factors, such as TGF β1, and insulin-like growth factors (IGF) are autoregulated (Kim *et al.*, 1989). Mechanisms involved in the expression of *pdgf a* in osteoblasts have not been established, but in nonskeletal cells they involve transcriptional control (Kavanaugh *et al.*, 1988; Starksen *et al.*, 1987). Post-transcriptional regulation of the *pdgf a* gene in skeletal cells has not been reported, and its rapid induction by PDGF and TGF β1 suggests that transcriptional mechanisms are involved in osteoblasts. The promoter region of the *pdgf a* gene contains multiple Sp-1 and Egr-1-binding sites, and negative regulatory or silencer elements (Lin *et al.*, 1992; Lin *et al.*, 1993; Takimoto *et al.*, 1991). A serum response element appears to be responsible

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TABLE I Factors Inducing PDGF Gene Expression in Osteoblasts

GENE pdgf a	pdgf b	pdgf c	pdgf d
Factors			
PDGF	TGF β	Unknown	Not Expressed
TGF β			
Phorbol esters			

for the autoregulation of *pdgf a* (Lin *et al.*, 1992). The use of alternate promoters, alternative splicing, and multiple polyadenylation signals results in the expression of multiple species of *pdgf a* transcripts (Rorsman *et al.*, 1988; Rorsman *et al.*, 1992). In contrast to the regulation of the *pdgf a* gene by selected growth factors in osteoblasts, systemic hormones do not modify its synthesis in skeletal cells (Rydziel *et al.*, 1992).

TGF β1 increases the expression of *pdgf b* gene transcripts in a time- and dose-dependent fashion in osteoblasts (Rydziel and Canalis, 1996). Other growth factors and systemic hormones do not regulate *pdgf b* gene expression in skeletal cells. TGF β induces *pdgf b* expression in osteoblasts and nonskeletal cells, where it acts at the transcriptional level (Daniel and Fen, 1988). The *pdgf b* gene promoter contains a classic TATA box, Sp-1-binding sites, and a transcriptional silencer region (Jin *et al.*, 1993; Pech *et al.*, 1989). The regulation of *pdgf a* and *b* gene expression in skeletal cells is analogous to their regulation in nonskeletal cells, indicating that there are no specific transcription factors responsible for the regulation of *pdgf a* or *b* in osteoblasts. Because both TGF-β and PDGF regulate *pdgf a* and *b* gene expression, and because TGF β and PDGF are present in platelets, their release following platelet aggregation should act as a central step in the control of PDGF synthesis in the skeleton. This may prove critical for fracture healing. The subsequent induction of PDGF by TGF β and PDGF in the bone microenvironment may be a mechanism to ensure adequate and more constant levels of PDGF in skeletal tissue following platelet aggregation. Under basal conditions there may be no need for skeletal cells to be exposed to significant concentrations of PDGF, and its levels are low (Rydziel *et al.*, 1992; Rydziel *et al.*, 1994).

TABLE II Effects of PDGF on Skeletal Cells

Increases cell replication
Inhibits the differentiated function of the osteoblast
Decreases collagen synthesis and type I collagen expression
Decreases bone formation
Increases bone resorption
Increases collagen degradation and collagenase 3 expression
Decreases osteonectin expression
Increases osteopontin expression

IN VITRO ACTIONS OF PDGF ON SKELETAL CELLS

PDGF AA, BB, and AB are the isoforms studied more extensively in skeletal cells. They have similar biological actions, although PDGF BB is more potent than PDGF AA, and PDGF AB has intermediate activity (Centrella *et al.*, 1991). PDGF stimulates bone cell replication and DNA synthesis, both in intact calvariae and in isolated rat osteoblasts. It appears that the primary effect of PDGF in bone is related to its mitogenic activity (Table II) (Canalis *et al.*, 1989). As a consequence of this effect, PDGF has the potential to increase a pool of cells of the osteoblastic lineage, which could differentiate and express the osteoblastic phenotype. The mitogenic effect of PDGF is observed primarily in the periosteal layer, a zone rich in fibroblasts and preosteoblasts. It is possible that preosteoblastic cells, replicating under the influence of PDGF, differentiate into mature osteoblasts. However, PDGF inhibits the differentiation of stromal cells into cells of the osteoblastic lineage (Tanaka and Liang, 1995). This would indicate that the dividing cells affected by PDGF could remain in a proliferative undifferentiated state. Some cells may respond to other local signals, and differentiate toward a mature functional osteoblast. It is important to note, that in accordance with the impaired differentiation of cells of the osteoblastic lineage, PDGF inhibits the expression of the mature osteoblastic phenotype, and decreases mineral apposition rate, a marker of osteoblastic function and bone formation (Canalis *et al.*, 1989; Centrella *et al.*, 1992; Hock and Canalis, 1994). PDGF may have direct and indirect effects on skeletal cells because it inhibits the synthesis of bone morphogenetic proteins (BMP) and IGF-I and II; factors that enhance the differentiation and the function of the osteoblast (Table III) (Canalis *et al.*, 1993; Gabbitas *et al.*, 1994). PDGF causes a time- and dose-dependent inhibition of IGF I and II mRNA and polypeptide levels (Canalis *et al.*, 1993; Gabbitas *et al.*, 1994; Gangji *et al.*, 1998), and inhibits BMP-4 expression in osteoblasts (Pereira *et al.*, 2000).

TABLE III Effects of PDGF on Cytokine Expression in Osteoblasts

Decreases IGF I and IGF II synthesis
Increases IGF I receptor binding
Decreases IGFBP-5 synthesis
Decreases BMP-4 expression
Increases IL-6 transcription
Increases HGF/SF expression

The inhibition of IGF I and II and BMP-4 expression by PDGF correlates with its inhibitory effects on the differentiated function of the osteoblast, although this does not prove that the decrease in IGF I and II or BMP-4 mediates the effect of PDGF on osteoblastic function. Some of the effects of PDGF on the osteoblast, such as the inhibition of type I collagen transcription, occur following a short exposure to PDGF, suggesting direct effects on the differentiated function of the osteoblast. However, persistent or chronic exposure of skeletal cells to PDGF could result in a significant reduction of IGF and BMP levels, which may be ultimately responsible for a decrease in osteoblastic function and bone formation. The inhibitory effect of PDGF on IGF expression is not directly related to its mitogenic activity. In addition to the decrease in IGF I and IGF II synthesis, PDGF inhibits the transcription of IGF-binding protein (IGFBP) 5, a protein reported to regulate bone cell growth and bone formation (Canalis and Gabbitas, 1995). The interactions of PDGF with IGF I in skeletal cells appear relevant to the effects of PDGF in bone. PDGF modifies the synthesis of IGF I and II and of IGFBP-5, and opposes the effects of IGF I on bone collagen synthesis (Canalis *et al.*, 1989).

By increasing the number of osteoclasts, PDGF enhances bone resorption. In accordance with its effects on bone resorption, PDGF increases the synthesis of matrix metalloproteinase (MMP)-13 or collagenase 3 by the osteoblast (Holliday *et al.*, 1997; Varghese *et al.*, 1996). MMP-13 is a proteinase capable of initiating the degradation of type I collagen at neutral pH, and is required for bone resorption (Zhao *et al.*, 1999). Mice with a mutation of the *collagenase 3* or of the *type I collagen* gene that causes resistance to collagenase 3 cleavage, fail to resorb bone following exposure to parathyroid hormone (Zhao *et al.*, 1999). However, other proteases, particularly those biologically active at acid pH, are relevant to the bone resorptive process.

PDGF enhances collagenase 3 expression by transcriptional and post-transcriptional mechanisms, an effect mediated by the activator protein-1 (AP-1) family of transcription factors (Varghese *et al.*, 1996). Targeted mutations of an AP-1 binding site in the promoter region of the *collagenase 3* gene decrease the basal and PDGF BB-induced transcriptional activity (Varghese *et al.*, 1996). Electrophoretic mobility shift assays reveal enhancement of AP-1 nuclear protein complexes by PDGF BB, and PDGF-dependent interactions of c-Fos, Fos B, Fra-2, c-Jun, Jun B, and Jun D with AP-1 sequences present in the *collagenase 3* gene. The mechanism involves a rapid induction of these transcription factors by PDGF (Rydziel *et al.*, 2000). The elements and cytosolic proteins responsible for the post-transcriptional regulation of *collagenase 3* have not been reported.

The induction or activation of members of the AP-1 family of transcription factors by PDGF BB is consistent with the mitogenic activity of the growth factor and with its stimulatory effects on bone resorption (Angel and Karin, 1991). c-Fos is essential for osteoclast differentiation and mice with null mutations of *c-fos* exhibit osteopetrosis and decreased bone remodeling (Grigoriadis *et al.*, 1994). PDGF may have direct and indirect effects on bone resorption, as it increases the transcription of *interleukin* (*IL*)*-6* in osteoblasts, and IL-6 enhances the recruitment of osteoclasts and is critical to the process of bone resorption (Jilka *et al.*, 1992). The effect of PDGF on *IL-6* transcription, like that on *collagenase 3*, is mediated by members of the AP-1 family of transcription factors (Franchimont *et al.*, 1999). Changes in IL-6 expression could play a role not only in the actions of PDGF in bone resorption, but also in its effects on collagenase 3 expression and matrix breakdown, as IL-6 is an inducer of *collagenase 3* expression in osteoblasts (Franchimont *et al.*, 1997).

PDGF RECEPTORS AND BINDING PROTEINS

PDGF can interact with either one of two PDGF receptors, namely the PDGF receptor (PDGFR) α and β. These receptors have differential binding specificity for the various PDGF dimers (Claesson-Welsh *et al.*, 1989; Gronwald *et al.*, 1988; Seifert *et al.*, 1989; Yarden *et al.*, 1986). PDGFR α ligates PDGF A, B, and C chains, and PDGFR β binds PDGF B and D chains (Fredriksson *et al.*, 2004; Reigstad *et al.*, 2005). The two PDGF receptors are structurally and functionally related, and PDGF binding results in receptor dimerization and the formation of PDGF αα, ββ, and αβ receptor dimers (Reigstad *et al.*, 2005). For receptor activation, PDGF AA and PDGF CC require PDGFR αα, or αβ dimers, PDGF DD requires PDGFR ββ, or αβ dimers, whereas PDGF AB and PDGF BB can activate PDGFR αα, αβ, or ββ dimers. Cells of the osteoblastic lineage express PDGF α and β receptors. PDGF binding to its osteoblast receptor results in receptor dimerization, and activation of tyrosine kinase activity, leading to activation of protein kinase C, and intracellular calcium signaling pathways (Claesson-Welsh, 1994; Heldin *et al.*, 1989).

PDGF activity in osteoblasts can be regulated by changes in PDGF receptor number and affinity. In rodent, but not in human osteoblasts, IL-1 increases PDGF α receptor transcripts and the binding and mitogenic activity of PDGF AA (Centrella *et al.*, 1992; Gilardetti *et al.*, 1991; Tsukamoto *et al.*, 1991). Fibroblast and epidermal growth factors increase PDGF α receptor expression in osteoblastic cell lines (Tsukamoto *et al.*, 1991). TGF-β decreases PDGF binding, and hormones have no effect on PDGF binding to osteoblasts (Kose *et al.*, 1996). Information on the activity of PDGF CC and PDGF DD and receptor regulation in skeletal cells is limited. PDGF CC interacts with PDGF αα and αβ receptor dimers, and has potent mitogenic activity for mesenchymal cells, as well as angiogenic properties (Gilbertson *et al.*, 2001). These properties of PDGF CC could be important during the vascularization of endochondral bone formation, as it has been reported for VEGF. PDGF DD interacts with PDGF β receptors, has mitogenic activity for vascular cells and induces tissue fibrosis, but its effects on the skeleton are not known (Ponten *et al.*, 2005).

Although there is considerable information about the existence and function of specific binding proteins for many growth factors, including IGFs and TGF β, less is known about specific PDGF binding proteins. PDGF BB binds to α_2-macroglobulin and osteonectin or secreted protein acidic and rich in cysteine (SPARC), and PDGF is stored in the extracellular matrix by its association to osteonectin, as well as to other extracellular matrix proteins (Crookston *et al.*, 1993; Kelly *et al.*, 1993; Lane and Sage, 1994; Raines *et al.*, 1992). The interactions between PDGF and osteonectin may be important, as osteonectin, by binding PDGF B chains, may modify its binding to specific receptors and activity or may prolong its half-life. Skeletal cells secrete substantial amounts of osteonectin, and osteonectin plays a role in bone remodeling and angiogenesis. *Osteonectin* null mice exhibit decreased bone formation and decreased osteoblast number, leading to decreased bone remodeling with a negative bone balance and osteopenia (Delany *et al.*, 2000). The activity of PDGF in the *osteonectin* null state has not been tested. It is possible that changes in the expression of osteonectin in skeletal cells play a role in the regulation of PDGF activity. In chondrocytes, PDGF increases osteonectin expression, whereas in osteoblasts, PDGF BB and fibroblast growth factor 2 (FGF 2) decrease the expression of this matrix protein (Chandrasekhar *et al.*, 1994; Delany and Canalis, 1998). FGF 2 acts by destabilizing osteonectin transcripts, but the mechanisms involved in the action of PDGF on osteonectin expression have not been explored.

IN VIVO ACTIONS OF PDGF ON THE SKELETON

Although there is considerable knowledge about the actions of PDGF *in vitro*, information about its effects on the skeleton *in vivo* is limited. Consistent with its mitogenic effects, the systemic administration of PDGF BB to ovariectomized rats prevents bone loss, and increases the number of osteoblasts and as a consequence bone formation (Mitlak *et al.*, 1996). This suggests that, *in vivo*, the mitogenic effects of PDGF on preosteoblasts result in an increased number of osteoblasts, which are capable of forming bone. Osteoclast number was not altered by systemically administered PDGF. This is in contrast to the stimulatory effects of PDGF on osteoclastogenesis *in vitro*, but may be related to the model used because ovariectomy causes a substantial increase in bone resorption and remodeling. This would preclude an additional effect by PDGF on bone resorption. Topical application of PDGF to craniotomy defects in rodents stimulates soft tissue repair, but not osteogenesis (Marden *et al.*, 1993). The effects of PDGF on endothelial cell proliferation and angiogenesis probably contribute to the process of wound healing (Deuel *et al.*, 1991). PDGF induces the expression of hepatocyte growth factor/scatter factor (HGF/SF) in osteoblasts, and HGF/SF plays a role in tissue repair (Blanquaert *et al.*, 1999; Blanquaert *et al.*, 2000; Strain, 1993). Consequently, its induction by PDGF may also be relevant to the process on wound healing.

Studies of gain and loss of function mutations in mice have provided important information on the physiological role of PDGF during development and after birth. Null mutations of *pdgf b*, *pdgf α* and *β* receptors are lethal before birth, and *pdgf a* null deletions cause prenatal and perinatal death (Betsholtz, 2004). Because of their lethality, these models have not allowed for the study of the function of PDGF in the adult skeleton. *Pdgf b* and *pdgf β* receptor null mice exhibit vascular defects and absent renal mesangial cells (Leveen *et al.*, 1994; Soriano, 1994). *Pdgf a* and *pdgf α* receptor null mutants display defective alveolar formation leading to emphysema, and reduced intestinal villi, thin dermis, and spermotogenic arrest, but *pdgf a* null mutants do not manifest a skeletal phenotype (Fruttiger *et al.*, 1999; Tallquist and Soriano, 2003). *Pdgf c* null mice exhibit neonatal lethality and various skeletal developmental abnormalities, including cleft palate and spina bifida (Ding *et al.*, 2004). Similar defects are observed in *pdgf α* receptor null mice, which exhibit a phenotype characterized by embryonic lethality, cleft face, spina bifida, and vascular and skeletal defects (Fruttiger *et al.*, 1999; Tallquist and Soriano, 2003). Although PDGF B can interact with the PDGF α receptor, loss of function mutations of the *pdgf b* gene do not resemble the *pdgf α* receptor null phenotype, indicating that the functions of the PDGF α and β receptors are not redundant (Leveen *et al.*, 1994; Soriano, 1994). The phenotype of the *pdgf d* gene deletion has not been reported, but overexpression of PDGF DD, like that of PDGF CC, results in tissue fibrosis (Campbell *et al.*, 2005; Ponten *et al.*, 2005).

ROLE OF PDGF IN SKELETAL FUNCTION

The mitogenic activity of PDGF and its release by platelets suggests a role in wound healing and fracture repair. This is supported by the demonstration of *pdgf a* and *b* gene expression at fracture sites, and the effects of PDGF on endothelial cell proliferation and angiogenesis. However, the effects of PDGF on wound healing are short-lived, and there is limited information regarding the possible effectiveness of PDGF in fracture repair. Because the *pdgf a* and *b* genes are expressed by a variety of malignant cells, including osteosarcoma, PDGF may play a role in tumorigenesis, a possibility supported by the near identity of the *pdgf b* gene and *p28-sis*. In addition, the Wilms tumor gene product WT1 represses transcription of the *pdgf a* gene, and a loss of WT1 or related represser activities could contribute to the pathogenesis of Wilms tumors (Gashler *et al.*, 1992). PDGF does not appear to play a role in the maintenance of bone mass in view of its inhibitory effects on the differentiated function of the osteoblast. Systemic administration of PDGF to estrogen-deficient rats prevents vertebral bone loss (Mitlak *et al.*, 1996). However, the mitogenic activity of PDGF and the fact that its systemic administration causes soft tissue fibrosis would limit its use. Topical application of PDGF to rat craniotomy defects results in increased soft tissue repair, but not increased osteogenesis, also placing limitations on the potential use of PDGF (Marden *et al.*, 1993).

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Chapter 51

Fibroblast Growth Factor (FGF) and FGF Receptor Families in Bone

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FIBROBLAST GROWTH FACTORS AND BONE

Studies have demonstrated an important role for members of the heparin-binding fibroblast growth factor (FGF) family and their receptors in normal limb development, bone growth, and fracture repair and the inherited human osteo- and chondrodysplastic syndromes with their attendant skeletal abnormalities. FGF-1 and FGF-2 are potent agonists with stimulatory effects on bone resorption and both stimulatory and inhibitory effects on bone formation. The inhibitory effects on collagen synthesis have been demonstrated in cell and organ culture and appear to involve inhibition of transcription of the collagen gene and to be independent of effects on cell replication. The resorptive effects of FGFs appear to be both prostaglandin dependent and independent and also involve the replication and differentiation of osteoclasts. *In vivo*, exogenous FGF-1 and FGF-2 increase bone formation by multiple mechanisms. The effects of FGF may be mediated in part by transforming growth factor β (TGF β), bone morphogenetic protein-2 (BMP-2), and prostaglandins (PGs), because the production of these factors can be regulated by FGFs. These studies emphasize the important effects of FGFs on the control of skeletal cells (Hurley *et al.*, 2002; Marie *et al.*, 2002). Extensive studies demonstrate an important role for FGF23 in phosphate transport and this topic is reviewed in a separate chapter.

EXPRESSION OF FGF RECEPTORS IN BONE

The expression of FGFs and FGF receptors (FGFRs) is regulated temporally and spatially during skeletal development. Several FGF members are expressed during early stages of development in mammals (Yamaguchi and Rossant, 1995; Niswander, 1996), and their expression is related to skeletal development (Johnson and Tabin, 1997; Naski and Ornitz, 1998). During intramembranous bone formation, FGF-2 transcripts are found in mesenchymal cells and osteoblasts (Mehrara *et al.*, 1998; Rice *et al.*, 2000), together with FGF-9 (Kim *et al.*, 1998). During skeletal development, FGF-1 is expressed in proliferating and hypertrophic chondrocytes; FGF2 is present in resting and proliferating chondrocytes whereas FGF17 and 19 are widely expressed (Krejci *et al.*, 2007). FGFs 2, 7,18 and 22 are expressed in postnatal growth plate (Lazarus *et al.*, 2007). In the developing bone, FGF-2, -9, -18, and -20 are expressed in the calvaria and limbs (Hajihosseini and Heath, 2002). FGF-2 is produced by and stored in the extracellular matrix (Globus *et al.*, 1989; Hurley *et al.*, 1994).

FGF's actions are dependent on the expression and interactions with FGFRs. During skeletal tissue development, the spatiotemporal pattern of FGFR-1, -2, and -3 expression differs (Hughes *et al.*, 1997; Orr-Urtreger *et al.*, 1991; Wanaka *et al.*, 1991; Peters *et al.*, 1992; Patstone *et al.*, 1993; Wilke *et al.*, 1997; Delezoide *et al.*, 1998). During endochondral development, FGFR-1 transcripts are expressed in the mesenchyme and later in hypertrophic cartilage and osteoblasts, whereas FGFR-2 is expressed at sites of ossification and later in cartilage, perichondrium, and periosteum (Peters *et al.*, 1992; Delezoide *et al.*, 1998).

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In contrast, FGFR-3 transcripts are found mainly in proliferating and prehypertrophic chondrocytes (Naski *et al.*, 1998) and are not found in mesenchymal cells or perichondral or periosteal cells until late stages of development (Peters *et al.*, 1993; Delezoide *et al.*, 1998). Consistently, the expression of FGFR-3 increases during the development of chondrogenesis *in vitro* (Szebenyi *et al.*, 1995; Molténi *et al.*, 1999a). FGF/FGFR3 signaling also has an essential role in mandibular skeletogenesis by facilitating the elongation of Meckel's cartilage, development of osteogenic condensations, and appositional growth of mandibular bones (Mina *et al.*, 2007).

In the human cranial vault, FGFR-1 immunostaining is localized in proliferating mesenchymal cells and preosteoblasts, whereas FGFR-2 and FGFR-3 immunostaining is found mainly with FGF-2 in preosteoblasts and osteoblasts (Delezoide *et al.*, 1998). In the mouse, FGFR-2 transcripts are present at the osteogenic fronts of the cranial suture (Iseki *et al.*, 1997; Kim *et al.*, 1998, Johnson *et al.*, 2000), and FGFR-2 expression is associated with endogenous FGF-2 expression (Mehrara *et al.*, 1998; Most *et al.*, 1998; Rice *et al.*, 2000). *In vitro*, FGFR-1 and FGFR-2 immunostaining is also found in neonatal rat calvaria cells (Molténi *et al.*, 1999a) and in human calvaria osteoblastic cells (Debiais *et al.*, 1998). Interestingly, FGFR1 and FGFR2 expression is age-related in calvaria cells (Cowan *et al.*, 2003). Although the spatiotemporal pattern of expression of FGF and FGFRs suggests that skeletal development is controlled by FGF/FGFR interactions, the FGF effects on bone cells may be modulated by the various affinity and specificity of FGF binding to the various alternative splice forms of FGFRs (Ornitz *et al.*, 1996).

FGF RECEPTORS AND HUMAN CHONDRODYSTROPHIES

FGFs have profound effects on chondrocyte development, proliferation and differentiation. The effects span stages of chondrocyte biology from chondrogenesis through the final steps of chondrocyte hypertrophy. Not surprisingly the effects of FGFs are complex and incompletely understood. The emerging data demonstrate that FGFs can both stimulate and suppress chondrocytes, depending on the stage of differentiation (Bobick and Kulyk, 2006; Bobick *et al.*, 2007; Mancilla *et al.*, 1998).

A number of human skeletal dysplasias, including chondrodysplasias and craniosynostosis syndromes have been linked genetically to mutations in FGFR1, FGFR2 and FGFR3 (Ornitz, 2005; Lajeunie *et al.*, 1999). Mutations in FGFR3 cause achondroplasia and others chondrodysplasias and dwarfism in humans. The mechanisms underlying these disorders are now better understood (Chen and Deng, 2005; Ornitz, 2005). The constitutive FGFR3 activation was found to be related to increased ligand stabilization, prolonged signaling, ligand-independent receptor activation or altered receptor conformation, resulting in constitutive activation of the receptor (Hart *et al.*, 2001; Sorokin *et al.*, 1994; Mohammadi *et al.*, 1996). In humans, mutations in FGFR-3 cause constitutive activation of FGFR-3, resulting in chondrodysplasia syndromes, including achondroplasia, the most common form of dwarfism, hypochondroplasia, and thanatophoric dysplasias (Wilkie, 2005), which demonstrates a role for FGFR-3 in the control of long bone cartilage growth. Several FGFR-3 mutations cause abnormal chondrogenesis in the mouse (Chen *et al.*, 1999; Segev *et al.*, 2000). Dwarfism induced by activating mutations in FGFR3 is associated with STAT activation, resulting in inhibition of proliferation of growth plate chondrocytes (Chen *et al.*, 1999; Li *et al.*, 1999). These skeletal abnormalities induced by FGFR3 activation result from abnormal activation of STAT1, STAT5 and p21 (Su *et al.*, 1997); Legeai-Mallet *et al.*, 1998; Sahni *et al.*, 1999; Yoon *et al.*, 2006). Additionally, FGFR3 mutants activate ERK1/2 signaling (Lievens *et al.*, 2005). Inhibition of MAPK pathway was found to rescue achondroplasia induced by FGFR3 activation in mice (Yasoda *et al.*, 2004). Additionally, pRb family members and PI3-kinase contribute to the effects of FGF receptor 3 on chondrocyte proliferation (Priore *et al.*, 2006; Raucci *et al.*, 2004). Stimulation of cultured chondrocytic cells with FGF causes rapid hypophosphorylation of the pRb family protein, p107. With slower kinetics, p130 and pRb are also dephosphorylated (Dailey *et al.*, 2003). Dephosphorylation of the Rb proteins inhibits proliferation by binding to E2F proteins and subsequently inhibiting expression of E2F target genes required for proliferation (Dailey *et al.*, 2003). Significantly, both the MAP kinase and PI3-kinase pathways regulate the dephosphorylation of pRb family proteins in response to FGF (Dailey *et al.*, 2003; Raucci *et al.*, 2004), suggesting that signals transmitted by FGF receptor 3 converge at this point.

Also, constitutive activation of FGFR-3 induced by the R248C mutation triggers premature apoptosis, which is mediated by activation of the STAT1 signaling pathway (Legeai-Mallet *et al.*, 1998). Human FGFR3 mutant chondrocytes also display increased apoptosis (Legeai-Mallet *et al.*, 1998) and treatment with IGF-1 can prevent the apoptosis induced by FGFR3 mutation through PI3K and MAPK pathways (Koike *et al.*, 2003). Thus, several mechanisms induced by FGFR3 signaling appear to be involved in the control of endochondral ossification (Ornitz, 2005).

Our understanding of how FGF signaling integrates with other cytokine signaling pathways during endochondral bone development is expanding. Of particular interest is the interaction of FGF and C-type natriuretic peptide (CNP). CNP is an essential regulator of endochondral bone development, as evidenced by the dwarfism resulting from disruption of the CNP gene of mice (Chusho *et al.*, 2001) or inherited mutations of the CNP receptor in humans (Bartels *et al.*, 2004). CNP regulates skeletal growth in part by antagonizing FGF signaling. This has been demonstrated both *in vivo* and *in vitro*. Acting through natriuretic peptide receptor B, CNP inhibits the activation of MAP kinases by FGFs. In this way, CNP reverses the dwarfing condition caused by an activated FGFR-3 (Yasoda *et al.*, 2004). Cell culture experiments have corroborated these findings. Through blockade of MAP kinase activation, CNP repressed the inhibition of proliferation caused by FGF (Krejci *et al.*, 2005; Ozasa *et al.*, 2005). Interestingly, CNP may also moderate the effects of FGF on chondrocyte differentiation. This is supported by data showing that CNP partially blocked the induction of matrix metalloproteinases caused by FGF-2 (Krejci *et al.*, 2005).

FGFs also interact directly with the Wnt signaling cascade. This is demonstrated by results showing that the expression of certain FGFs like FGF-18 requires canonical Wnt signaling. The Wnt pathway increases the levels of the transcription co-activator β-catenin. β-Catenin induces FGF18 expression through binding to TCF-Lef transcription factors localized to the transcription start site (Reinhold and Naski, 2007). Significantly, canonical Wnt signaling inhibits chondrocyte development and differentiation (Chimal-Monroy *et al.*, 2002; Enomoto-Iwamoto *et al.*, 2002; Hartmann and Tabin, 2000, 2001; Stott *et al.*, 1999). Because FGFs are induced by Wnt signaling and FGFs inhibit early chondrogenesis (Bobick *et al.*, 2007; Buckland *et al.*, 1998; Moftah *et al.*, 2002), the inhibitory actions of Wnt may be a consequence of FGF induction. Consistent with this, the inhibitory effects of a Wnt mimetic on chondrocyte proliferation were abrogated in bone rudiments from FGFR-3 null mice (Kapadia *et al.*, 2005). In the future, it will be important to know which of the broader activities of Wnt signaling are mediated through the induction of FGFs and signaling via FGFRs.

FGF RECEPTORS AND HUMAN CRANIOSYNOSTOSIS

The important role of FGFRs during cranial development is supported by genetic evidence that FGFR mutations induce abnormal ossification of the cranial sutures (craniosynostosis) in humans (Lajeunie *et al.*, 1999; Ornitz and Marie, 2002; Wilkie, 2005). Several mutations in FGFR 1 or FGFR2 induce craniofacial abnormalities, causing Apert, Crouzon, Pfeiffer, and Jackson-Weiss syndromes (Wilkie, 2005). Genetic analyses indicated that mutations in the Ig III domain or in the linker between the Ig II and Ig III domain of FGFR-1 and FGFR-2 induce constitutive activation of the receptor (Neilson and Friesel, 1995, 1996; Robertson *et al.*, 1998; Plotnikov *et al.*, 2000). In Crouzon syndrome, the C342Y mutation in FGFR-2 results in the activation of FGFR-2 signaling and decreased binding of FGF-2 to the receptor (Mangasarian *et al.*, 1997). In Apert syndrome, FGFR-2 mutations enhance receptor occupancy by FGF ligands, prolongation of the duration of receptor signaling (Park *et al.*, 1997; Anderson *et al.*, 1998), enhancement of ligand binding (Ibrahami *et al.*, 2001, 2004) or loss of ligand binding specificity to FGFR2 (Yu *et al.*, 2000). A clue to the cellular and molecular mechanisms underlying the phenotype induced by FGFR genetic mutations came from studies in humans with FGFR2 mutations (Marie *et al.*, 2002). FGFR-2 mutations in Apert syndrome accelerate subperiosteal osteogenic differentiation without affecting cell proliferation (Fragale *et al.*, 1999; Lomri *et al.*, 1998; Lemonnier *et al.*, 2000; Tanimoto *et al.*, 2004), a phenotype also found in human nonsyndromic craniosynostosis (DePollack et al 1996; Fragale *et al.*, 1999). Apert mutations of FGFR-2 constitutively increase osteoblast marker genes in calvaria preosteoblasts in part through activation of PKC expression, phosphorylation and activity (Fragale *et al.*, 1999; Lemonnier *et al.*, 2000), resulting in increased N-cadherin–mediated cell-cell adhesion (Lemonnier *et al.*, 2001a). The S252W Apert FGFR2 mutation also downregulates the expression and activity of Src family members Fyn and Lyn in human osteoblasts, which contributes to the premature differentiation phenotype (Kaabeche *et al.*, 2004). This phenotype and underlying signaling mechanisms are consistent with the *in vivo* phenotype in fused cranial sutures in humans (Marie *et al.*, 2002; Wilkie, 2005).

In mice, activating FGFR mutations induce variable phenotypes. *In vitro* analyses showed that activating FGFR2 mutations stimulate calvaria bone cell proliferation, but inhibit mineralization (Mansukhani *et al.*, 2000; Ratisoontorn *et al.*, 2003). *In vivo*, unchanged or increased osteoblast proliferation and differentiation were reported in mice expressing FGFR1 or FGFR2 mutations (Zhou *et al.*, 2000; Chen *et al.*, 2003). Also, activating Apert and Crouzon FGFR2 mutations increase cell proliferation and decrease osteoblast differentiation in mice (Mansukhani *et al.*, 2005). However, conditional inactivation of FGFR2 affects the proliferation of osteoprogenitors, but not the differentiation, of mature osteoblasts in mice (Yu *et al.*, 2003). Moreover, the cell phenotype induced by FGFR2 activation varies with the sutures examined (Wang *et al.*, 2005). Although there are discrepancies in the phenotype induced by point mutations in mice and natural mutations in humans, which may relate to the distinct environmental factors or genetic background, the resulting effect is increased osteogenesis (Marie *et al.*, 2002; Wilkie, 2005).

Several data suggest that the accelerated osteogenesis induced by activation of FGFR signaling may result in part from increased expression of Runx2. Activating FGFR1 and FGFR2 mutations, or a gain-of-function mutation in FGFR2c in the mouse causes premature osteoblast differentiation associated with increased expression of Runx2 (Zhou *et al.*, 2000; Baroni *et al.*, 2004; Eswarakumar *et al.*, 2004). The P253R and S252W FGFR2 mutations were also found to increase Runx2 expression in human calvarial osteoblasts from Apert patients (Tanimoto *et al.*, 2004; Baroni *et al.*, 2005) and increased FGFR2 expression also correlates with Runx2 expression in human cranial osteoblasts (Guenou *et al.*, 2005). Other transcription factors may however be involved downstream of FGFR signaling. For example, FGFR2 activating mutations increase Sox2 expression in murine osteoblasts (Mansukhani *et al.*, 2005). Recent analysis of cell signaling in murine osteoblasts expressing Apert or Crouzon FGFR2 mutations revealed that activation of FGFR downregulates Wnt target genes (Mansukhani *et al.*, 2005) suggesting that Wnt signaling may also be involved in the phenotype induced by FGFR2 activation. Although this may provide a molecular mechanism for the premature osteoblast differentiation and cranial suture ossification induced by FGFR2 signaling, the role of transcription factors or Wnt signaling in human syndromic craniosynostosis is unknown.

Another issue in FGFR signaling is the role of FGFR downregulation in the control of skeletal cells. Constitutive activation of FGFR2 in Apert syndrome accelerates FGFR downregulation in mutant osteoblasts *in vitro* and *in vivo* (Lemonnier *et al.*, 2000), which is consistent with the decreased expression of FGFR-2 in Crouzon syndrome (Bresnick *et al.*, 1995). The ubiquitin ligase Cbl was found to control FGFR1 degradation after ligand activation (Wong *et al.*, 2002). Similarly, FGFR2 activation in Apert syndrome induces Cbl-mediated FGFR2 proteasome degradation, as well as Src protein downregulation, which results in increased expression of early markers of osteoblast differentiation (Kaabeche *et al.*, 2004). Activated FGFR2 also shows increased binding to the adaptor protein FRS2 (Hatch *et al.*, 2006), an important molecule involved in the negative feedback mechanism induced following FGFR stimulation (Eswarakumar *et al.*, 2006). Uncoupling between the docking protein FRS2 alpha and activated Crouzon-like FGFR2c mutant results in normal skeletal development in mice (Eswarakumar *et al.*, 2006). These studies emphasize the role of FRS2 alpha and Cbl in the attenuation of signals induced by FGFR activation. In marked contrast, activated FGFR3 in achondroplasia escapes Cbl-mediated ubiquitination and lysosomal degradation, resulting in amplification of the FGFR3 signal in the growth plate (Cho *et al.*, 2004). The kinase activity of mutant FGFR3 affects receptor trafficking, resulting in increased signaling capacity (Lievens *et al.*, 2005).

FGFR2 mutations also promote apoptosis in mature osteoblasts *in vitro* and *in vivo*. Constitutive activation of FGFR2 signaling by the C342Y Crouzon and the S252W Apert FGFR-2 mutations promote apoptosis in mouse or human cranial osteoblasts (Chen *et al.*, 2003; Mansukhani *et al.*, 2000; Lemonnier *et al.*, 2001b; Kaabeche *et al.*, 2005). The increased apoptosis in Apert osteoblasts is mediated by increased IL-1 and Fas expression, activation of caspase-8 and increased Bax/Bcl-2 levels (Lemonnier *et al.*, 2001b). FGFR2 activation also results in Cbl-mediated alpha 5 integrin ubiquitination and degradation, which contributes to osteoblast apoptosis (Kaabeche *et al.*, 2005). It is unclear whether the increased apoptosis is a cause or a consequence of the increased osteogenesis. The upregulation of apoptosis by constitutive FGF signaling may be a significant mechanism controlling osteoblast number and osteogenesis. Alternatively, apoptosis in mature osteoblasts may be a necessary event compensating for the accelerated osteoblast differentiation induced by FGFR2 signaling (Lemonnier *et al.*, 2001b). Further studies are required to determine the precise role of apoptosis induced by FGFR signaling during cranial suture formation. Microarray analyses revealed that apoptotic genes are altered by FGFR2 activation in human (Lomri *et al.*, 2001) and murine osteoblasts (Mansukhani *et al.*, 2005), but it is not known whether these genes are involved in the observed phenotype.

FGF AND CARTILAGE

FGF-2 is expressed in chondrocytes *in vivo* (Twal *et al.*, 1994) and may be an autocrine growth factor (Luan *et al.*, 1996). Exogenous FGF-2 stimulates chondrocyte cell growth and inhibits chondrocyte differentiation (Kato *et al.*, 1990; Wroblewski *et al.*, 1995), *In vitro*, several FGF members stimulate chondrocyte proliferation (Praul *et al.*, 2002). Recent data indicate that FGF-18 stimulates the proliferation of cultured chondrocytes through activation of FGFR3 (Liu *et al.*, 2007) and p38 MAPK signaling (Shimoaka *et al.*, 2002). *In vivo*, FGF-2 overexpression induces chondrodysplasia and shortening of long bones in mice (Coffin *et al.*, 1995; Lightfoot *et al.*, 1997; Sobue *et al.*, 2005). Consistently, FGFRs were shown to be key regulators of endochondral bone growth *in vivo*. Although FGFR1 induces a mitogenic signal in cultured chondrocytes, activated FGFR1 signaling *in vivo* suppresses growth plate chondrocytes mitogenesis, resulting in achondroplasia-like dwarfism *in vivo* (Wang *et al.*, 2001). Recent data indicate that the mesenchymal FGFR-3c isoform is responsible for controlling chondrocyte proliferation and differentiation that mediate normal skeletal development, whereas the epithelial FGFR-3b isoform does not contribute toward this process (Eswarakumar and Schlessinger, 2007). The negative role of FGFR-3 in endochondral growth is emphasized by the finding that FGFR-3 $-/-$ mice show excessive long bone growth, associated with increased chondrocyte proliferation (Colvin *et al.*, 1996; Deng *et al.*, 1996). Overexpression of activated FGFR-3 in mice reduces chondrocyte cell proliferation (Naski *et al.*, 1998; Ornitz, 2000), showing that activated FGFR-3 signaling inhibits chondrocyte proliferation and decreases differentiation (Li *et al.*, 1999; Rozenblatt-Rosen *et al.*, 2002). The expression in the growth plate of Ihh and BMP4 was found to be downregulated in mice overexpressing FGFR-3 and increased in mice lacking FGFR-3, suggesting that FGFR-3 may act on multiple signaling molecules to control endochondral bone growth (Naski *et al.*, 1996; Ornitz, 2000b). Conversely, BMPs antagonize FGF signaling pathways in chondrocytes (Yoon *et al.*, 2006). The balance between proliferating and differentiating chondrocytes is likely to be controlled by interactions between FGF and BMP signaling (Minina *et al.*, 2002).

FGFs have far-reaching effects on cartilage, and these effects vary depending on the stage of differentiation. A principal example is the effect of FGFs on chondrocyte proliferation. During formative stages of cartilage development FGFs stimulate chondrocyte proliferation (Liu *et al.*, 2007), whereas during later stages FGFs inhibit proliferation. A number of studies using cultured chondrocytes, which probably represent early or immature chondrocytes , show that FGFs stimulate proliferation. For example FGF-2 is expressed in chondrocytes *in vivo* (Luan *et al.*, 1996) and when added to cultured chondrocytes stimulates cell replication (Kato *et al.*, 1990; Wroblewski *et al.*, 1995). Other FGFs including 4, 9, and 18 also strongly induce chondrocyte proliferation (Praul *et al.*, 2002; Shimoaka *et al.*, 2002). These data compare with the effects of FGFs in the established epiphyseal growth plate where signaling through FGFR-3 clearly inhibits proliferation. Overexpression of FGF-2 induces a dwarfing condition that in many respects phenocopies that caused by an activated form of FGFR-3 (Coffin *et al.*, 1995; Lightfoot *et al.*, 1997; Naski *et al.*, 1998a; Ornitz, 2000). Further evidence that FGFR-3 inhibits chondrocyte proliferation comes for studies of FGFR-3 null mice that show enhanced chondrocyte proliferation and excessive long bone growth (Colvin *et al.*, 1996; Deng *et al.*, 1996). These effects are dictated almost exclusively by the FGFR-3c isoforms that results from alternative splicing the C-terminal portion of the extracellular domain (Eswarakumar and Schlessinger, 2007). FGFs also directly affect the postproliferative stages of chondrocyte development. Chondrocyte hypertrophy is inhibited by FGF signaling. This is shown both in *ex vivo* chondrocyte cultures (Kato *et al.*, 1990) and in mouse models expressing activated FGFRs (Naski *et al.*, 1998; Li *et al.*, 1999). The FGF-dependent pathways that regulate chondrocyte hypertrophy are incompletely understood and require further investigation. However, several studies show genetic or biochemical interactions of FGF with BMP- or CNP-dependent processes (Li *et al.*, 1999; Yoon *et al.*, 2006; Minina *et al.*, 2002; Yasoda *et al.*, 2004). These complex interactions are likely to form a self-regulating circuit that adjust the rate of differentiation in step with chondrocyte proliferation.

FIBROBLAST GROWTH FACTOR PRODUCTION AND REGULATION

Although there are 23 members of the fibroblast growth factor family (Fig. 1), only FGF-1, FGF-2, FGF-18, and FGF 23 have been studied extensively in bone. FGF-2

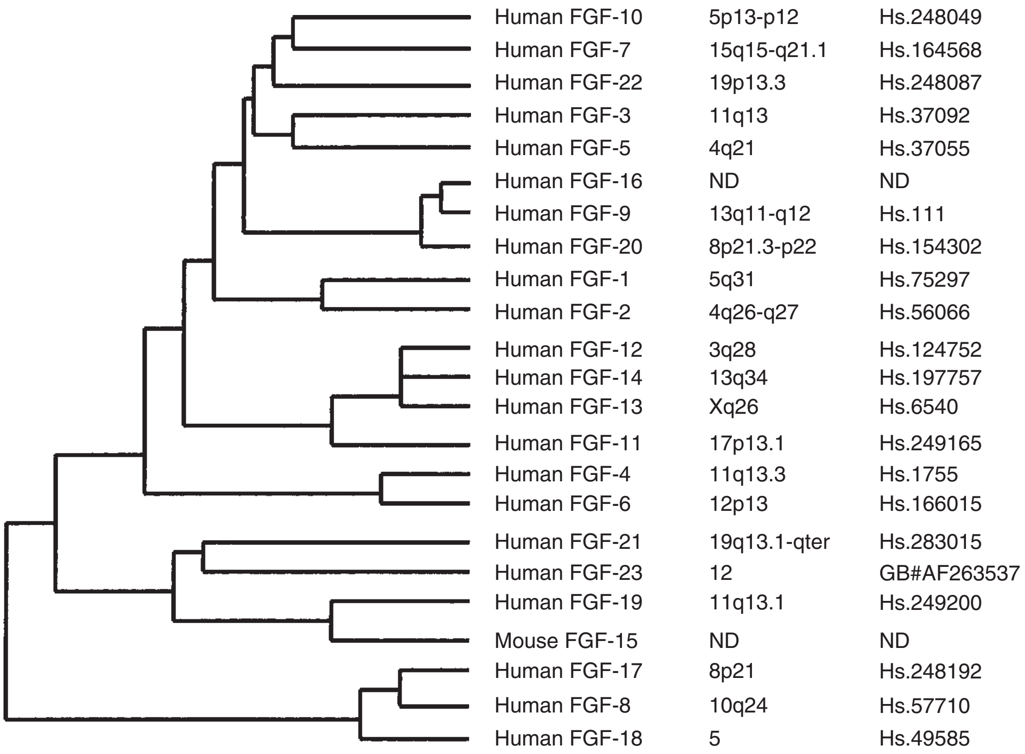
Human FGF-10 5p13-p12 Hs.248049
Human FGF-7 15q15-q21.1 Hs.164568
Human FGF-22 19p13.3 Hs.248087
Human FGF-3 11q13 Hs.37092
Human FGF-5 4q21 Hs.37055
Human FGF-16 ND ND
Human FGF-9 13q11-q12 Hs.111
Human FGF-20 8p21.3-p22 Hs.154302
Human FGF-1 5q31 Hs.75297
Human FGF-2 4q26-q27 Hs.56066
Human FGF-12 3q28 Hs.124752
Human FGF-14 13q34 Hs.197757
Human FGF-13 Xq26 Hs.6540
Human FGF-11 17p13.1 Hs.249165
Human FGF-4 11q13.3 Hs.1755
Human FGF-6 12p13 Hs.166015
Human FGF-21 19q13.1-qter Hs.283015
Human FGF-23 12 GB#AF263537
Human FGF-19 11q13.1 Hs.249200
Mouse FGF-15 ND ND
Human FGF-17 8p21 Hs.248192
Human FGF-8 10q24 Hs.57710
Human FGF-18 5 Hs.49585

FIGURE 1 The human FGF gene family is made up of 23 genes. A dendrogram depicting the sequence relationship between members of the family is shown along with their human chromosomal location and corresponding unigene number. For FGF-15, no human homologue has been published, and the chromosomal location of FGF-16 has not yet been determined.

and FGF-1 are found in the bone matrix (Hauschka *et al.*, 1986) and are produced by bone cells, with FGF-2 being 10-fold higher than FGF-1 when measured by radioimmunoassay (Seyedin *et al.*, 1985; Globus *et al.*, 1989). FGF 23 has been shown to be an important regulator of phosphate homeostasis (reviewed by Fukimoto and Yamashita, 2007). FGF 23 mRNA was detected in many fetal and adult tissues, with the highest expression observed in adult calvariae, femurs, and incisors. Immunoreactive FGF2 23 was primarily found in osteoblasts, cementoblasts, and odontoblasts, although sporadic labeling was detected in chondrocytes, osteocytes, and cementocytes as well as osteoclasts (Yoshiko *et al.*, 2007).

There are few data on the regulation of FGF expression in bone. In cultures of mouse osteoblastic MC3T3-E1 cells, exogenous FGF-2 increases its own mRNA (Hurley *et al.*, 1994). TGF-β also increases FGF-2 mRNA and protein in MC3T3-E1 cells (Hurley *et al.*, 1994). TGF-β increases FGF-2 protein in both the cytoplasm and the nucleus. However, there is no measurable FGF-2 in the conditioned medium. The significance of TGF-β regulation of FGF-2 in osteoblasts is not clear. Amplification of the responses to both FGF and TGF-β could occur by an increase in endogenous FGF. In addition, parathyroid hormone (PTH; Hurley *et al.*, 1999) and prostaglandins (Sabbieti *et al.*, 1999) increase FGF-2 mRNA and protein levels in bone cells, suggesting that anabolic factors for bone may act in part by stimulating endogenous FGF-2 in osteoblasts. Impaired bone formation (Hurley *et al.*, 2006)and hypercalcemic response (Okada *et al.*, 2003) to PTH in Fgf2 null mice support an important role for endogenous FGF-2 in the actions of PTH in bone. Interestingly, FGF-2 is an immediate-early gene induced by mechanical stress in osteoblasts, an effect that results in activation of the PKA and MERK1/2 pathways, suggesting that FGF-2 may mediate some of the osteogenic effects induced by mechanical forces (Li *et al.*, 2006).

Studies have shown that 1,25$(OH)_2D_3$ increased FGF23 expression in fetal rat calvarial cell cultures (Yoshiko *et al.*, 2007).

A DISTINCTIVE TRANSLATION INITIATION MECHANISM MEDIATES EXPRESSION OF FGF-2 AND FGF-3

Although all FGFs are encoded by single copy genes, multiple isoforms of both FGF-2 (Florkiewicz and Sommer, 1989; Prats *et al.*, 1989) and FGF-3 (Dickson and Acland, 1990) have been detected. The novel mechanism by which this occurs distinguishes these FGFs from all other known growth factors and ultimately may prove to be a critical point from which FGF-2 and FGF-3 gene expression is qualitatively regulated. Each of the multiple isoforms is a primary translation product with no precursor–product relationship. The larger isoforms are colinear amino-terminal extended versions of the shorter isoforms. Moreover, translation of each of the four high-molecular-weight (HMW) human FGF-2 isoforms (22, 23, and 24 kDa) is initiated with an unconventional CUG translation initiation codon; a 34-kDa isoform of FGF-2 has also been described (Arnaud *et al.*, 1999). In contrast, translation of the 18-kDa isoform is initiated with a classical AUG codon located downstream of the four CUG codons. Multiple HMW isoforms of rodent FGF-2 also initiate translation with CUG codons (Powell and Klagsbrun, 1991). Curiously, the rainbow trout FGF-2 gene appears to encode only an 18-kDa translation product, even though preferential CUG translation initiation from transfected human cDNA expression vectors does occur (Hata *et al.*, 1997). Likewise, the larger isoform of FGF-3 (31.5 kDa) utilizes a CUG codon, whereas the 28.5-kDa isoform uses an AUG codon to initiate translation (Dickson and Acland, 1990). In the case of FGF-2, CUG-initiated HMW isoforms are not minor translation products, and in some cases the HMW isoforms are expressed at levels equal to or greater than the 18-kDa isoform (Coffin *et al.*, 1995). Thus, the unusual utilization of CUG codons to initiate the translation of HMW FGF-2 effectively diversifies this single copy gene.

The uncommon use of CUG codons to initiate FGF-2 translation prompted speculation that selecting a CUG as opposed to AUG translation initiation codon may represent a physiologically relevant mechanism regulating FGF-2 gene expression. Three observations support this possibility. The first is that the CUG-initiated translation products of FGF-2 and FGF-3 localize to the nucleus preferentially, whereas their respective AUG-initiated isoforms are predominantly cytosolic or extracellular (Florkiewicz *et al.*, 1991). Second, the HMW isoforms of FGF-2 are expressed in a tissue-specific pattern distinct from the 18-kDa isoform that is maintained in transgenic model systems (Coffin *et al.*, 1995), as well as during development of the central nervous system (Giordano *et al.*, 1992). Third, partially purified HMW and 18-kDa FGF-2 isoforms have similar *in vitro* extracellularly mediated mitogenic activities (Florkiewicz and Sommer, 1989), but when synthesized in continuously expressing transfected cells, the HMW isoforms are transforming whereas the 18-kDa isoform is not (Quarto *et al.*, 1991). Although FGF-2 appears to be widely expressed when detected by immunohistochemical techniques and *in situ* hybridization, it is likely that the different molecular isoforms are expressed in a spatially and temporally restricted manner. Therefore, in order to put the complete FGF-2 picture into perspective, it will be necessary to determine the qualitative, as well as quantitative, patterns of expression.

As noted previously, the HMW isoforms of human FGF-2 initiate translation from CUG codons that are differentially recognized in a *de novo* pattern that is tissue specific. Regulating translation is one mechanism by which eukaryotic cells control gene expression, including start site selection followed by formation of a highly ordered translation initiation complex (Hershey *et al.*, 1996). In most circumstances, ribosomes arrive at the site of translation initiation after establishing contact and assembling at the 5′ end of eukaryotic mRNAs and then scan 3′ until encountering the most favorable combination of mRNA structure and sequence to initiate protein synthesis. However, in some cases, ribosomes recognize translation initiation sites directly without scanning, termed internal ribosome entry (Le and Maizel, 1997). The CUG-mediated translation initiation of FGF-2 HMW isoforms is an example of internal ribosome entry that is also maintained in a tissue-specific manner (Creancier *et al.*, 2000; Vagner *et al.*, 1995). A number of factors are likely to be involved in this process (Kevil *et al.*, 1995; Touriol *et al.*, 2000). Although the sophisticated cellular mechanism mediating CUG-initiated translation of HMW FGF-2 is being elaborated, their physiological significance remains an enigma. Interestingly, it is proposed that age-related changes in FGF2 isoform expression might affect osteogenesis. Studies in primary osteoblasts derived from calvariae of neonatal and adult rats demonstrated that adult osteoblasts produced more FGF2 and preferentially translated the 22-kDa isoform (Cowan *et al.*, 2003). Ultimately, selected expression in transgenic animal model systems will be necessary for a thorough analysis. In addition to the isoform-specific phenotypes reported previously, other publications show that 24-kDa FGF-2 modulates interleukin-6 promoter activity (Delrieu *et al.*, 1999) and cell migration (Piotrowicz *et al.*, 1999).

EXPORT OR SECRETION OF FGFs?

FGFs are known to be released from cells by two clearly distinct intracellular pathways: one that is or likely to be endoplasmic reticulum (ER)/Golgi dependent (FGF-3 through 10 and 15 through 23) and another that is ER/Golgi independent (FGF-1 and -2). Although considered to be prototypic members of the FGF family, FGF-1 and FGF-2 are distinctive in that they are found extracellularly but do not possess hydrophobic signal peptide sequences. Historically, this has been one of the most intriguing features of FGFs because their signal-transducing receptors are extracellular (Rifkin and Moscatelli, 1989). In contrast, with the exception of FGFs 11–14, the other FGFs contain typical or noncleaved internal hydrophobic amino acid signal sequences that function as ER-targeting motifs. Consequently, FGF-3 through 10 and 15 through 23 are secreted proteins. However, an alternative exocytic ER/Golgi-independent trafficking pathway must also exist because FGF-1 and FGF-2 are detected routinely on the cell surface or in the extracellular matrix (Bashkin *et al.*, 1992; Florkiewicz *et al.*, 1991; Folkman *et al.*, 1988; Gonzalez *et al.*, 1990), in vitreous fluid (Baird *et al.*, 1987), in cell culture media (Florkiewicz *et al.*, 1995), and in the urine (Nguyen *et al.*, 1994) and serum (Dietz *et al.*, 2000) of patients with cancer. Several key publications have demonstrated unequivocally the existence of an energy- and plasma membrane–dependent "export" pathway for FGF-2 pictured in Figure 2 (Florkiewicz *et al.*, 1991, 1995; Mignatti *et al.*, 1992). Evidence has also been presented for the involvement of a heat shock response mechanism in the export of FGF-1 (Jackson *et al.*, 1992). A number of proteins have been characterized as functional components of the intracellular trafficking "export" pathway for FGF-2 and FGF-1 (Friesel and Maciag, 1999). In addition, a critical FGF-1 and FGF-2 targeting signal(s) that mediates trafficking through the export pathway has been identified (Shi *et al.*, 1997). It is particularly interesting to note that interleukins 1α and β, which share structural homology with the FGF family, are also exported through a yet to be defined ER/Golgi-independent pathways (Muesch *et al.*, 1990). In any instance, it is likely that the export pathway(s) of FGF-1, FGF-2, and the interleukins is clinically important and of therapeutic value. Inhibitors of export would be predicted to interfere with the bioavailability of these potent growth-promoting polypeptides (Florkiewicz *et al.*, 1995).

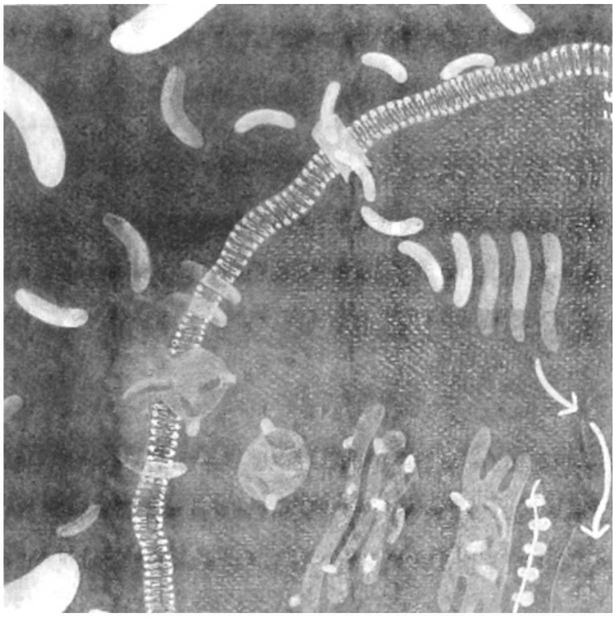

FIGURE 2 Two pathways traffic and release proteins from cells. One requires ER/Golgi, vesicular intermediates, and is signal sequence dependent. A second pathway exists for proteins without hydrophobic signal peptide sequences, such as 18-kDa FGF-2. These proteins are "exported" directly through a translocation apparatus located at the plasma membrane. The amino-terminally extended high-molecular-weight FGF-2 isoforms are targeted to the nucleus.

FIBROBLAST GROWTH FACTORS AND BONE FORMATION

Osteoblast Proliferation

FGF-1 and FGF-2 stimulate bone cell replication (Canalis 1980; Rodan *et al.*, 1987; Globus *et al.*, 1988, 1989; McCarthy *et al.*, 1989; Shen *et al.*, 1989). In osteosarcoma-derived ROS 17/2.8 cells, which express osteoblastic features, Rodan *et al.* (1989) found that FGF-2 stimulated cell proliferation when cells are seeded at low density in serum-free media but not when seeded at high density in the presence of serum. In contrast, in mouse osteoblastic MC3T3-E1 cells, FGF-2 stimulates cell proliferation in the presence of serum at both high and low density (Hurley *et al.*, 1993). FGF-1 and FGF-2 also activate the growth of rat and human stromal bone cells (Noff *et al.*, 1989; Pitaru *et al.*, 1993; Locklin *et al.*, 1995; Hanada *et al.*, 1997; Berrada *et al.*, 1995; Pri-Chen *et al.*, 1998). In human calvaria cells, FGF-2 also promotes cell proliferation at an early stage of differentiation (Debiais *et al.*, 1998). It is noteworthy that the mitogenic effect of FGF-2 on rat calvaria cells decreases with age (Kato *et al.*, 1995; Tanaka *et al.*, 1999). Recent data indicate that FGF-18, a novel member of the FGF family, stimulates the proliferation of cultured osteoblasts through activation of ERK signaling (Shimoaka *et al.*, 2002). Collective data suggest that FGF/FGFR signaling expands committed osteogenic population (Fakhry *et al.*, 2005).

Osteoblast Differentiation

In vitro FGFs inhibit markers of the osteoblast phenotype. FGFs reduce differentiation-related markers such as alkaline phosphatase (Rodan *et al.*, 1989; Shen *et al.*, 1989). FGF-2 inhibits parathormone responsive adenylcyclase activity (Rodan *et al.*, 1989). Type I collagen is the major product of the osteoblast, and FGF-2 affects its production. Both stimulatory and inhibitory effects of FGF-1 and FGF-2 on collagen synthesis in osteoblasts have been reported (Canalis *et al.*, 1988; McCarthy *et al.*, 1989; Shen *et al.*, 1989). Short-term treatment with FGF-2 results in the stimulation of collagen synthesis. However, continuous or chronic FGF treatment inhibits type I collagen synthesis. FGF-2 appears to have independent effects on osteoblast proliferation and differentiation. In bone organ cultures, the inhibitory effect of FGF-2 on collagen synthesis is similar in the presence or absence of aphidicolin, an inhibitor of cell replication (Hurley *et al.*, 1992b). Rodan *et al.* (1989) found that under conditions where FGF-2 had no effect on the proliferation of ROS 17/2.8 cells, there is a reduction in alkaline phosphatase activity, osteocalcin synthesis, and type I collagen mRNA. A similar dissociation is observed in chondrocytes where terminal differentiation and calcification are inhibited by FGF-2 independent of cell proliferation (Kato and Iwamoto, 1990).

The inhibitory effect of FGF-2 on collagen synthesis appears to be transcriptional (Rodan *et al.*, 1989; Hurley *et al.*, 1993) In studies in osteoblastic MC3T3-E1 cells, it has been shown that FGF-2 reduces the transcription of the type I collagen gene by nuclear run-on analysis (Hurley *et al.*, 1993). Studies on transcription of the collagen gene suggest that a DNA locus between −3.5 and −2.3 kb of the collagen promoter is negatively regulated by FGF-2 (Hurley *et al.*, 1993). The mechanism(s) by which FGF-2 negatively regulates collagen gene transcription is unknown.

FGF-2 also affects the expression of other bone matrix proteins. FGF-2 stimulates fibronectin expression through phospholipase C gamma, protein kinase C alpha, c-Src, NF-κB, and p300 pathway in osteoblasts (Tang *et al.*, 2007). FGF-2 also stimulates osteopontin and modulates osteonectin in rat osteosarcoma cells (Rodan *et al.*, 1989; Shiba *et al.*, 1995; Delany and Canalis, 1998a). Interestingly, osteonectin mRNA levels were reduced in osteoblasts from Fgf2 −/− and Fgf2 +/− mice (Naganawa *et al.*, 2006). Although FGF-1 inhibits osteocalcin expression in rat calvaria cells (Tang *et al.*, 1996), it enhances osteocalcin in bovine bone cells (Schedlich *et al.*, 1994) and directly affects osteocalcin transcription in mouse calvaria cells (Boudreaux *et al.*, 1996; Newberry *et al.*, 1996). In these cells, FGF-2 and cAMP have synergistic effects on osteocalcin expression, and some elements conferring FGF-2 responsiveness in the osteocalcin promoter have been identified (Schedlich *et al.*, 1994; Newberry *et al.*, 1997). FGF may also affect osteoblast function by acting on functional membranous proteins. FGFs downregulate the expression of connexin-43 associated with decreased gap junction-mediated communication (Shiokawa-Sawada *et al.*, 1997), and FGF-2 upregulates the expression of N-cadherin in osteoblasts (Debiais *et al.*, 2001).

Osteoblast Apoptosis

FGF signaling also controls osteoblast apoptosis. *In vitro*, FGF signaling protects from apoptotic effects induced by serum starvation (Hill *et al.*, 1997) and inhibits osteoblast death induced by peroxynitrite (Kelpke *et al.*, 2001). It was then found that FGF-2 induces osteoblast survival through a phosphatidylinositol 3-kinase-dependent pathway (Debiais *et al.*, 2004). Studies by Agas *et al.* (2007) showed that PGF2alpha increased osteoblast survival via endogenous FGF2/FGFR1 enhancement of Bcl-2/bax ratio. In contrast, FGF treatment induces apoptosis in more differentiated osteoblasts *in vivo* (Mathijssen *et al.*, 2001), and overexpression of FGF2 signaling in transgenic mice leads to increased apoptosis in mouse calvaria (Mansukhani *et al.*, 2000). Constitutive FGFR2 activation also induces apoptosis in more mature human calvaria osteoblasts (Lemonnier *et al.*, 2001b). This dual effect of FGF signaling on osteoblast apoptosis may be an important mechanism controlling osteoblastogenesis (Marie *et al.*, 2003).

FGF REGULATION OF GROWTH FACTORS IN BONE

FGF-2 has been shown to affect the production of several growth factors in osteoblasts. Continuous treatment with FGF-2 decreases IGF-I mRNA and protein levels in mouse osteoblastic MC3T3-E1 cells (Hurley *et al.*, 1992a) and rat bone cell cultures (Canalis *et al.*, 1993). This effect occurs independently of cell replication. Also, FGF-2 decreases the mRNA and protein levels for several IGF-binding proteins *in vitro*, in MC3T3-E1 cells (Hurley *et al.*, 1995a). In contrast to the studies of Chen *et al.* (1993), 24-hour treatment with FGF-2 does not inhibit IGF-binding protein mRNA in rat osteoblast-like cells. The inhibitory effects on IGFs and their binding proteins could play a role in the inhibition of bone formation by FGF. However, intermittent FGF-2 treatment increased bone nodule formation *in vitro* that was associated with increased IGF-1 expression (Zhang *et al.*, 2002). In addition, in ovariectomized rats, *in vivo* administration of FGF-2 caused a significant increase in IGF-I after 2 to 10 days of treatment (Power *et al.*, 2004).

Both FGF-1 and FGF-2 increase the production of TGF*β* *in vitro* and *in vivo* (Noda and Vogel, 1989). In addition, TGFβ and FGF-1 and FGF-2 interact to modulate their mitogenic effects in osteoblasts (Globus *et al.*, 1989). In human calvaria cells, FGF-2 decreases TGFβ2 production in immature cells but increases TGFβ2 synthesis by more differentiated cells (Debiais *et al.*, 1998). However, FGF-2 increases vascular endothelial cell growth factor (VEGF) expression in rat and mouse calvaria cells independently of TGFβ synthesis (Saadeh *et al.*, 2000). FGF-2 also induces hepatocyte growth factor expression in osteoblasts (Blanquaert *et al.*, 1999), which may play a role in bone repair. Additionally, FGF-2 modulates the release of VEGF through Erk1/2, p38, and SAPK/JNK signaling in osteoblasts (Takai *et al.*, 2007). Overall, the data suggest that these factors may perhaps mediate some of the actions of FGF on osteogenesis.

BONE FORMATION *IN VITRO* AND *IN VIVO*

Studies have shown that FGF-2 has both stimulatory and inhibitory effects on bone formation *in vitro*. FGF-1 reduces bone nodule formation in the rat calvaria system (Tang *et al.*, 1996), whereas FGF-2 inhibits osteoblast differentiation markers in human marrow stromal cells (Rifas *et al.* 1995; Berrada *et al.*, 1995; Martin *et al.*, 1997). However, FGF-2 increases cell differentiation and matrix mineralization in rat bone marrow stromal cells (Noff *et al.*, 1989; Pitaru *et al.*, 1993) and in dexamethasone-treated rat (Locklin *et al.*, 1995) and human bone marrow stromal cells (Pri-Chen *at al.*, 2000). Recent data indicate that FGF-8 also promotes the differentiation of mesenchymal stem cells into osteoblasts (Valta *et al.*, 2006). In human calvaria cells, FGF-2 initially reduces osteocalcin synthesis, whereas a prolonged treatment increases both osteocalcin synthesis and matrix mineralization, indicating that the effects of FGF-2 are dependent on the osteoblast maturation stage (Debiais *et al.*, 1998). Therefore, FGF signaling appears to regulate osteoblast proliferation and differentiation positively in the long term *in vitro*.

Multiple studies showed that FGF signaling enhances bone formation *in vivo*. In the cranial vault, the proliferation and differentiation of skeletal cells is tightly modulated by endogenous FGF signaling (Greenwald *et al.*, 2001; Moore *et al.*, 2002). Local injection of FGF-1 also promotes calvaria bone formation (Mundy *et al.*, 1999). Consistently, implantation of FGF-2 or FGF-4 accelerates suture closure in mice (Iseki *et al.*, 1997; Kim *et al.*, 1998). Interestingly FGF-9 overexpression in cranial mesenchymal cells can promote chondrogenesis (Govindarajan and Overbeek, 2006). At the endosteal level, FGF-2 at low dose also stimulates bone formation in growing rats (Aspenberg *et al.*, 1991; Nakamura *et al.*, 1995; Kawaguchi *et al.*, 1994; Mayahara *et al.*, 1993; Nagai *et al.*, 1995). In normal rabbits, intraosseous application of FGF-2 increases bone formation and bone mineral density (Nakamura *et al.*, 1997). A comparative study of the bone restorative efficacy of PTH, FGF-2, and a selective agonist for prostaglandin E receptor subtype in aged ovariectomized rats revealed that FGF-2 was the most effective in reversing severe cancellous osteopenia, followed by PTH. EP4 and PTH caused a similar increase in cortical bone mass (Iwaniec *et al.*, 2007).

Exogenous FGF-1 and FGF-2 appear to act by increasing the recruitment of osteoblast precursor cells, which then differentiate into osteoblasts (Nakamura *et al.*, 1995). It is also possible that FGF-2 acts indirectly by increasing the expression of TGF*β* in osteoblasts (Noda and Vogel, 1989; Nakamura *et al.*, 1995; Kawaguchi *et al.*, 1994). Additionally, FGF-2 enhances BMP-2- induced ectopic bone formation in mice by altering the expression of BMP receptors (Nakamura *et al.*, 2005), indicating that FGF-induced bone formation may be in part indirect.

Recent data point to an important role of FGF-18 in chondrogenesis and osteogenesis during skeletal development (Xiao *et al.*, 2004; Haque *et al.*, 2007). FGF-18 acts as a physiological ligand for FGFR3 in chondrocytes and through FGFR1/FGFR2 in osteoblasts (Liu *et al.*, 2002). FGF-18 deficiency in mice results in increased proliferation and differentiation, and conversely, decreases osteoblast proliferation and differentiation (Ohbayashi *et al.*, 2002). One mechanism of action appears to involve suppression of noggin expression and subsequent activation of BMP effects on chondrocyte differentiation (Reinhold *et al.*, 2004). Recent data indicate that FGF-18 may induce osteoblast recruitment through VEGF expression and subsequent neovascularization (Liu *et al.*, 2007). The positive role of FGF-18 in osteogenesis is supported by the finding that FGF-18 is a direct target of Wnt signaling and Runx2, two important pathways controlling osteogenesis (Kapadia *et al.*, 2005; Reinhold and Naski, 2007).

There is evidence that FGF and BMP signaling cooperate to control osteogenesis. FGF acts with BMP4 to promote osteogenesis in rats (Kubota *et al.*, 2002). Moreover, FGF2 and FGFR2 inhibit the expression of noggin in the patent cranial suture, resulting in increased BMP4 activity and suture fusion (Warren *et al.*, 2003). It is likely that the biological activities of FGFs in bone is dependent on the presence of other signaling molecules such as BMPs.

Genetic evidence indicates that several FGFs are important factors controlling bone formation *in vivo*. Overexpression of FGF2 in mice induces abnormal long bone formation (Coffin *et al.*, 1995; Sobue *et al.*, 2005) whereas FGF2 invalidation inhibits it (Montero *et al.*, 2000), pointing to an important role of FGF2 in the control of osteogenesis. The role of FGFRs in the control of bone formation is much more complex. For example, the impact of FGFR1 inactivation depends on the cell type. Inactivation of FGFR1 in osteo-chondro progenitor cells increases proliferation and delays osteoblast maturation whereas inactivation in differentiated osteoblasts accelerates differentiation, possibly through increased expression of FGFR3 (Jacob *et al.*, 2006). Conditional inactivation of FGFR2 alters osteogenesis and bone mass in mice, as a result of defective proliferation of osteoprogenitor cells and anabolic function of mature osteoblasts, indicating that FGFR2 plays an essential role in osteoblastogenesis (Yu *et al.*, 2003). In mice, a secreted soluble dominant-negative FGFR-2 causes skull abnormalities resembling those induced by FGFR mutations (Celli *et al.*, 1998), suggesting that increased or decreased FGFR signaling induces skeletal abnormalities. Activation of FGFR3 inhibits chondrocyte proliferation and differentiation in mice (Wang *et al.*, 1999), whereas mice lacking FGFR3 exhibit skeletal overgrowth, indicating that FGFR3 acts as a negative regulator of endochondral bone growth (Colvin *et al.*, 1996). Osteoblasts express little FGFR3, but mice lacking FGFR3 display decreased bone density (Valverde-Franco *et al.*, 2004). Some FGFR3 mutation may also affect osteogenesis in addition to chondrogenesis (Guitz, 2005). FGFR1, FGFR2, and FGFR3 exist as two splice variants, IIIb and IIIc. The mesenchymal splice variant of FGFR2 (Fgfr2IIIc) is expressed in early mesenchymal condensates and later in sites of endochondral and intramembranous ossification where it interacts with FGF18 (Eswarakumar *et al.*, 2002). The recessive phenotype of Fgfr2IIIc(−/−) mice is characterized by decreased expression of Runx2 and retarded long bone ossification, suggesting that Fgfr2IIIc is a positive regulator of ossification (Eswarakumar *et al.*, 2002). Interestingly, FGFR2 and FGFR1 expression is markedly impaired in osteoblasts from Fgf2 null mice (Naganawa *et al.*, 2006).

Fibroblast Growth Factors in Fracture Repair

Fibroblast growth factors appear to play an important role in fracture repair in rats (Kawaguchi *et al.*, 1994) and in humans (Wildburger *et al.*, 1994). During fracture repair, FGF-1 and -2 are expressed in the granulation tissue (Bollander, 1992), suggesting that FGFs increase cell migration and angiogenesis at early stages of bone repair. At later stages, FGF-1 expression increases during callus formation, whereas FGF-2 expression is stable (Joyce *et al.*, 1991). Several studies indicate that exogenous FGF-2 can accelerate bone repair (Kawaguchi *et al.*, 2001; Komaki *et al.*, 2006). In various animal models of bone defects, FGF-2 at low doses was found to stimulate chondrogenesis and bone formation (Kato *et al.*, 1998; Tabata *et al.*, 1999; Zellin and Linde, 2000), suggesting that FGF may improve bone regeneration (Radomsky *et al.*, 1998). Part of the effects of FGF on bone repair may involve the release of HGF (Blanquaert *et al.*, 1999) or VEGF (Saadeh *et al.*, 2000) by osteoblasts. Because FGF2 expands mesenchymal cell progenitors *in vitro* (Solchaga *et al.*, 2005), this may be used to obtain a large number of cells for bone tissue repair (Bianchi *et al.*, 2003).

FGF Signaling in Bone Cells

The signaling mechanisms involved in the regulation of skeletal cells by FGF/FGFRs are complex because FGF signaling (Fig. 3) controls a large number of genes such as transcription factors, soluble factors, membranous and matrix proteins (Marie, 2003). The nature of the cellular response depends on the ligand, receptor, and integration of signaling pathways targeting specific genes in skeletal cells (Dailey *et al.*, 2005). Moreover, the cellular response to FGFR activation is dependent on the stage of osteoblast maturation (Debiais *et al.*, 2004; Mansukhani *et al.*, 2005). In the growth plate, a number of signaling events are activated by FGF signaling to coordinate chondrocyte growth arrest and differentiation (Laplantine *et al.*, 2002; Dailey *et al.*, 2003). FGF-mediated growth arrest of chondrocytes is mediated by ERK1/2 and p38 MAPK pathways (Raucci *et al.*, 2004). Additionally, FGF signaling and activating FGFR-3 mutations activate STAT1 signaling pathway, leading to growth arrest and apoptosis (Sahni *et al.*, 1999, 2001; Li *et al.*, 1999; Legeai-Mallet *et al.*, 1998; Su *et al.*, 1997).

In cultured osteoblasts, FGF-2 activates MAP kinase signal transduction and extracellular signal-regulated kinase 2 (ERK2; Hurley *et al.*, 1996b; Newberry *et al.*, 1997; Chaudhary and Avioli, 1997). Erk signaling pathway mediates the FGF-2 mitogenic effect and effects on procollagen, OP, OC, and VEGF expression in osteoblasts

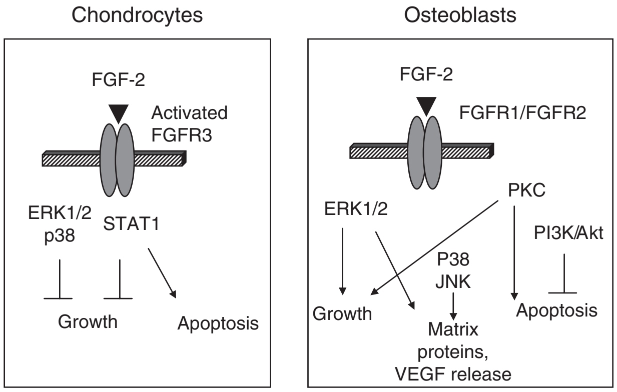

FIGURE 3 Signaling pathways involved in the response to FGF/FGFR in cartilage and bone. In chondrocytes, FGF-2-mediated growth arrest of chondrocytes is mediated by ERK1/2 and p38 MAPK pathways. FGF and activating FGFR-3 mutations activate the STAT1 signaling pathway, leading to growth arrest and apoptosis. In osteoblasts, ERK2 signaling mediates the FGF-2 effects on cell growth, matrix proteins (procollagen, OP, OC) and VEGF expression. p38 and JNK MAPKs also regulate FGF-2-stimulated VEGF release. The PKC and PI3K pathways are involved in osteoblast growth, differentiation or apoptosis induced by FGF2 or FGFR2 signaling (see text for details).

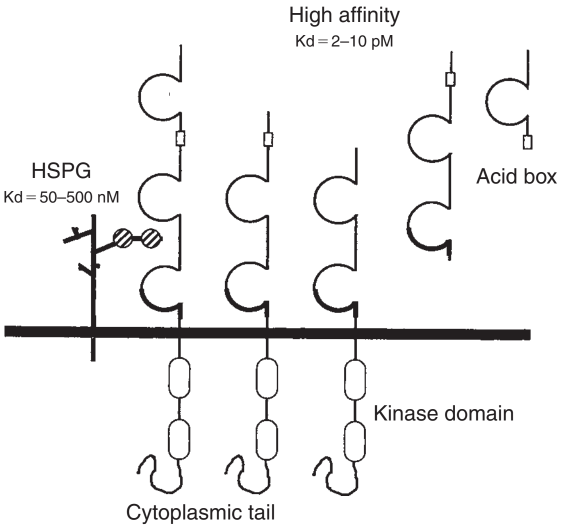

FIGURE 4 Cell surface FGF receptors. The low-affinity HSPG type, which protects and stabilizes FGF (hatched circles) in a conformationally dependent manner for presentation to its high-affinity receptor. Second, the high-affinity receptor containing two or three extracellular immunoglobulin-like loop structures. The characteristic location of an extracellular acid box is labeled (shaded box). Soluble splice variants of the high-affinity receptor without transmembrane domains are also diagrammed. Intracellularly, the high-affinity receptor contains a split tyrosine kinase domain and cytoplasmic tail. The third type, cysteine-rich receptor or MG-160, is found in the Golgi apparatus and is not shown here.

(Chaudhary and Avioli, 2000; Xiao *et al.*, 2002; Shimoaka *et al.*, 2002). Erk1/2, p38, and JNK MAPKs regulate FGF-2- stimulated VEGF release in osteoblasts (Takai *et al.*, 2007). The PKC pathway is another important pathway regulating FGF actions in osteoblasts. FGF-2 activates PKC activity in human osteoblasts, and this pathway is implicated in the expression of N-cadherin expression induced by FGF-2 (Debiais *et al.*, 2001). The PKC pathway is also involved in the growth response (Hurley *et al.*, 1996b), increased fibronectin expression (Tang *et al.*, 2007), and differentiation marker expression in osteoblasts (Lemonnier *et al.*, 2001a, 2001b) induced by FGF2 or FGFR2 signaling.

The effects of FGF signaling may be in part mediated by transcription factors. In mouse calvaria cells, FGF-2 and cAMP have synergistic effects on OC expression by acting on specific elements in the OC promoter (Schedlich *et al.*, 1994; Boudreaux *et al.*, 1996; Newberry *et al.*, 1996). Another direct effector of FGF signaling is Runx2 (Kim *et al.*, 2006). FGF2 increases Runx2 expression and activity in osteoblasts (Choi *et al.*, 2005; Kim *et al.*, 2006). FGF-2 also increases Runx2 protein nuclear accumulation in osteoblasts (Naganawa *et al.*, 2006). Moreover, Runx2 is phosphorylated and activated by FGF-2 via Erk1/2 (Xiao *et al.*, 2002), indicating that Runx2 is an important target of FGF signaling in osteoblasts.

Heparin sulfate proteoglycans are made by osteoblasts (Beresford *et al.*, 1987), and Globus *et al.* (1989) suggested that the FGF–heparin sulfate complex is released in an active form, resulting in osteoblast proliferation and new bone formation. Studies suggest a direct role for heparin in modulating the biological effects of FGF-1 and FGF-2 in bone (Simmons *et al.*, 1991; Hurley *et al.*, 1992b). Heparin enhances the mitogenic effect, in fetal rat long bone cultures (Simmons *et al.*, 1991). In contrast, heparin does not enhance the mitogenic effect of FGF-2 in fetal rat long bone or in fetal rat calvarial cultures (Simmons *et al.*, 1991; Hurley *et al.*, 1992b).

Low-affinity membrane-associated heparan sulfate proteoglycans (HSPGs) (Fig. 4) act as low-affinity co-receptors to modulate FGF actions (Ornitz, 2000a; Schlessinger *et al.*, 2000). Syndecans are cell surface proteoglycans that bind FGF via their heparin sulfate moieties and interact with FGF binding and signaling (David, 1993; Rapraeger, 1993; Bernfield *et al.*, 1993). During development, syndecan-1 is expressed in limb bud (Solursh *et al.*, 1990) and affects bone cell differentiation (Dhodapkar *et al.*, 1998). Syndecan-2 is abundant in prechondrogenic cells and is expressed in the perichondrium and periosteum at the onset of *in vitro* osteogenesis (Molténi *et al.*, 1999a). Syndecan-3 is expressed in mesenchymal cells during limb bud formation and is regulated by FGFs (Gould *et al.*, 1992). Thereafter, precartilage cells and immature chondrocytes express syndecan-3, which may control chondrocyte proliferation during endochondral ossification (Shimazu *et al.*, 1996). Syndecan-4 is strongly expressed in chondrocytes

(Kim *et al.*, 1994) and osteoblasts (Molténi *et al.*, 1999a) and is upregulated by FGF-2 (Song *et al.*, 2007). Coexpression of syndecans and FGFRs and functional analyses during osteogenesis suggests a role for these HSPGs in the control of bone formation. For example, rat chondrocytes coexpress FGFR-3, syndecan-2, and syndecan-4, and their expression decreases during *in vitro* differentiation. In contrast, rat calvaria osteoblasts express syndecan-1, -2, and -4 with FGFR-1 and FGFR-2, indicating that syndecans can interact with FGFRs to control FGF actions during *in vitro* chondrogenic and osteogenic differentiation (Molténi *et al.*, 1999a, 1999b; Jackson *et al.*, 2007), indicating that syndecans and other HSPGs may be important co-receptors modulating FGF/FGFR interactions in bone cells. Consistent with an important role of glycosaminoglycans in the control of FGFR signaling, it was found that specific GAGs can antagonize the overactive FGFR2 S252W mutation *in vitro* (McDowell *et al.*, 2005).

FIBROBLAST GROWTH FACTORS AND BONE REMODELING

FGF and Bone Resorption

FGF-1 induces bone resorption in fetal rat long bone via a prostaglandin-mediated mechanism (Shen *et al.*, 1989). FGF-2 is also a potent bone-resorbing agent in the fetal rat long bone model (Simmons *et al.*, 1991). However, in fetal rat long bones, the effects of FGF-2 on bone resorption appear to be independent of endogenous prostaglandin production (Simmons *et al.*, 1991). In contrast to the report by Shen *et al.* (1989), Simmons *et al.* (1991) reported that FGF-1 induces calcium release only in the presence of heparin and that this effect is not blocked by indomethacin. FGF-2 increases bone resorption in neonatal mouse calvariae, which is partially blocked by indomethacin, suggesting that the resorptive effects of FGF-2 are mediated in part by prostaglandins. In the presence of both an inhibitor of DNA synthesis and prostaglandin production, FGF-2-induced bone resorption is completely blocked. These results suggest that FGF-2 has direct effects on resorption through its effects on osteoclasts precursor proliferation and an indirect effect that is mediated by prostaglandin (Kawaguchi *et al.*, 1995a). These findings are important because prostaglandins are potent local regulators of bone metabolism (Kawaguchi *et al.*, 1995b).

FGF Regulation of the Collagenase Gene in Bone

FGF may also modulate bone matrix degradation by regulating collagenase expression and activity. Interstitial collagenase is required to initiate the degradation of type I collagen (Delaissé *et al.*, 1988), and inhibitors of metalloproteinases inhibit bone resorption (Delaissé *et al.*, 1988). In rat calvaria bone cells, FGFs increase interstitial collagenase-1 and -3 expression by a prostaglandin- and PKC-dependent pathway (Varghese *et al.*, 1995; Tang *et al.*, 1996). In contrast, in mouse cells the effects of FGF-2 on the collagenase gene are not affected by either indomethacin or staurosporine (Hurley *et al.*, 1995b). Similar to FGF-2, FGF-1 also upregulates the collagenase gene (M. M. Hurley, unpublished observation). FGF-2 transcriptionally regulates collagenase gene expression in mouse calvaria cells, and the tyrosine kinase inhibitors genistein and herbimycin A completely block the effect of FGF-2 on the collagenase promoter (Hurley *et al.*, 1995b). FGF response elements in the collagenase promoter have been identified (Newberry *et al.*, 1997). FGF-2 stimulates collagenase-3 gene transcription through an effect on the AP-1 site on the promoter (Varghese *et al.*, 2000) and induces the expression of tissue inhibitors of metalloproteinases (TIMP) 1 and 3 (Varghese *et al.*, 1995), which regulate collagenase activity. Although FGF-2 decreases stromelysin-3 mRNA stability, it increases gene transcription (Delany and Canalis, 1998b), which may contribute to the control of the bone matrix degradation.

FGF and Osteoclastogenesis

Histologic examination reveals that FGF-1 treatment increases osteoclast number in fetal rat long bones (Shen *et al.*, 1989) and in 21-day fetal rat calvariae (Hurley *et al.*, 1992b), suggesting a role for FGFs in osteoclastogenesis. Osteoclasts are derived from a hematopoietic precursor that is common to the granulocyte and the macrophage. The immediate progenitor of the osteoclast is the CFU-GM (colony-forming unit for granulocytes and macrophages; Mundy and Roodman, 1995), and studies show that FGF-2 stimulates the formation of CFU-GM in human marrow cultures (Gabrilove *et al.*, 1994). The production of osteoclasts from progenitor cells is regulated by cytokines and requires or is enhanced by interactions between marrow progenitor cells and either osteoblastic or stromal cells (Suda *et al.*, 1992). Interestingly, FGF-2 is a potent mitogen for stromal cells (Oliver *et al.*, 1990), which are multipotential mesenchymal cells in the marrow environment that are the precursors of osteoblasts, chondroblasts, fibroblasts, and other connective tissue cells in bone. FGF-2 is produced by bone marrow stromal cells (Brunner *et al.*, 1993) and hence may be autostimulatory.

FGF-2 has been found to increase the formation of multinuclear-resorbing osteoclast-like cells in mouse bone marrow cultures, an effect mediated by PGE_2 production (Hurley *et al.*, 1998; Kawaguchi *et al.*, 2000). Indeed, FGF-2 stimulates PGE_2 production rapidly in mouse calvaria cells through the transcriptional regulation of PGHS-2 (Kawaguchi *et al.*, 1995a). FGF-2 also increases osteoclast formation by a mechanism involving COX-2 stimulation and prostaglandin formation, leading to increased osteoclast differentiation factor (ODF) production (Nakagawa *et al.*, 1999). Apart from this indirect effect of FGF-2 through osteoblastic cells, FGF-2 acts directly on mature osteoclasts to stimulate bone resorption (Kawaguchi *et al.*, 2000) through the activation of FGFR-1 and MAPK activation (Chikazu *et al.*, 2000). Similar to FGF1 and FGF-2, FGF-18 also induces osteoclast formation through receptor activator of nuclear factor-κB ligand and COX-2 (Shimoaka *et al.*, 2002). Interestingly, in the absence of endogenous FGF-2, PTH, RANKL, and interleukin-11 induced osteoclastogenesis is markedly impaired (Okada *et al.*, 2003).

HEPARIN, FIBROBLAST GROWTH FACTORS, AND OSTEOPOROSIS

In humans, the prolonged use of heparin is associated with the development of clinical osteoporosis and spontaneous fractures (Megard *et al.*, 1982). Heparin is the predominant glycosaminoglycan in mast cells and may play a role in the osteoporosis often seen in mast cell disease (Chines *et al.*, 1991). The bone marrow of postmenopausal women who are osteoporotic show increased numbers of heparin-rich mast cells (Frame and Nixon, 1968). *In vivo* studies in rats treated with heparin for 33 to 65 days reveal significant reduction in bone mineral mass (Thompson, 1973). In studies in bone organ culture, heparin decreases collagen synthesis (Hurley *et al.*, 1990), and this effect is reversed by exogenous IGF-I (Hurley *et al.*, 1992a). Heparin also potentiates the inhibitory effect of FGF-2 on collagen synthesis and procollagen mRNA levels in fetal rat calvariae (Hurley *et al.*, 1992b). In bone, heparin augments FGF-1 and FGF-2-induced bone resorption (Simmons *et al.*, 1991). Studies by Thompson *et al.* (1990) revealed that heparin infusion into rabbits results in an increase in FGF-2-like activity in plasma. The mechanisms by which heparin induces osteoporosis are not known. It is possible that modulation of the availability or action of local factors such as IGF-I and FGF-2 is important in heparin-induced osteoporosis.

The role of fibroblast growth factors in the development of osteopenia and postmenopausal osteoporosis in humans has not been defined. However, the studies of Montero *et al.* (2000) clearly documented a role for FGF-2 in bone remodeling and maintenance of bone mass (Figs. 5 through 8, Table I). These studies show that disruption of the Fgf 2 gene in mice results in decreased osteoblast replication, decreased mineralized nodule formation in bone marrow cultures, and decreased new bone formation *in vivo*.

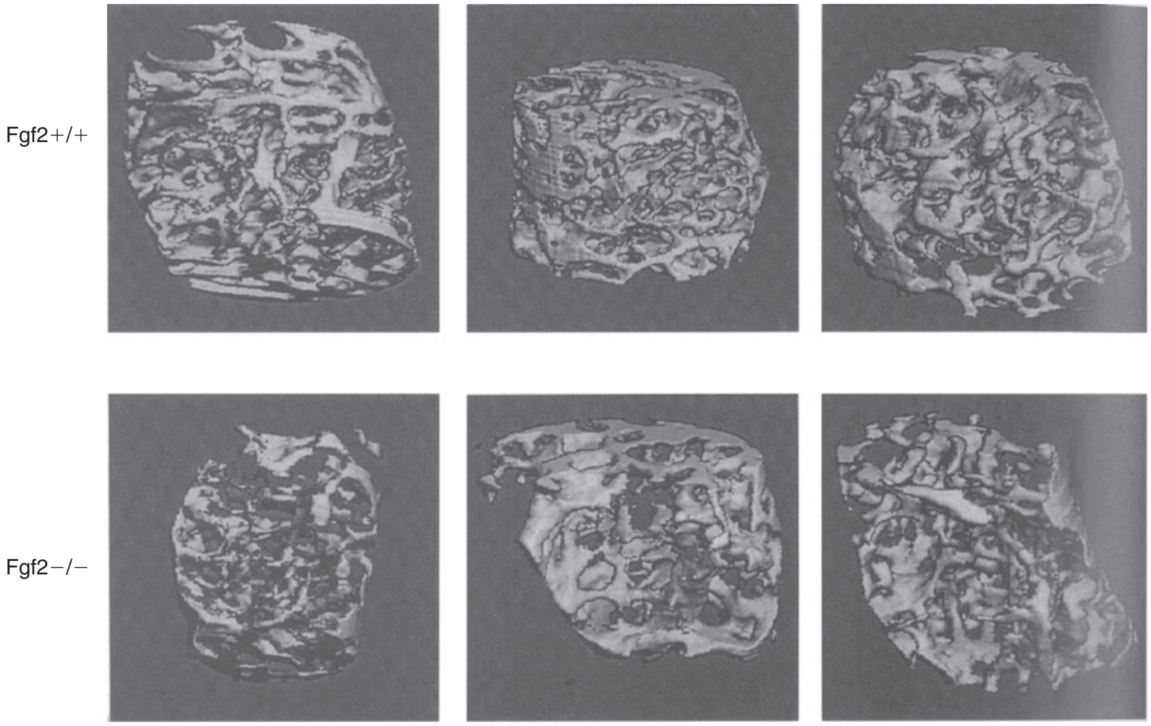

FIGURE 5 Morphological study by micro-CT scanning of trabecular bone of femurs of 4.5-month-old Fgf2 +/+ and Fgf2 −/− mice. The three-dimensional trabecular bone architecture of distal femoral metaphysis of 4.5-month-old male Fgf2 +/+ and Fgf2 −/− mice was analyzed by micro-CT. Note that the plate-like architecture of the trabecular bone is reduced markedly and the connecting rods of trabeculae are disrupted in Fgf2 −/− mice compared with Fgf2 +/+.

TABLE I Three-dimensional Parameters of Micro-CT of Femora from 8-Month-Old Fgf2+/+ and Fgf2−/− Mice

Genotype	*n*	Bone volume (%)	Trabecular number (mm^{-1})	Trabecular thickness (μm)	Trabecular separation (μm)
Fgf2+/+	3	22.0 (4.8)	3.9 (0.3)	57.0 (11.1)	203 (22)
Ffg2−/−	3	7.4 (2.4)[a,b]	1.8 (0.7)[a,b]	41.7[a,b] (3.2)	564[a,b] (190)

[a] $P < 0.05$, *significantly different from Fgf+/+ group*, Mann−Whitney U *test.*

[b] $P < 0.05$, *significantly different from Fgf+/+ group*, Tukey−Kramer *multiple comparison test* (ANOVA).

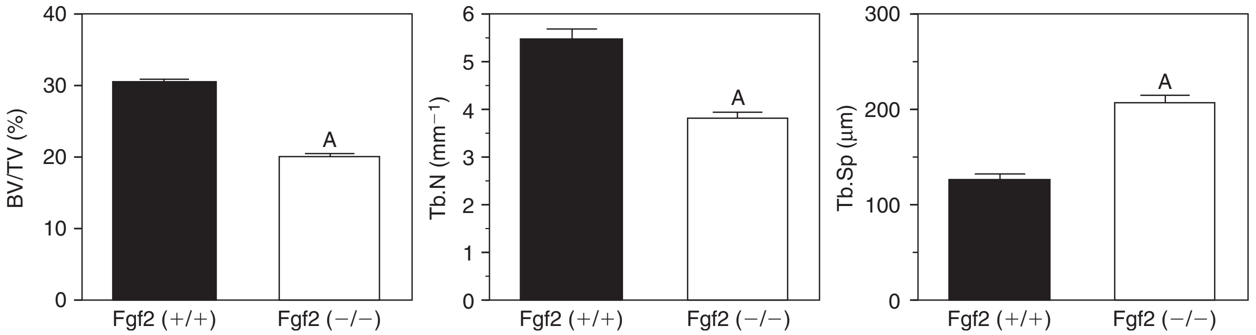

FIGURE 6 Three-dimensional microstructural parameters calculated using two-dimensional data obtained from micro-CT of femoral bones from 4.5-month-old Fgf2 +/+ and Fgf2 −/− mice. Calculated morphometric indices included bone volume density [bone volume (BV)/trabecular volume (TV)] trabecular number [Tb.N = (BV/TV)/Tb.Th], and trabecular separation [Tb.Sp = (1/Tb.N) – Tb.Th]. [A]Significantly different from Fgf2 +/+ group; $P < 0.05$, Tukey–Kramer multiple comparison test (ANOVA).

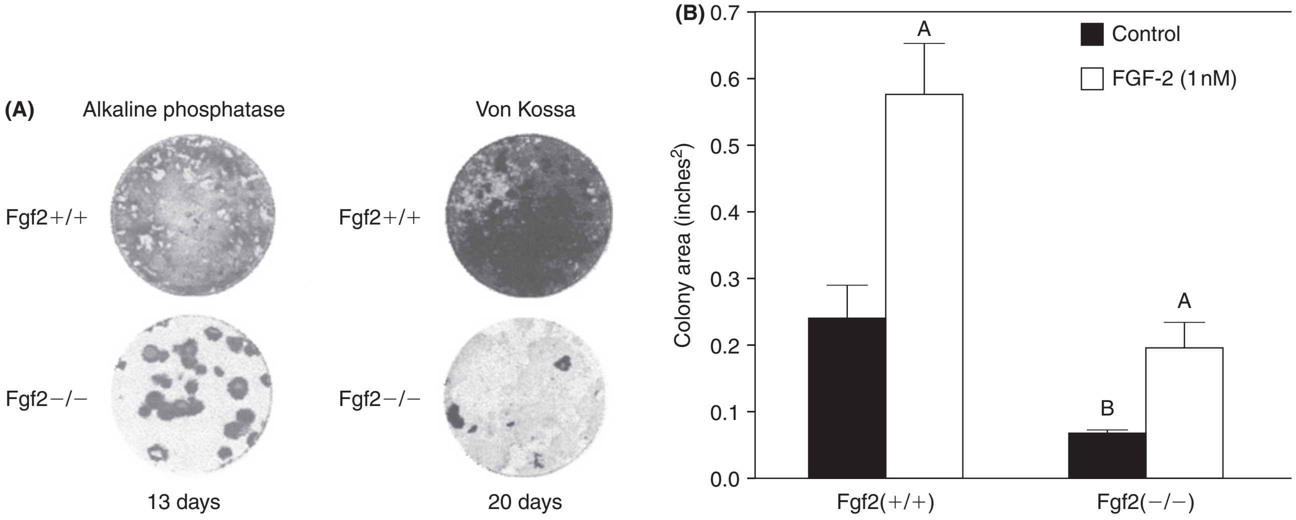

FIGURE 7 **(A)** Comparison of the ability to form ALP colonies and mineralized nodules as determined by von Kossa staining in mouse bone marrow cultures from Fgf2 +/+ and Fgf2 −/− mice. Cells were plated at a density of 20 million cells per well in αMEM containing penicillin/streptomycin and 10% heat-inactivated fetal calf serum (FCS). On day 3, media were changed and cells were cultured in differentiation media (αMEM, 10 n*M* dexamethasone, 10% FCS, 8 m*M* β-glycerophosphate, 50 μg/mL ascorbic acide) for the indicated times. (See also color plate) **(B)** Effect of FGF-2 on colony area in mouse bone marrow cultures from Fgf2 +/+ and Fgf2 −/− mice. Cells were plated at a density of 1 million cells per well in αMEM containing penicillin/streptomycin and 10% heat-inactivated FCS in the absence or presence of FGF-2 (10 n*M*). On day 3, media were changed and cells were cultured in differentiation media (αMEM, 10 n*M* dexamethasone, 10% FCS, 8 m*M* β-glycerophosphate, 50 μg/ml ascorbic acid) for another 11 days. [A]Significantly different from control cultures; $P < 0.05$. [B]Significantly different from Fgf2 +/+; $P < 0.05$.

TABLE II Effects of FGF/FGFR Interactions in Skeletal Cells

Chondrogenesis	Bone Formation	Bone Resorption/Degradation
Modulate chondroblast proliferation	Increase osteoblast proliferation	Promote osteoclast formation
Inhibit chondrocyte differentiation	Decrease collagen synthesis	Promote osteoclast formation
Promote chondrocyte apoptosis	Inhibit alkaline phosphatase	Regulate collagenase-1 and −3
	Modulate osteopontin, osteonectin, and osteoclacin	Promote TIMP 1and 3
	Downregulate connexin-43, upregulate N-cadherin	
	Increases Na-dependent P_i transport	
	Increase osteogenesis by marrow osteoprogenitors	
	Promote osteoblast apoptosis	
	Increase bone formation and promote bone repair *in vivo*	

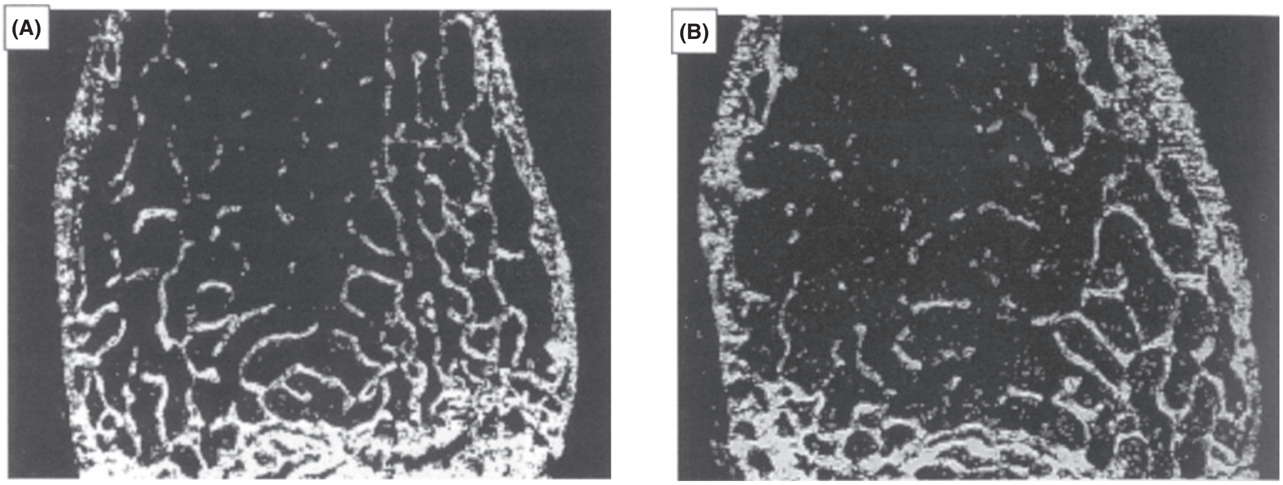

FIGURE 8 Undecalcified sections of the distal femur of **(A)** an 8-month-old Fgf2 + /+ mouse and **(B)** a FGF − /− mouse (dark-field illumination). Trabecular number is decreased in the secondary spongiosa of the Fgf2 − /− mouse.

There was a progressive decrease in trabecular number and trabecular bone volume as these mice aged. These data are the first to demonstrate an important role for endogenous FGF-2 in maintaining bone mass, as well as bone formation. These data further suggest that the redundancy of members of the FGF family cannot compensate to prevent bone loss when the Fgf 2 gene is disrupted in mice.

A therapeutic role for FGFs in osteopenic states is suggested by studies in animal models. In osteopenic mice, FGF-2 stimulates endocortical bone modeling (Nagai *et al.*, 1999). In ovariectomized rats, FGF-1 or FGF-2 administration also stimulates bone formation and restores bone volume (Nakamura *et al.*, 1997; Dunstan *et al.*, 1999; Liang *et al.*, 1999). The mechanism of this effect has not been studied. Studies are needed to examine the effects of menopause as well as oophorectomy on FGF expression in bones.

CONCLUSION

Multiple experimental and genetic evidence indicates that FGFs and FGFRs play important roles in the control of endochondral and intramembranous bone formation (Table II). However, the cellular and molecular effects of FGF and FGFRs in bone cells are not fully understood. Identification of the signal transduction pathways that are activated by FGF/FGFR interactions and that lead to the expression of specific genes in skeletal cells may help to better understand the regulatory effects of FGFs during osteogenesis *in vivo*, and may allow for the development of therapeutic approaches to stimulate bone formation and to improve bone regeneration.

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Chapter 52

Vascular Endothelial Growth Factor and Osteogenic-Angiogenic Coupling

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INTRODUCTION

Bone never forms without vascular interactions. However, the complex and enlightening biology behind this simple statement of fact has only recently been studied in detail. Studies from the fields of developmental biology, molecular genetics, and clinical pharmacology have converged to highlight the central role of the vasculature in bone physiology. The vasculature provides the conduit for calcium, phosphate, hematopoietic, hormonal, and nutrient flux necessary for matrix synthesis, mineralization, and calcium mobilization. It provides the organizational structure and rate-limiting "point-of-reference" for Haversian bone formation, remodeling, and repair (Eriksen *et al.*, 2007). Moreover, the vasculature provides a sustentacular niche and source of adult mesenchymal stem cells, including osteoprogenitors (Shi and Gronthos, 2003). During fracture repair, paracrine endothelial-mesenchymal signaling interactions are activated (Bouletreau *et al.*, 2002), recapitulating features of the epithelial-mesenchymal interactions that drive bone morphogenesis during embryonic development (Bandyopadhyay *et al.*, 2006). BMP2 and BMP4 stimulate the production of vascular endothelial growth factor (VEGF) by osteoblasts (Deckers *et al.*, 2002; Huang *et al.*, 2004) and placental growth factor (PlGF) by mesenchymal progenitors (Marrony *et al.*, 2003; Raida *et al.*, 2006), while VEGF actions on the endothelium enhance production of BMP2 and E-series prostaglandins (Bouletreau *et al.*, 2002; Kaigler *et al.*, 2005). Furthermore, recent evidence suggests that circulating, marrow-derived endothelial progenitor cells (EPCs) may be delivered by the vasculature to sites of bone formation—and contribute not only to mature endothelial populations, but also to microvascular smooth muscle cells and osteoblast lineages during skeletal growth and fracture repair (Eghbali-Fatourechi *et al.*, 2005, 2007; Matsumoto *et al.*, 2006). A better understanding of mechanisms coupling bone and vascular physiology will provide insights useful for devising novel strategies to address the mounting unmet needs in bone and mineral disease (Towler, 2007).

We know relatively little about how the vasculature integrates and conveys signals during skeletogenesis. However, vascular endothelial growth factor-A (VEGF, or VEGF-A) has emerged as the prototypic osteogenic-angiogenic coupling factor for bone-vascular interactions. In the previous edition of this volume, Harada and Thomas provide an outstanding, comprehensive overview of the VEGF family biochemistry and molecular biology (Bilezikian *et al.*, 2002). The reader is referred to that earlier edition, and recent reviews by Shibuya (Takahashi and Shibuya, 2005) and Claesson-Welsh (Olsson *et al.*, 2006), for a detailed foundation in VEGF molecular biology. Only a very brief overview and update will be given; in this edition, the focus will be on VEGF bone biology (Maes *et al.*, 2002, 2004, 2006; Wang *et al.*, 2007; Zelzer *et al.*, 2002)—incorporating selected but critical aspects of PlGF bone biology (Maes *et al.*, 2006)—to highlight the roles and regulation of VEGF receptor signaling during bone development, osteogenic-angiogenic coupling, matrix remodeling, fracture repair, and skeletal mineral metabolism.

VEGF MOLECULAR BIOLOGY

The VEGF gene is encoded on human chromosome 6; approximately 15 kb encompasses 5′ and 3′ regulatory elements, eight exons, and seven introns (Takahashi and Shibuya, 2005). Alternative splicing generates at least nine subtypes in humans: VEGF121, VEGF145, VEGF148,

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VEGF162, VEGF165, VEGF165b, VEGF183, VEGF189, and VEGF206. VEGF121, VEGF165, and VEGF189 predominate, and VEGF165b is an inhibitory form of VEGF, which binds to VEGFR2 with affinity approximating that of VEGF165. In the mouse, the three major isoforms are one amino acid shorter—namely, VEGF120, VEGF164, and VEGF188—encoded on mouse chromosome 17. (Unless otherwise stated, the human isoform numbering will be preferentially used.) VEGF165 is the major soluble isoform that is secreted and circulates as a homodimer of apparent molecular weight of 46 kDa. Like VEGF189, VEGF165 also binds to heparin and heparan sulfate proteoglycans such as glypican (Gengrinovitch *et al.*, 1999) and collagen XVIII/endostatin (Moulton *et al.*, 2004; Pufe *et al.*, 2005; Sipola *et al.*, 2006). However, VEGF189 is not soluble and very tightly associates with extracellular matrix proteoglycans—requiring proteolysis, heparanolysis, or heparin displacement for release (Houck *et al.*, 1992; Robinson *et al.*, 2006). By contrast, VEGF121 lacks the two heparan sulfate binding domains encoded by exons 6 and 7; this isoform is thus also found in the circulation. The details of how VEGF splicing is regulated with development and disease are poorly understood, but it is clearly important to determine them.

Embryonic lethality is observed in mice lacking even one copy of the VEGF gene (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). Mice capable of expressing only VEGF120 (VEGF120/120 mouse) survive to birth but die shortly thereafter from ischemic cardiomyopathy (Carmeliet *et al.*, 1999) and pulmonary hypoplasia (Galambos *et al.*, 2002; Zelzer *et al.*, 2002). By contrast, VEGF165/165 mice are phenotypically normal, whereas VEGF188/188 mice are dwarfed and have aberrant retinal vascularization (Maes *et al.*, 2004).

In the absence of any VEGF, bone never forms. When VEGF120, VEFG164, or VEGF188 are present, murine intramembranous and endochondral bone formation can ensue. However, VEGF120/120 (Zelzer *et al.*, 2002) mice and VEGF188/188 (Maes *et al.*, 2004) mice have skeletal defects, highlighting the importance of VEGF164 to skeletal growth and development. In the VEGF120/120 mouse (Zelzer *et al.*, 2002), early embryonic lethality is bypassed, but profound delay in mid-diaphyseal vascular invasion and primary ossification center formation. In VEGF188/188 mice (Maes *et al.*, 2004), formation of primary ossification centers occurs; the process of MMP9-dependent matrix turnover in the remodeling primary spongiosa is posited to liberate sufficient VEGF to recruit endothelial cells and osteoprogenitors for metasphyseal mineralization (Engsig *et al.*, 2000; Gerber *et al.*, 1999; Heissig *et al.*, 2002). However, secondary ossification centers are profoundly deficient in VEGF188/188 mice; there is severe reduction in bone marrow cavity and long bone length. Skeletal hypoxia—revealed by pimonidazole administration and hypoxic adduct immunolocalization with EF5—was expanded throughout the epiphysis (Maes *et al.*, 2004). Epiphyseal chondrocyte hypertrophy is completely absent, with subsequent concomitant ablation of secondary ossification centers. Abnormalities in epiphyseal maturation resulted in joint dysplasia and arthritis (Maes *et al.*, 2004). A large, hypocellular core segment extending from the developing long bone joint surface to the metaphysis was seen due to apoptosis in VEGF188/188 skeletons (Maes *et al.*, 2004). Because conditional deletion of cartilaginous VEGF results in epiphyseal chondrocyte apoptosis (Zelzer *et al.*, 2004), this gap in VEGF188/188 mice indicates that diffusible VEGF isoforms mediate this important epiphyseal survival signal. Without this signal, epiphyseal dysplasia and joint dysplasia ensue (Maes *et al.*, 2004).

VEGF165b is a very recently identified variant produced by multiple cell types and also circulates in human plasma (Bates *et al.*, 2002; Konopatskaya *et al.*, 2006; Woolard *et al.*, 2004). This variant is generated by alternative splicing within exon 8 that alters only the last six C-terminal amino acid residues (CDKPRR → SLTRKD). VEGF165b is not angiogenic, but rather inhibits VEGF165-dependent angiogenesis. VEGF165b binds to VEGFR2/Flk1 with affinity identical to that of VEGF165 and antagonizes VEGFR2-dependent angiogenesis. Moreover, VEGF165b expression is decreased in malignant prostate tissue relative to benign prostate issue (Bates *et al.*, 2002; Konopatskaya *et al.*, 2006; Woolard *et al.*, 2004). Regulation and bioactivity in bone cell physiology have yet to be characterized. Whether VEGF165b inhibits TNFR2-dependent activation of VEGFR2 via the Bmx/Etk kinase pathway is as yet unknown also see later discussion).

As compared to the complexity of VEGF gene splicing, the transcriptional regulation of the VEGF is relatively simple. Transcriptional regulators of the hypoxia induced factor (HIF) family are critical determinants of VEGF gene expression. HIF-dependent transcription is covered in detail elsewhere in this volume (Chapter 34). Briefly, there are three HIF family members, HIF-1α, HIF-2α, and HIF-1β, that bind to DNA via their basic helix–loop–helix domains. Heterodimerization between alpha and beta subunits is required for function. HIF heterodimers recognize the DNA cognate TACGTG, which in the VEGF gene is located at basepairs −947 to −939 relative to the transcription initiation site (Forsythe *et al.*, 1996). Transcriptional coactivators of the CBP/p300 and p160/SRC1 family are then recruited; these proteins not only acetylate histones to open VEGF chromatin structure, but also recruit the megadalton, multiprotein complexes that orient and phosphorylate RNA polymerase II as necessary for VEGF transcription initiation (Cho *et al.*, 2007; Fath *et al.*, 2006). The main stimulus for HIF activation and VEGF transcription is hypoxia (Semenza, 2001). Prolyl hydroxylation of HIF-alpha subunits in oxygen sensing domains regulates ubiquitination and HIF turnover; under conditions of low oxygen tension, HIF subunits are not hydroxylated and are stabilized in an active form (Jaakkola *et al.*, 2001). Intriguingly, it appears that most of the physiologic stimuli relevant to bone formation—including osteoanabolic growth factors and mechanical stimuli—function as "secretagogues" for VEGF production by the osteoblast (Towler, 2003). Moreover, as occurs with hypoxia, VEGF transcriptional induction by these other stimuli utilize HIF-alpha signaling for robust VEGF induction in osteoblasts (Akeno *et al.*, 2001, 2002). However, other Sp1-like binding cognates have now been described that convey responses to EP2 and EP4 prostaglandin receptor agonists in smooth muscle, and EGF receptor/Akt-dependent pathway induction in breast cancer cells (Abdelrahim and Safe, 2005; Bradbury *et al.*, 2005). Whether PGEs utilize specific Sp1 family members to support VEGF expression in osteoblasts is as yet unknown (Harada *et al.*, 1994). The tumor suppressor WT1 has been identified as one zinc-finger transcription factor that recognizes and represses VEGF expression via these Sp1 binding cognates (Hanson *et al.*, 2007). However, expression of VEGF in hypertrophic chondrocytes during endochondral bone formation requires Runx2/Cbfa1 (Himeno *et al.*, 2002; Zelzer *et al.*, 2001). Clearly, a detailed study of the protein – DNA interactions supporting VEGF gene transcription during chondrocyte and osteoblast development needs to be undertaken. Nonetheless, the recent data of Clemens and co-workers (Wang *et al.*, 2007) elegantly demonstrates that HIF-alpha will be the primary determinant of VEGF in normal osteoblasts, and the subsequent osteogenic/angiogenic coupling (Fig. 1).

Post-transcriptional regulation of VEGF expression also occurs; besides the alternatively splicing noted earlier, translation of the VEGF mRNA and stability of the mRNA transcript have been studied, although seldom in cells types immediately relevant to bone biologists (Yoo *et al.*, 2006). Both VEGF120 and VEGF164 transcripts are upregulated

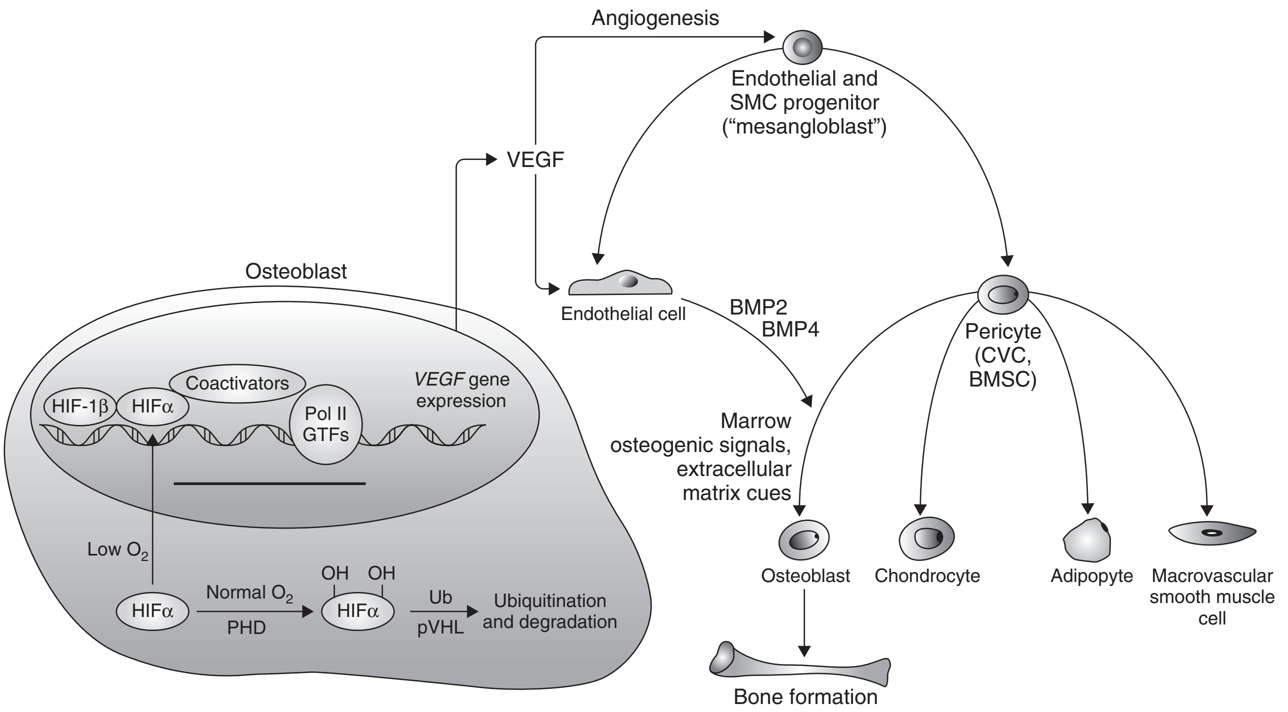

FIGURE 1 Working model of osteogenic-angiogenic coupling in trabecular bone. Recent data from multiple laboratories have indicated that microvascular smooth muscle cells known as pericytes represent osteoprogenitors capable of bone formation when placed in the correct microenvironment. Pericytes (Dellavalle *et al.*, 2007; Doherty *et al.*, 1998) appear to arise from a vessel-associated stem cell progenitor (mesoangioblast; Esner *et al.*, 2006; Tagliafico *et al.*, 2004)—and during the process of mesoderm growth and angiogenesis, this VEGFR2-expressing stem cell undergoes expansion. Not shown is the capacity of VEGF (or PlGF) to induce proliferation and osteogenic differentiation of mesenchymal progenitors via VEGFR1 (Maes *et al.*, 2006). Clemens and colleagues (Wang *et al.*, 2007) recently demonstrated that osteoblast HIF-alpha subunits, transcriptional regulators of VEGF expression, represent rate-limiting components of osteogenic-angiogenic coupling and trabecular bone formation. Augmentation of osteoblast HIF-alpha expression and bone formation was achieved by conditionally deleting Vhl, the gene encoding pVHL—the E3 ubiquitin ligase necessary for HIF-alpha degradation. Bone formation requires VEGF-mediated paracrine signals in bone that stimulated angiogenesis. Because VEGF can expand VEGFR2-expressing mesoangioblast numbers during angiogenesis (Brunelli *et al.*, 2004; Cossu and Bianco, 2003), this process may drive the increase in osteoblast numbers that promotes massive trabecular bone formation in the osteogenic marrow environment. PHD enzyme activity is required for HIF-alpha degradation, oxidatively "tagging" HIF-alpha for recognition by pVHL. In addition to low oxygen levels (as shown here), mechanical stimuli, TNF-alpha, and reactive oxygen species can also upregulate HIF-alpha expression. See text for details. BMSC, bone marrow stromal cell; CVC, calcifying vascular cell; GTF, general transcription factor; Pol II, RNA polymerase II; Ub, ubiquitin. Reprinted from Towler (2007) with permission.

in bone with fracture healing (Pufe *et al.*, 2002). LaFage-Proust and colleagues demonstrated that mechanical strain controls splicing of VEGF isoforms in MG63 human osteosarcoma cells (Fuare *et al.*, 2006); a low-frequency regimen (0.05 Hz for 150 min) stimulated soluble VEGF 121 and 165 production, whereas a high-frequency regimen (5 Hz for 1.5 min) stimulated matrix-bound VEGF 145, 165, 189, and 206 isoform production (Fuare *et al.*, 2006). Reactive oxygen species control VEGF translation in renal proximal tubule in response to angiotensin (Feliers *et al.*, 2006). Reactive oxygen species may control VEGF expression in osteoblasts via HIF-alpha signaling that is independent of hypoxia (Kim *et al.*, 2002), but this remains to be studied in detail.

VEGF RECEPTORS IN BONE BIOLOGY

Three classic VEGF receptors have been described—VEGFR1 (Flt1), VEGFR2 (Flk1), and VEGFR3—that are all members of the receptor tyrosine kinase superfamily. In addition, other “co-receptors”—namely, neuropilin 1, neuroplin 2, and glipican 1—have been identified that augment VEGF signaling in a ligand-specific, VEGFR-specific, and cell type–specific fashion (see later discussion). Detailed review of the combinatorial complexity of this receptor signaling system is beyond the scope of this chapter but has been recently reviewed (Takahashi and Shibuya, 2005). However, given the prominent contributions of VEGFR2 and VEGFR1 to skeletal physiology, those functional relationships and the ligands clearly relevant to the signaling of these receptors will be presented. The structural features of VEGFR structure and ligand-dependent signaling has been well discussed by Harada and Thompson (Bilezikian *et al.*, 2002); the reader is referred to the previous edition for this excellent overview.

VEGFR1

VEGFR1 is also known as Flt1 and, for simplicity, will be referred to as VEGFR1. The VEGFR1 is a 1338-amino-acid-long receptor tyrosine kinase, encoded in 30 exons spanning about 150 kbp of genomic DNA. The major ligands of VEGFR1 directly germane to bone biology are VEGF and PlGF—but PlGF and VEGF-B are VEGFR1-specific ligands. In addition to homodimerization, VEGFR1 forms ligand-induced heteromeric complexes with VEGFR2 and neuropilin; the signaling properties of these heteromers are poorly understood (Pan *et al.*, 2007b). However, neuropilin-1 enhances VEGF165 binding affinity for VEGR1 at least 10-fold (Fuh *et al.*, 2000), and. Mice completely null for the VEGFR1 gene are embryonic lethal around E8.5; this arises from abnormal vascular morphogenesis without impairment of endothelial cell differentiation; indeed, the VEGFR1 −/− embryo exhibits increased endothelial cell proliferation numbers in the yolk sac and nascent endomyocardium (Fong *et al.*, 1999). This may relate to the important role of the extracellular domain of VEGFR1 to bind and sequester VEGF and PlGF; indeed, a soluble, secreted form of VEGFR1 is elaborated during development, and is thought to be central to the pathobiology of preeclampsia (Ahmad and Ahmed, 2004; Koga *et al.*, 2003; Levine *et al.*, 2004). It had been appreciated that VEGFR1 was highly expressed in the osteoclast lineage, and that VEGF could replace M-CSF/CSF-1 in the development and activation of osteoclasts in concert with RANKL *in vitro* (Niida *et al.*, 1999). These contributions were robustly established via the analysis of VEGFR1(TK−/TK−) mice. Because the extracellular domain of VEGR1 is biologically active in the absence of intracellular tyrosine kinase domain (see earlier discussion), Shibuya and colleagues characterized a mutant form of the VEGFR1 gene in which the intracellular kinase domain was compromised without affecting the coding of the extracellular domain. This mouse, known as the VEGFR1(TK−/TK−) mouse, is a hypomorph that survives into adulthood (Niida *et al.*, 2005). No major defects in vasculogenesis or angiogenesis with development were observed; however, postnatal reductions in the chemotaxis of monocytes/macrophages to VEGF were noted with reductions in inflammation in VEGFR1(TK−/TK−) mice in arthritic disease models (Murakami *et al.*, 2006). Because monocytes and macrophages strongly express VEGFR1, it was suggested that this lineage—including the osteoclast—might be affected. Osteoclast numbers were modestly reduced in VEGFR1(TK−/TK−) mice; however, severe osteoclast abnormalities became clearly evident when VEGFR1(TK−/TK−) genotype was superimposed on the CSF-1 deficiency of the osteopetrotic op/op mouse. Thus, the VEGFR1 signaling system is clearly important to the regulation of bone-resorbing osteoclast physiology.

Other functions of VEGFR1 signaling relevant to bone marrow stromal cell physiology also emerged in these early studies. Marrow fibrosis was a significant finding in the VEGFR1(TK−/TK−) mice on the op/op background (Niida *et al.*, 2005), prompting the investigative team to propose that VEGFR1 signaling participates in other aspects of bone marrow function. These notions have now been confirmed. Recently, Nakamura and colleagues showed that bone mass, bone strength, and bone formation rates were reduced in VEGFR1(TK−/TK−) mice (Otomo *et al.*, 2007). Intriguingly, although osteoblast numbers were not increased, mineralizing surface, bone formation rate, and mineral apposition rates were reduced. No significant change in osteoclast surface was noted, although the trend was downward, consistent with coupling. Cultured bone marrow stromal cells exhibited reduced nodule mineralization in culture, suggesting that at least part of the deficit was cell autonomous (Otomo *et al.*, 2007). Thus, VEGFR1 may play an important role in mature osteoblast synthetic function, maintaining synthetic activity necessary for optimal bone mass accrual and bone strength.

Carmeliet and colleagues very recently identified another aspect of VEGFR1 biology that reflects activation by placental growth factor (PlGF), a highly selective ligand for VEGFR1 (Maes *et al.*, 2006). Like VEGF, PlGF is spliced to form four variants denoted as PlGF1 (PlGF131), PlGF2 (PlGF152), PlGF3 (PlGF203), and PlGF4 (PlGF 224). Although PlGF −/− mice are developmentally viable (Carmeliet *et al.*, 2001), detailed studies subsequently demonstrated that PlGF potentiates the actions of VEGF, augmenting VEGFR1 signaling postnatally during wound repair and tumor angiogenesis (Carmeliet *et al.*, 2001; Maes *et al.*, 2006). Of note, treatment of mesenchymal stem cell with the powerful bone morphogen BMP2 upregulates PlGF expression (Marrony *et al.*, 2003; Raida *et al.*, 2006). PlGF recruits mesenchymal VEGFR1+ stem cells from the marrow environment to promote fracture healing (Maes *et al.*, 2006). In a definitive series of experiments, Carmeliet and colleagues demonstrated that PlGF deficiency compromises fracture healing in mice (Maes *et al.*, 2006). Moreover, the turnover of calcified cartilage—a component of fracture healing necessary for lamellar bone formation—is also severely compromised in VEGFR1(TK–/TK–) mice, to the extent that nonunion occurs (Niida *et al.*, 2005). In some aspects, this phenotype resembles that of mesenchymal BMP2 deficiency (BMP2$^{cko/cko}$; Prx1::cre); importantly, BMP2 is a potent stimulus that sustains PlGF1 and PlGF2 production by mesenchymal stem cells (Marrony *et al.*, 2003; Raida *et al.*, 2006). Thus, *in toto*, the results of Carmeliet, Shibuya, and colleagues indicate that VEGFR1 activation by PlGF not only is important in bone mass accrual via osteoblast synthetic activity, but also is critical to the bone metabolic processes necessary for fracture repair and other pathologic responses (Maes *et al.*, 2006; Murakami *et al.*, 2006; Niida *et al.*, 2005).

VEGFR2

VEGFR2 is critical to the developmental organization of vascular endothelial cells (Shalaby *et al.*, 1995). Mice completely null for the VEGFR2 gene are embryonic lethal around E8.0 to E8.5 because of lack of vasculogenesis and blood island formation that are dependent on the hemangioblast—a VEGFR2+ progenitor of both endothelial and hematopoietic cells (Choi *et al.*, 1998). Like VEGFR1, this 1356-amino-acid receptor tyrosine kinase possesses (a) seven immunoglobulin-like extracellular domains, (b) a transmembrane domain, (c) a tyrosine kinase with an about 70-amino-acid kinase insert domain; and (d) a carboxy-terminal effector domain that interacts with PLC-gamma and other signal transducing molecules (Takahashi and Shibuya, 2005). VEGFR2 also associates with the co-receptor neuropilin dependent on VEGF165 ligand-induced binding; mice with transgenic augmentation of neuropilin-1 (NRP1) exhibit embryonic lethality with excess capillary growth (Kitsukawa *et al.*, 1995). The glycosaminoglycan modification of NRP1 modulates VEGFR2 signaling in a modification-specific and cell type specific fashion (Shintani *et al.*, 2006). Activation of PI3K cell survival signals, ERK/MAPK-dependent mitogenicity, and the focal adhesion kinase pathways necessary for cell migration are also provided by the VEGFR2 carboxy-terminal domain. Moreover, VEGF recruitment of EPCs to sites of injury and active angiogenesis is dependent on VEGFR2 signaling (Li *et al.*, 2006).

VEGFR2 is classically activated by ligand-induced homodimerization that activates receptor tyrosine kinase activity, leading to VEGFR2 autophosphorylation and recruitment of cytosolic signaling adapters that elaborate second messengers (Takahashi and Shibuya, 2005). Ligands important for VEGFR2 action in bone include VEGF and VEGF-E, with plasmin-activated VEGF-C and VEGF-D also contributing (McColl *et al.*, 2003; Rissanen *et al.*, 2003) as VEGF-A actions are truncated (Roth *et al.*, 2006). Unlike VEGF, the parapoxivirus protein VEGF-E is a ligand specific to VEGFR2; VEGF-E can activate angiogenesis with much less edema and inflammation (Ogawa *et al.*, 1998). The fact that VEGF-E has little if any direct effect on osteoblasts has been taken to indicate that the expression of VEGFR2 on the osteoblast surface is of questionable consequence (Mayr-Wohlfart *et al.*, 2002). However, by promoting VEGFR1-mediated phosphorylation and activation of VEGFR2, PlGF binding to VEGFR1 can recruit active signaling via VEGFR2 (Autiero *et al.*, 2003). Moreover, elegant work by Min, Alitalo, and colleagues has identified another mechanism for activation of VEGFR2 signaling—via TNFR2 activation (He *et al.*, 2006; Luo *et al.*, 2006; Pan *et al.*, 2002; Zhang *et al.*, 2003). TNF-alpha activation of TNFR2 activates Etk/Bmx, a tyrosine kinase that phosphorylates VEGFR2 at Tyr-1175 and Tyr-801, residues necessary for PI3K/Akt signaling that promote endothelial cell migration, survival, and vascular permeability (Luo *et al.*, 2006; Zhang *et al.*, 2003). The TNFR2-Bmx/Etk pathway appears to be critical for angiogenic responses in response to tissue ischemia. In both Tie2-Cre;Bmx$^{CKO/CKO}$ (He *et al.*, 2006) and TNFR2–/– mice (Goukassian *et al.*, 2007), arteriogenesis and capillary formation necessary for hindlimb recovery following ischemia were markedly impaired; in the absence of endothelial and marrow TNFR2 → Bmx→ VEGFR2 signaling, limbs could not generate adequate angiogenic responses to ischemia, and limb necrosis and autoamputation ensued.

Because of the critical importance of vascular supply to tumor growth, VEGFR2 signaling has been successfully targeted to improve survival in patients with colorectal and renal malignancies (Dallas *et al.*, 2007). Moreover, sunitinib—a kinase inhibitor that targets VEGFR2, PDGFR-beta, and c-kit—has proven useful in the treatment of gastrointestinal stromal cell tumors (Dallas *et al.*, 2007; Takahashi and Shibuya, 2005). However, mechanism-based toxicity to bone health and fracture may occur as well, dependent on the physiological demands placed on the patient's skeleton. In the growing skeleton, VEGF-VEGFR2 signaling is critical to the vascular invasion of growth plate cartilage necessary for endochondral bone formation (Gerber *et al.*, 1999; Maes *et al.*, 2002, 2004; Peng *et al.*, 2005; Zelzer *et al.*, 2002). As noted earlier, expression of VEGF—regulated by Runx2/Cbfa1 in hypertrophic chondrocytes—depends on the activity of the osteochondrogenic transcription factor, Runx2 (Zelzer *et al.*, 2001). Inhibition of VEGF bioactivity using a faux receptor-soluble fragment of VEGFR1 inhibits growth plate vascularization, decreases hypertrophic chondrocyte mineralization, reduces chondroclast recruitment and primary spongiosa remodeling, and thus impairs trabecular bone formation (Gerber *et al.*, 1999). VEGF expression and bioactivity figures prominently in fracture repair, whether occurring via endochondral or intramembranous ossification mechanisms. Gerstenfeld, Einhorn, and colleagues studied angiogenic responses in detail in distraction osteogenesis, a model of fracture repair where intramembranous ossification is stimulated by mechanical tension; little if any significant endochondral bone forms. In this model VEGF is upregulated in the distraction gap by the graded mechanical stimuli that promote intramembranous repair. Although the precise mechanisms are as yet unclear, HIF-alpha, a critical regulator of osteogenic-angiogenic coupling via paracrine VEGF production (Wang *et al.*, 2007), is also upregulated with appropriate mechanical distraction (Carvalho *et al.*, 2004; Mori *et al.*, 2006). Moreover, recent QTL mapping of loci contributing to bone anabolism response to mechanical load identified a region on mouse chromosome 17 that encompasses VEGF (and Runx2) genes (Kesavan *et al.*, 2007).

Neuropilin 1, Neuropilin 2

Neuropilins are transmembrane proteins initially identified as receptors for the semaphorin family of axonal guidance molecules, and VEGF itself does indeed participate in axonal guidance processes (Kolodkin *et al.*, 1997; Schwarz *et al.*, 2004). However, as outlined throughout the text, neuropilin 1 (NRP1; VEGF165R) and 2 (NRP2) are critical components of the receptor complexes that mediated VEGF and PlGF signaling during physiological and pathological angiogenesis (Staton *et al.*, 2007). Mice lacking either NRP1 or NRP2 exhibit profound abnormalities in vasculogenesis, and disruption of both genes results in very early embryonic lethality with completely avascular yolk sacs (Takashima *et al.*, 2002). Lowik and colleagues first identified that NRP1 is expressed by the KS483 osteoprogenitor cell line, with mRNA accumulation peaking at the point of maximal mineralization in culture (Deckers *et al.*, 2002). However, because soluble VEGFR1—a ligand for both VEGF and PlGF—did not inhibit mineralization of this cell culture model (Deckers *et al.*, 2002), the role and regulation of NRP1 in osteoblast cell physiology has yet to be clarified *in vivo*. Since NRP1 regulation of VEGF receptor signaling is cell-type specific and completely dependent on the type of NRP1 glycosaminoglycan modification (Shintani *et al.*, 2006), comprehensive studies must include an analysis of NRP1 post-translational modifications during osteoblast differentiation.

Recently, data from Watts and colleagues (Pan *et al.*, 2007b) and Neufeld and colleagues (Shraga-Heled *et al.*, 2007) have identified that a direct role for VEGF121 interactions with NRP1—independent of VEGFR2 bridging—is important for endothelial cell migration and capillary sprouting. An inhibitor antibody designed to selectively target NRP1 (Pan *et al.*, 2007a) not only inhibited VEGF165 binding and signaling, but antagonized VEGF121 actions (Pan *et al.*, 2007b); the latter was not expected, because the original working models of NRP1 signaling posited that NRP1 would facilitate VEGR2 signaling by ligands such as VEGF165. Indeed, NRP1 does enhance VEGF165—but not VEGF121—binding to VEGFR2 in surface plasmon resonance assays (Pan *et al.*, 2007b). However, residues encoded by VEGF amino acids 109 to 121 mediate direct interactions between VEGF and NRP1 that is independent of the NRP1–VEGF heparin binding domain interactions of VEGF165. This tail region, present in both VEGF165 and VEGF121, has been called Tuftsin and directly binds NRP1 (von Wronski *et al.*, 2006). Thus, VEGF121—independent of NRP1-VEGFR2 bridging—mediates key components of paracrine VEGF signaling via NRP1. Early data suggests that both NRP1 and NRP2 can enhance VEGF121-induced phosphorylation of VEGFR2 (Shraga-Heled *et al.*, 2007). The intracellular signaling mechanisms conveying NRP-VEGF121 activation of VEGFR2 have yet to be elucidated.

VEGF RECEPTOR SIGNALING AND SKELETAL REPAIR: SUMMARY AND FUTURE DIRECTIONS

The biological mechanisms whereby VEGFR signaling promotes bone mass accrual and restores skeletal health following injury are better understood—but is by no means comprehensive. Osteogenic-angiogenic coupling (see Fig. 1) is necessary for skeletal growth and repair (Gerber *et al.*, 1999; Maes *et al.*, 2006; Peng *et al.*, 2005; Street *et al.*, 2002; Zelzer *et al.*, 2002). The actions of VEGF and PlGF signaling on tissue angiogenesis (VEGFR2), recruitment of mesenchymal progenitors (VEGFR1), recruitment of multipotent EPCs capable of endothelial as well as osteogenic cell fates (VEGFR2, VEGFR1), and inhibition of osteoblast apoptosis (VEGFR2) have much to do with the skeletal benefits of activating VEGF signaling (Li *et al.*, 2006; Maes *et al.*, 2006) in preclinical models of fracture nonunion. Studies by Huard and colleagues demonstrated synergistic enhancement of osteogenic differentiation and bone repair in critical calvarial defect models (Peng *et al.*, 2002). An optimal ratio of BMP4 to VEGF was required to ensure efficient cartilage induction, followed by VEGF-dependent vascularization and endochondral bone formation. Street and colleagues made similar observations in a rabbit radius gap defect model, and highlighted that VEGF promotes bone repair by promoting angiogenesis, remodeling, and ossification of fracture the callus. Inhibition of VEGF and PlGF actions with soluble VEGFR1 impaired fracture healing (Street *et al.*, 2002). Krebsbach and colleagues subsequently showed that radiation-induced compromise of surgically induced calvarial defects was also reversed by local VEGF administration on biodegradable scaffolds; recovery of bone formation closely tracked recovery of blood flow (Kaigler *et al.*, 2005, 2006). Of note, with skeletal growth and following fracture, circulating CD34+ EPCs with osteogenic potential increase; Khosla and colleagues first proposed that these EPCs would be recruited to sites of skeletal injury and thus accelerate bone repair (Eghbali-Fatourechi *et al.*, 2005). Asahara and co-workers directly demonstrated this fact; they showed that human CD34+ EPCs enhanced angiogenesis and osteogenesis—and promoted femoral fracture healing when transplanted intravenously in a xenograft-tolerant nude rat model of fracture nonunion (Matsumoto *et al.*, 2006). Since VEGFR1 and VEGFR2 are required to recruit circulating EPCs to sites of angiogenesis (Li *et al.*, 2006), the local production of VEGF elaborated during fracture is likely to be critical to rapid repair and remodeling of the injured skeleton.

VEGFR signaling—including VEGFR1, VEGFR2, and NRP1—is central to the cross-talk between cells of endothelial, mesenchymal, and histocyte lineages (Fig. 2) that give rise to the endothelium, osteoblasts, and osteoclasts in the bone remodeling compartment (Eriksen *et al.*, 2007). Propelled by the seminal data of Harada, Rodan, and Rodan (Harada *et al.*, 1994), recent data from multiple laboratories have now clearly established the important

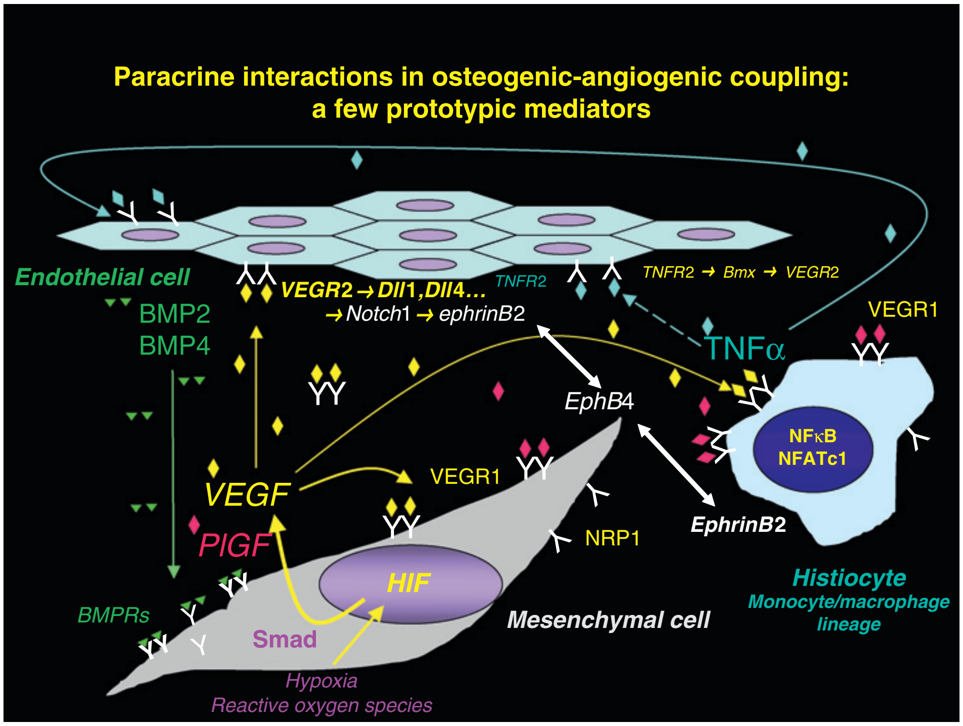

FIGURE 2 The central role of VEGF receptor signaling in osteogenic-angiogenic coupling. Recent studies of orthotopic and heterotopic mineral deposition have established that functional interactions between endothelium, mesenchyme (pericytes, bone marrow stromal cells, osteoblasts), and tissue histiocytes (monocytes, macrophages, osteoclasts) coordinate the deposition and removal of extracellular calcified matrix. Paracrine signals provided by VEGF and PlGF (Autiero *et al.*, 2003; Li *et al.*, 2006; Maes *et al.*, 2006; Marrony *et al.*, 2003; Raida *et al.*, 2006) convey and control osteogenic morphogens such as BMPs, Delta-Notch, and bidirectional ephrinB2–EphB4 signaling (Erber *et al.*, 2006; Hainaud *et al.*, 2006; Scehnet *et al.*, 2007; Thurston *et al.*, 2007; Zhao *et al.*, 2006) that control bone formation in the bone remodeling compartment (Eghbali-Fatourechi *et al.*, 2007; Eriksen *et al.*, 2007). Not shown are the numerous other paracrine signals—e.g., those provided by Wnt, FGF, Ihh, prostaglandin, or matrix cytokines—that are necessary for intramembranous and endochondral bone metabolism. See text for details. BMPR, type I and type II bone morphogenetic protein receptors; Bmx, bone marrow kinase X-linked; Dll, delta-like; HIF, hypoxia induced factor; NRP, neuropilin; PlGF, placental growth factor; TNFR, tumor necrosis factor receptor; VEGFR, vascular endothelial growth factor receptor. (See plate section)

role for VEGF signaling in all aspects of bone physiology (Gerber *et al.*, 1999; Maes *et al.*, 2006; Zelzer *et al.*, 2002). Enlightened by this knowledge, novel strategies for enhancing bone formation under conditions of impaired wound healing have emerged. Because between 10% and 15% of fractures exhibit impaired healing and nonunion, strategies that augment VEGF and PlGF—with diminished inflammatory responses—may accelerate fracture repair in these therapeutically difficult settings (Damany *et al.*, 2005; Kalra *et al.*, 2006). Low-turnover bone disease—a serious complication in a subset of patients with end-stage kidney disease that increases risk for avascular necrosis—might be addressed in part via augmentation of PlGF-dependent EPC and osteoclast lineage activation (Kalra *et al.*, 2006). Future studies will no doubt address how pharmacologic manipulation of VEGFR1, VEGFR2, and NRP1 signaling cascades can address the burgeoning unmet clinical needs in musculoskeletal medicine (Towler, 2007).

It is important to note that strategies useful for reducing VEGF signaling in neoplasia (Takahashi and Shibuya, 2005) will compromise skeletal reparative potential to varying extents—dependent on drug, dose, disease, and skeletal metabolic status. Because the high-dose aminobisphosphonates utilized in patients with malignancy suppress angiogenesis and accumulate in the skeleton (Wood *et al.*, 2002), some cases of osteonecrosis of the jaw following dental procedures in these patients may in fact reflect drug-induced impairment of osteogenic angiogenic coupling (Khosla *et al.*, 2007). This notion has yet to be rigorously tested. The effects of more specific VEGF-VEGFR2 signaling inhibitors on skeletal homeostasis in patients have not been reported; based on the impaired fracture healing observed with VEGF antagonism in preclinical models, these therapeutics may also compromise skeletal reparative capacity in cancer patients. Better modalities are necessary to functionally image bone metabolism and blood flow in all skeletal venues (Leitha *et al.*, 1996; Schmitz *et al.*, 2002; Yang *et al.*, 2002). As our fundamental understanding of osteogenic-angiogenic coupling in specific physiological contexts becomes more comprehensive, personalized strategies will emerge for optimizing clinical outcomes and skeletal health to the benefit of our patients.

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Chapter 53

Transforming Growth Factor-β

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TGFβ SUPERFAMILY

Transforming growth factor beta-1 (TGFβ-1) is the prototype and founding member of the TGFβ superfamily. This family has grown to include more than 40 members, including the TGFβ isoforms, activins and inhibins, Mullerian inhibitory substance, growth and differentiation factors (GDFs), and an ever-increasing number of bone morphogenetic proteins (BMPs) (for reviews see Chang *et al.*, 2002; Chen *et al.*, 2004; Alliston *et al.*, 2008). Members of this superfamily appear to mediate many key events in growth and development and have been maintained evolutionarily from fruit flies to mammals. TGFβ superfamily members are structurally related dimeric molecules. They share a set of conserved cysteine residues that, through disulfide bonding, form a three-dimensional "cysteine knot structure." These proteins are produced as precursor molecules that contain a signal sequence, prodomain and C-terminal mature growth factor domain. Their actions are mediated primarily through a family of serine/threonine kinase transmembrane receptors. Members of the TGFβ superfamily have important regulatory roles in a number of cellular functions, including proliferation, differentiation, chemotaxis, apoptosis, and tumor suppression. Many members of this superfamily also have profound effects on skeletal tissues, both during development and postnatally (reviewed in Janssens *et al.*, 2005; Alliston *et al.*, 2008).

ISOFORMS OF TGFβ

The mammalian TGFβ isoforms include TGFβs 1 through 3, which are closely related and are thought to have arisen by duplication of a common ancestor gene. Much greater homology exists between the mature growth factor regions of the molecules as compared with their precursor regions, suggesting that the propeptides are under less selection pressure and/or that the precursor regions may provide distinct functions. The isoforms of TGFβ have often been "lumped" together and their effects on bone generalized, which must be kept in mind when reviewing the literature in this field. However, in spite of their similarity, the TGFβs1 through 3 are expressed differentially in mammalian tissues. Each binds with different affinities to TGFβ receptors and appears to have slightly different biological effects in *in vitro* assays. Gene knockout studies of the different isoforms have revealed major differences in their phenotypes, emphasizing their nonoverlapping *in vivo* functions (see Section X).

The dramatic differences in tissue distribution of the three mammalian TGFβ isoforms suggest specific functions for each isoform. For example, 80% to 90% of TGFβ in bone is the TGFβ-1 isoform (reviewed in Bonewald, 1999), but the major form produced by kidney glomerular mesangial cells (approximately 50%) is TGFβ-2 (Marra *et al.*, 1996). Prostate produces 30 to 70 times more TGFβ-2 than 1 (Dallas *et al.*, 2005). Even within the skeleton, the expression of the different isoforms is highly regulated, with unique but overlapping expression patterns for each isoform (reviewed in Alliston *et al.*, 2008). Accordingly, the promoter regions for TGFβ-1, TGFβ-2, and TGFβ-3 show little similarity (Kim *et al.*, 1989; Lafyatis *et al.*, 1990; Malipiero *et al.*, 1990). TGFβ genes also appear to be regulated post-transcriptionally, which may account for the lack of correlation between mRNA expression and protein secreted from some cell types.

In addition to having different expression patterns, the TGFβ isoforms have different functions. In many of the *in vitro* assays in which the isoforms have been tested, small variations in biological effects have been observed (reviewed in Centrella *et al.*, 1994). However, the different TGFβ isoforms can have very different effects *in vivo*,

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which may depend on tissue and receptor expression. An example of different opposing effects of the isoforms can be found in the cranial suture of rat calvaria, where removal of TGFβ-3 results in obliteration of the suture, but this is prevented by removing TGFβ-2 (Opperman *et al.*, 1999). Similarly, in models of wound healing, reduced scarring is observed following inhibition of TGFβ-1 and -2, but the same effect can be achieved by addition of TGFβ-3 (reviewed in Ferguson and O'Kane, 2004). On the other hand, TGFβ isoforms can exhibit some functional redundancy, as shown by the observation that defective fusion of the palatal shelves in organ cultures from TGFβ-3 null mice can be partially rescued by application of TGFβ-1 and TGFβ-2 (Taya *et al.*, 1999).

The various TGFβ isoforms bind with different affinities to TGFβ receptors. Investigators are now involved in unraveling the specificity of each isoform for each receptor by determining the three dimensional structures of the ligands and receptors in their bound and free states (for review, see Lin *et al.*, 2006). The TGFβ-2 tertiary structure was determined by x-ray crystallography (Daopin *et al.*,1992) and the structure of TGFβ-1 by nuclear magnetic resonance imaging (Hinck *et al.*, 1996). The shape of TGFβ isoforms has been described as resembling an outstretched hand with curled fingers. Hydrophobic patches are on the "heel" of the "hand" and on the "fingertips." When the two TGFβ monomers bind together to form the homodimer, the hydrophobic "heel" of one molecule touches the hydrophobic "fingers" of the other molecule. The central core of the hydrophobic molecule also contains four molecules of water of unknown function. The further analysis of the three-dimensional structure of the ligand and its receptors should lead to the production of agonists and antagonists for pharmaceutical use in diseases in which TGFβ is misregulated (see Section XI).

PROCESSING AND LATENCY OF TGFβ

Control of TGFβ latency, activation and deposition in the extracellular matrix are all critical steps in the regulation of TGFβ in the skeleton. TGFβs are secreted by most cells in one or more biologically latent forms (reviewed in Annes *et al.*, 2003; Hyytiainen *et al.*, 2004). Release of the mature TGFβ 25kDa homodimer from these latent complexes is necessary for TGFβs to bind to receptors and exert their effects on target cells. Of the three TGFβ isoforms, TGFβ-1 has been the most widely studied. The insights gained into its processing, storage and activation may form a paradigm for the other isoforms and possibly other members of the superfamily.

TGFβ-1 is produced as a 390 amino acid precursor (prepro-TGFβ-1), which consists of a 29-amino-acid signal peptide, a 249-amino-acid propeptide (also known as latency-associated peptide, or LAP), and a 112-amino-acid mature growth factor peptide. A series of post-translational modifications occur as summarized schematically in Fig. 1. First, the signal peptide is cleaved off, then two monomers dimerize through disulfide bonding at positions 223 and 225 in the propeptide and position 356 in the mature growth factor. Following dimerization the precursor is cleaved by furin at position 278 to produce the mature 25-kDa TGFβ homodimer and the 75-kDa LAP. After cleavage, the mature TGFβ and LAP remain associated noncovalently, forming a complex known as the small latent TGFβ complex (SLC), in which the TGFβ is inactive and unable to bind to its receptors. Activation of TGFβ requires dissociation of the mature dimeric growth factor from its propeptide (see Section IV).

Most cells secrete the majority of their TGFβ as a "large latent complex" (LLC) in which proteins called latent TGFβ-binding proteins (LTBPs) are linked to LAP. This linkage occurs via disulfide bonding between cysteine 33 of the LAP and the third 8-cysteine repeat domain of the LTBP. LTBPs do not appear to be required for latency, as the small latent complex is, by itself, latent (Gentry and Nash, 1990). However, these proteins can facilitate the correct folding and secretion of TGFβ (Miyazono *et al.*, 1991) and they appear to play a major role in targeting latent TGFβ for storage in the extracellular matrix (Taipale *et al.*, 1994; Dallas *et al.*, 1995; Olofsson *et al.*, 1995). To date, four LTBP isoforms (LTBPs 1–4) have been identified, ranging in size from 125 to 240 kDa (for reviews see Hyytiainen *et al.*, 2004; Rifkin, 2005).

Although most cell types secrete TGFβ predominantly as part of the large latent complex, containing LTBPs, bone cells are unique in that they are the only known cell type that secretes a significant proportion (approximately 50%) of their TGFβ in the small latent complex form (Bonewald *et al.*, 1991; Dallas *et al.*, 1994). The SLC may therefore have a unique function in bone, perhaps by acting as a soluble, more readily activated form.

ACTIVATION OF LATENT TGFβ

Because both TGFβ and its receptors are ubiquitously expressed and TGFβ has such potent effects on many cell types, activation of the latent forms must be tightly controlled and is likely one of the major mechanisms for regulation of TGFβ functions in skeletal and other tissues. Latent TGFβ can be activated physicochemically by transient acidification or alkalinization, by the action of chaotropic agents, or by heat treatment (Lawrence *et al.*, 1985; Pircher *et al.*, 1986; Brown *et al.*, 1990) . These treatments presumably induce conformational changes in the LAP that destabilize the complex, releasing the mature growth factor. Although the precise physiological mechanisms by which TGFβ is activated *in vivo* remain unclear, several potential mechanisms have been proposed, based mainly on *in vitro* studies using purified native or recombinant

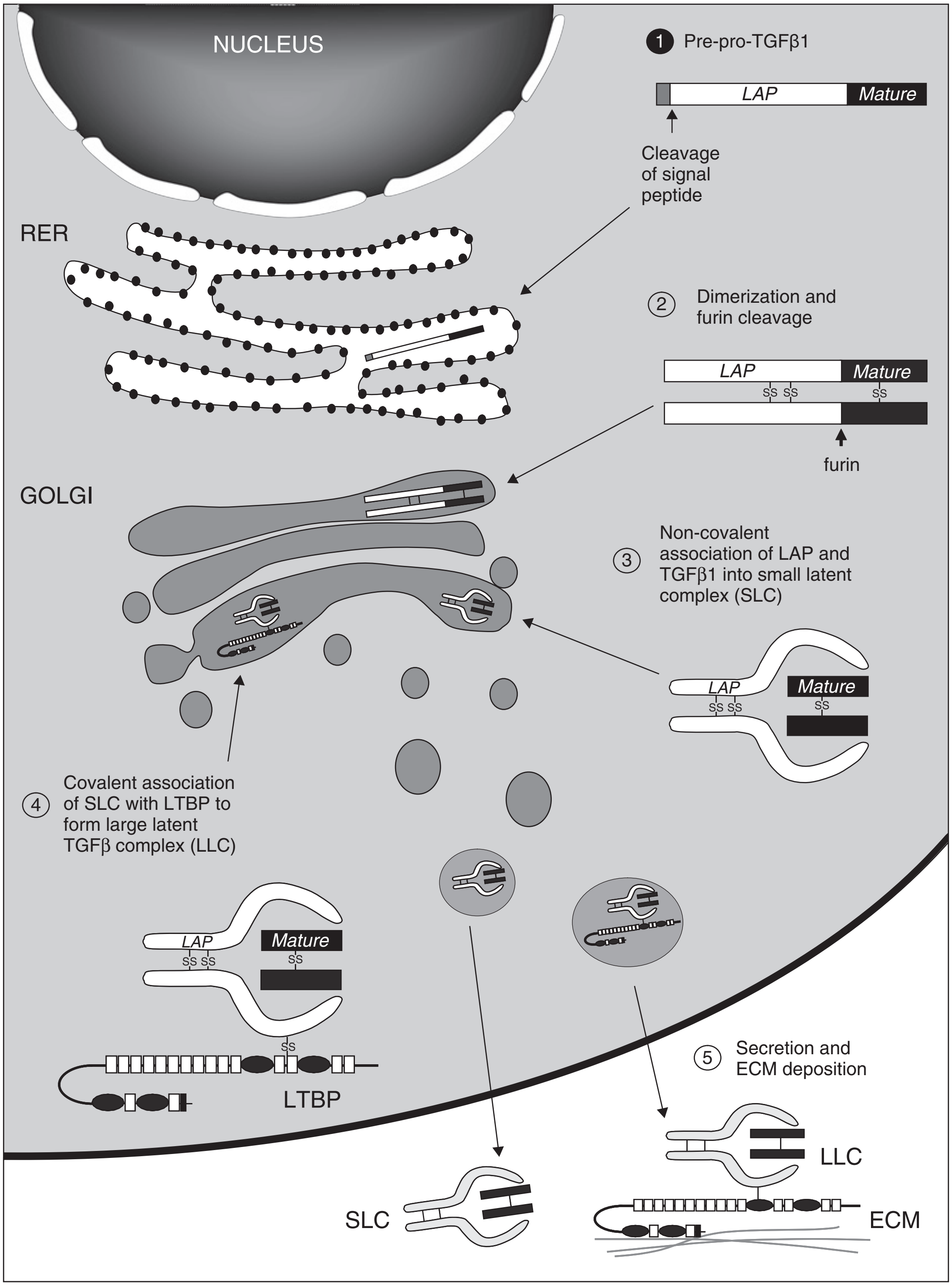
NUCLEUS
① Pre-pro-TGFβ1
LAP
Mature
Cleavage of signal peptide
RER
② Dimerization and furin cleavage
LAP
Mature
SS SS
SS
furin
GOLGI
③ Non-covalent association of LAP and TGFβ1 into small latent complex (SLC)
LAP
Mature
SS SS
SS
④ Covalent association of SLC with LTBP to form large latent TGFβ complex (LLC)
LAP
Mature
SS SS
SS
SS
LTBP
⑤ Secretion and ECM deposition
LLC
SLC
ECM

FIGURE 1 Schematic diagram depicting the major steps in the synthesis, processing and secretion of TGFβ-1. (1) TGFβ-1 is synthesized as a 390 a.a. precursor (prepro-TGFβ-1), consisting of 29 a.a. signal peptide, a 249 a.a. propeptide (also known as latency-associated peptide, or LAP), and a 112 a.a. mature growth factor peptide. A series of post-translational modifications occur as it traffics through the rough endoplasmic reticulum (RER) and Golgi and is secreted into the extracellular space. First, the signal peptide is cleaved off, and (2) two TGFβ-1 monomers dimerize through disulfide bonding at positions 223 and 225 in the propeptide and position 356 in the mature growth factor. Following dimerization, the precursor is cleaved by furin at position 278 to produce the mature 25-kDa TGFβ homodimer and the 75-kDa LAP homodimer. (3) After cleavage, the mature TGFβ and LAP remain associated noncovalently, forming a complex known as the small latent TGFβ complex (SLC), in which the TGFβ is inactive and unable to bind to its receptors. (4) Proteins called latent TGFβ-binding proteins (LTBPs) are linked to LAP via disulfide bonding between cysteine 33 of the LAP and the third 8-cysteine repeat domain of the LTBP to form the "large latent TGFβ complex" (LLC). (5) Once secreted from the cell, the LLC binds to the extracellular matrix, primarily through binding of the N terminus of LTBPs to ECM components. Bone cells also secrete significant amounts of SLC, which may represent a soluble, more readily activatable form. Modified from Janssens *et al.* (2005). *Endocr. Rev.* **26**, 743–744.

proteins and/or cell culture systems (reviewed in Annes *et al.*, 2003; Rifkin, 2005). When reviewing the literature it is important to consider the source of the TGFβ being activated (i.e., SLC versus LLC, recombinant versus native protein) as well as whether the latent TGFβ is in solution or may be bound to the matrix.

Proposed *in vivo* activation mechanisms fall into three main categories, any or all of which may be relevant in bone: (1) proteolytic degradation of the LAP, resulting in release of mature TGFβ, (2) induction of a conformational change in LAP (e.g., through interaction with cell surface integrins or thrombospondin), resulting in destabilization of the complex; or (3) disruption of the noncovalent interactions between LAP and mature TGFβ. It also seems likely that these mechanisms may work in combination, for example a conformational change, followed by proteolytic cleavage at a previously cryptic site. Other steps, such as conversion of matrix-bound LLC into soluble form(s) of LLC, may also be key events in the activation pathway.

Lyons and colleagues were the first to demonstrate proteolytic activation of recombinant SLC by plasmin, which cleaves LAP at multiple sites (Lyons *et al.*, 1990). Plasmin-mediated activation of latent TGFβ was also shown in cocultures of pericytes and smooth muscle cells (Sato and Rifkin, 1989), and subsequently, matrix metalloproteinases (MMPs) 2 and 9 were reported to activate latent TGFβ *in vitro* (Yu and Stamenkovic, 2000). Not only is LAP a target for proteolysis, but the protease-sensitive hinge regions of LTBPs are also potential targets for protease action to release from the matrix a soluble form of LLC containing a cleaved fragment of LTBP. This released LLC presumably then requires further processing for activation. Several studies have confirmed that proteases including plasmin, elastase, and MMPs can cleave LTBP1 to release LLC from the matrix (Taipale *et al.*, 1995; Dallas *et al.*, 2000, 2002). In the case of plasmin, the concentrations required to release bone matrix bound LLC appear to be 10- to 100-fold less than those required to activate the SLC (Dallas *et al.*, 2002), suggesting that a more physiological function of plasmin may be to release the latent complex from the matrix rather than to activate soluble forms. Elastase will cleave matrix-bound LTBP1 (Taipale *et al.*, 1995; Dallas *et al.*, 2002) but does not appear to activate the SLC (Dallas *et al.*, 2002). Whereas both MMP-9 and MMP-2 activate SLC (Yu and Stamenkovic, 2000), MMP-9, but not MMP-2, appears to be able to cleave LTBP1 (Dallas *et al.*, 2002). Other studies have shown that stromelysin-1 (MMP3) can cleave LTBP1 and release LLC from the matrix for subsequent activation by other proteases (Maeda *et al.*, 2002). Together, these observations suggest that combinations of proteases may be involved in the activation of TGFβ, i.e., activation may occur via a series of sequential proteolytic cleavage events that may be different in specific tissues.

Thrombospondin-1 (TSP-1) is a matricellular protein that activates both small and large latent TGFβ by inducing a conformational change in LAP (reviewed in Murphy-Ullrich and Poczatek, 2000). This occurs via a two-step process involving binding of TSP-1 to LAP, followed by release of active TGFβ. A KRFK amino acid motif in the type I repeats of TSP-1 binds to a conserved LSKL sequence in the N terminus of LAP. A synthetic KRFK peptide by itself can activate latent TGFβ (Schultz-Cherry *et al.*, 1995) and, conversely, a synthetic LSKL peptide acts as an antagonist to prevent TSP-1 activation of latent TGFβ. There are obvious potential therapeutic applications for such inhibitory or stimulatory peptides in diseases of TGFβ misregulation (see Section XI). A recent study showed that the LSKL sequence in LAP is also essential for LAP binding to TGFβ (Young and Murphy-Ullrich, 2004). Presumably, the mechanism by which TSP-1 activates latent TGFβ is by occupying this site, thereby preventing TGFβ and LAP from forming a stable complex. In support of an *in vivo* role for TSP-1 in TGFβ activation, the TGFβ-1 null and TSP-1 null mice share a partially overlapping phenotype (Crawford *et al.*, 1998). Furthermore, treatment of TSP-1 null mice with the KRFK peptide that activates TGFβ rescues several of the abnormalities in these mice. Although TSP-1 is known to be expressed in skeletal tissues, future studies are needed to determine whether it contributes to TGFβ activation in the skeleton.

Another proposed mechanism for TGFβ activation *in vivo* is via interactions with cell surface integrins (reviewed in Sheppard, 2005). Munger and colleagues identified the $\alpha_v\beta_6$ integrin as an activator of TGFβ (Munger *et al.*, 1999), and Mu *et al.* (2002) reported activation of latent TGFβ by $\alpha_v\beta_8$ integrins through a mechanism that also requires MT1-MMP activity. In the case of $\alpha_v\beta_6$, the activation occurs through binding between the RGD sequence in the TGFβ-1 LAP and the $\alpha_v\beta_6$ integrin. This would theoretically allow TGFβ to be activated in a spatially restricted manner that would prevent the free diffusion of the activated growth factor away from the cell surface (Sheppard, 2001). These authors also generated mutant forms of the β_6 subunit that bound LAP efficiently but did not activate the latent complex, suggesting that integrin ligation alone is not sufficient for latent TGFβ activation. They presented evidence that the cytoskeleton is required. One possibility is that, after binding of LAP to cell surface integrins, a mechanical force may be applied to LAP via the actin cytoskeleton in a manner analogous to the mechanical stretching of fibronectin during its assembly on the cell surface (Pankov *et al.*, 2000). This may induce a conformational change in LAP to allow activation of the growth factor. In support of an *in vivo* role for $\alpha_v\beta_6$ integrins in TGFβ activation, integrin β_6 null mice showed greatly reduced lung fibrosis compared to wild-type animals, presumably due to less activation of TGFβ (Munger *et al.*, 1999). However, as $\alpha_v\beta_6$ integrin expression is limited to epithelia, this activation mechanism may be cell-type specific. Although an integrin-mediated TGFβ activation mechanism seems likely in skeletal tissues, the specific integrins that may be involved in such a mechanism have yet to be determined.

The mechanisms for activation of TGFβ in bone are not yet fully understood, and much work remains to be done to define these important pathways. Isolated avian osteoclasts upon treatment with retinol were shown to activate latent TGFβ (Oreffo *et al.*, 1989), but this activation was not dependent on plasmin (Bonewald *et al.*, 1997). Similarly, Oursler (1994) showed that avian osteoclast-like cells produced and activated latent TGFβ but saw no significant inhibition of activation using a series of protease inhibitors, including those that block plasmin. However, a combination of five protease inhibitors significantly blocked activation, suggesting a potential role for proteases. Osteoclasts also appear to be able to cleave LTBP1 via the action of serine proteases and/or MMPs to release a soluble LLC from bone ECM, which may facilitate activation of bone matrix bound TGFβ (Dallas *et al.*, 2002). Interestingly, bone is one of the few tissues for which an acid-mediated activation mechanism may be physiological. A mild acid pH (4.5) is sufficient for activation of latent TGFβ (Lyons *et al.*, 1988), which is similar to the pH generated within the osteoclast resorption lacunae (Silver *et al.*, 1988). Thus, resorbing osteoclasts may be able to activate latent TGFβ within their acidic microenvironment (Oreffo *et al.*, 1989). Much less is known concerning the mechanisms of latent TGFβ activation in osteoblasts. Human osteoblast-like cells treated with glucocorticoids activate latent TGFβ (Oursler *et al.*, 1993). Parathyroid hormone-treated osteoblast-like cells, UMR-106–01 cells, and neonatal mouse calvarial cells appear to activate latent TGFβ using the plasminogen activator system (Yee *et al.*, 1993). Karsdal and colleagues have shown that TGFβ activation via MT1-MMP expressed on the cell surface of osteoblasts may play a role in maintaining their viability during the transition from osteoblast to osteocyte (Karsdal *et al.*, 2002). A noncellular mechanism has been described in cartilage in which latent TGFβ is activated by extracellular organelles called matrix vesicles that are associated with matrix calcification (Boyan *et al.*, 1994). Alone, these organelles have no effect on latent TGFβ; however, upon pretreatment of matrix vesicles with $1,25(OH)_2D_3$, both recombinant latent TGFβ-1 and TGFβ-2 are activated. Matrix vesicles contain proteases, such as plasminogen activator, and therefore one of the nongenomic effects of $1,25D_3$ on the membranes of these organelles may be the release of TGFβ-activating proteases

To date, there does not appear to be an extensive literature on potential isoform-specific differences in activation of latent TGFβ complexes. However, a recent study has shown that prostate-specific antigen (PSA) preferentially activates latent TGFβ-2 but not latent TGFβ-1 (Dallas *et al.*, 2005). In the same study, PSA was shown not to cleave LTBP1 or release bone matrix-bound latent TGFβ. Clearly, further studies are needed to better define the complex and potentially tissue-specific and isoform-specific *in vivo* activation mechanisms for TGFβ in the skeleton, as well as in other cell systems, as this may represent a key step in the regulation of these potent growth factors.

LATENT TGFβ-BINDING PROTEINS (LTBPs)

The TGFβs may be unique among growth factors because of their intimate association with and regulation by the LTBPs (see Fig. 1). The LTBPs are a family of four extracellular glycoproteins ranging in size from 125 to 240 kDa. They share homology and structural similarities with the fibrillins and are therefore grouped together as members of a larger superfamily including the fibrillins 1–3 and LTBPs 1–4 (reviewed in Hyytiainen *et al.*, 2004; Todorovic *et al.*, 2005). LTBPs are well established as major regulators of TGFβ actions and appear to exert control at multiple levels in the TGFβ growth factor pathway. Thus, LTBPs may facilitate secretion of latent TGFβ from the cell (Miyazono *et al.*, 1991), provide a mechanism for targeting of TGFβ to the matrix (Taipale *et al.*, 1994; Dallas *et al.*, 1995; Olofsson *et al.*, 1995), and may also provide a mechanism for release of the latent growth factor through proteolytic cleavage of LTBP (Dallas *et al.*, 1995, 2002; Taipale *et al.*, 1995). LTBPs have also been shown to play a role in latent TGFβ activation in certain cell systems (Flaumenhaft *et al.*, 1993; Annes *et al.*, 2004). In particular, ECM targeting of TGFβ, via LTBPs, appears to be essential for latent TGFβ activation via $\alpha_V\beta_6$ integrins on the cell surface (Annes *et al.*, 2004; Fontana *et al.*, 2005).

Independent of their role in regulation of TGFβ, the LTBPs may have other functions. For example, it is now known that, unlike the other LTBP isoforms, LTBP2 does not interact with TGFβ (Gleizes *et al.*, 1996; Saharinen *et al.*, 1996). Therefore its role may be primarily as an extracellular matrix protein. Ninety percent of the LTBP1 in osteoblasts is produced without association with TGFβ, and LTBP1 colocalizes with fibrillin-1 in microfibrillar structures (Dallas *et al.*, 2000). Therefore, independent from its association with TGFβ, LTBP1 appears to function as an extracellular matrix protein required for new bone formation. Both antibodies and antisense oligonucleotides to LTBP1 inhibit the formation of bone-like nodules by fetal rat calvarial cells (Dallas *et al.*, 1995), which cannot be mimicked by antibodies to TGFβ.

The LTBPs, like the fibrillins, have a repeating domain structure consisting predominantly of cysteine-rich repeats of two types. These include 6-cysteine (EGF-like) repeats, similar to motifs found in epidermal growth factor (EGF) precursor, and 8-cysteine repeats (termed "TB repeats"). The TB repeats are unique to the LTBPs and fibrillins and importantly, the site for binding with LAP is located within the third TB repeats in LTBPs 1, 3, and 4 (Gleizes *et al.*, 1996; Saharinen *et al.*, 1996). Specificity for LAP binding is conferred by a four-amino-acid intervening sequence between the sixth and seventh conserved cysteines in the third TB repeat (Saharinen and Keski-Oja, 2000), which is not present in LTBP2. LTBP4 binds more weakly than LTBPs 1 and 3, which is likely due to sequence differences in this four amino acid motif (Saharinen and Keski-Oja, 2000; Chen *et al.*, 2005).

Although all four LTBP isoforms are widely expressed, they do have different tissue distributions, and LTBPs 1 and 3 may be particularly important in bone. In the skeleton, LTBP1 is expressed in early and late differentiated osteoblasts and colocalizes with fibrillin-1 in osteoblast extracellular matrix *in vitro* (Dallas *et al.*, 2000). *In vivo* it is present in cartilage matrix and is colocalized with fibrillin-1 in the periosteum and perichondrium (Dallas *et al.*, 2000). LTBP3 is expressed in osteoblasts, periosteal cells, chondrocytes, and perichondrial cells of the developing skeleton, as well as in articular cartilage and within the presumptive growth plate (Yin *et al.*, 1995). As will be described later in this chapter, gene deletion of LTBP3 results in skeletal abnormalities.

TGFβ RECEPTORS

Members of the TGFβ superfamily signal through a receptor system consisting of two type I and two type II transmembrane receptors with serine/threonine kinase activity. Type III receptors have also been identified that may indirectly regulate signaling through modulation of ligand binding specificity. The TGFβ receptor structure and signaling serves as a model for the rest of the TGFβ family, including BMPs, GDFs, and activins, each of which also plays important roles in the skeleton.

Essentially all cells express receptors for TGFβ (reviewed in Heldin *et al.*, 1997; Hu *et al.*, 1998), and all three TGFβ receptor types are expressed throughout the skeleton, including in cells of the osteoblast and osteoclast lineages (Horner *et al.*, 1998; Kaneda *et al.*, 2000). The type II receptor (TβRII) is responsible for binding to the TGFβ1, TGFβ2, or TGFβ3 ligands. The ligand-bound TβRII then recruits type I receptors to form a heterotetrameric complex, which mediates downstream signaling. Of the three type I receptors that can interact with the TGFβ/TβRII complex (ten Dijke and Hill, 2004), only Alk5 (called TβRI) has yet been implicated in TGFβ signaling in bone. In addition to the type I and II receptors, both of the known type III receptors, betaglycan and endoglin, are present in bone (Segarini *et al.*, 1989).

The TGFβ receptors are transmembrane glycoproteins with a conserved protein structure. Both type I and type II receptors have short extracellular domains with a long cytoplasmic region that mainly consists of a serine/threonine kinase domain. TGFβ binds directly to the type II receptor, which is a constitutively active kinase. The ligand-bound TβRII is then recognized by the type I receptor. These form a complex in which the type II receptor phosphorylates the type I receptor, initiating its kinase activity. Phosphorylation propagates signaling to downstream substrates. For example, phosphoserines in the conserved SGSGSGLP motif of the type I receptor bind Smad proteins. TβRI then phosphorylates Smads, resulting in their release and the subsequent activation of the TGFβ signaling cascade (Attisano *et al.*, 1994).

The type III receptor, betaglycan, can bind to all three TGFβ isoforms and may play a role in presenting TGFβ to the type II receptor (Lopez-Casillas *et al.*, 1993). Glycosylation of the extracellular domain of betaglycan is regulated and likely affects whether these receptors promote or antagonize TGFβ signaling. Glycosylated type III receptor facilitates the presentation of ligand to TβRII. This appears to be particularly important for the TGFβ2 isoform, which cannot efficiently bind to TβRII independently of betaglycan (Stenvers *et al.*, 2003). However, addition of certain glycosaminoglycan chains to betaglycan can inhibit formation of TβRI/TRII complexes (Eickelberg *et al.*, 2002). Betaglycan has a short intracellular domain with no kinase activity. This domain binds β-arrestin following phosphorylation by TβRII, and this interaction is thought to mediate receptor endocytosis, thereby downregulating TGFβ signaling (Chen *et al.*, 2003). The other type III receptor, endoglin, binds TGFβ1 and β3 in the presence of the type II receptor and may also regulate TGFβ signaling (Cheifetz *et al.*, 1992). Mice null for endoglin show a phenotype with many similarities to the TGFβ-1 and TβR11 knockout mice, suggesting an important role in TGFβ signaling *in vivo* (Bourdeau *et al.*, 1999). Further research is needed to fully understand the regulation and role of type III receptors in TGFβ signaling in bone.

The ratio and expression of the different TGFβ receptors can be modified by osteotropic factors. Studies on bone cells using crosslinking of radiolabeled TGFβ showed that PTH and BMP enhanced, whereas glucocorticoids reduced the binding of TGFβ to TβRI (Centrella *et al.*, 1991, 1995). The authors suggested that downregulation of the TGFβ receptor might be essential for osteoblast differentiation. At least part of this regulation has been explained at the transcriptional level. The osteoblast-specific transcription factor, Runx2, binds to the TβRI promoter and confers glucocorticoid dependent regulation of TβRI (Chang *et al.*, 1998; Ji *et al.*, 1998). Because Runx2 integrates signals from several osteotropic pathways, including estrogen, parathyroid hormone, and BMPs, this mechanism may couple TGFβ signaling to the progression of osteoblast differentiation (Chang *et al.*, 1998; McCarthy *et al.*, 2003).

TGFβ SIGNALING PATHWAYS

TGFβ family receptors have multiple signaling effectors. Among the direct substrates for TGFβ type I receptors, the Smad family of intracellular signaling molecules appear to play a central role in TGFβ signaling and have been investigated most extensively (reviewed in Shi and Massague, 2003; ten Dijke and Hill, 2004). Additionally, TGFβ activates signaling by Smad-independent pathways, including signaling via MAP-kinase (MAPK), ERK, p38 MAPK and G protein-mediated pathways to regulate AP-1 and other downstream effectors in osteoblast differentiation (for review, see de Caestecker *et al.*, 2000). Many other proteins that bind TGFβ receptors or Smads facilitate TGFβ crosstalk with other signaling pathways, which may also be important in the intricate cellular regulation of the skeleton. However, this discussion focuses on proteins that participate directly in the TGFβ signaling pathway, with particular emphasis on those that have been shown to impact bone.

The Smad proteins are widely viewed as the major effectors of TGFβ signaling. These proteins are highly conserved and share structural homology through two domains called MH-1 and MH-2. Of the receptor-activated Smads (R-Smads), TβRI specifically phosphorylates and activates Smads 2 and 3. These two R-Smads bind to Smad4, a common mediator Smad (Co-Smad), to form a trimeric complex that can translocate to the nucleus to regulate the transcription of target genes (Feng and Derynck, 2005). Smad4 appears to be the only co-Smad available for TGFβ signaling and genome-wide searches have failed to identify any new Smad family members (Attisano *et al.*, 2001). TGFβ signaling is negatively regulated by the inhibitory Smads (I-Smads), Smad6 and Smad7 (Shi and Massague, 2003). I-Smads can compete with R-Smads for binding to the TGFβ receptor, but can also inhibit signaling via other mechanisms, including the recruitment of ubiquitin ligases to degrade components of the TGFβ signaling pathway.

The expression level, tissue localization, subcellular distribution, and stability of R-Smads, co-Smads, and I-Smads are tightly regulated (ten Dijke and Hill, 2004). The expression of Smads during skeletogenesis mirrors sites and times of known TGFβ family function (Sakou *et al.*, 1999). Both Smad2 and Smad3 are present in osteoblasts and osteoclasts and are required for normal skeletogenesis. Once activated, the R-Smads, in complex with Smad4, translocate to the nucleus to activate or repress gene transcription. The nuclear translocation of Smads is regulated in part by LEMD3, an inner nuclear membrane protein (Hellemans *et al.*, 2004; Lin *et al.*, 2005). In humans, loss of LEMD3 function causes osteopoikilosis, a skeletal dysplasia characterized by symmetric but unequal distribution of hyperostotic bone (Hellemans *et al.*, 2004).

In the nucleus, Smad DNA binding occurs with relatively low affinity and specificity. Therefore, Smads typically interact with other sequence-specific transcription factors to confer TGFβ-responsive gene transcription. For example, Smad3 interacts specifically with the vitamin D nuclear receptor, VDR, at a VDR consensus DNA binding site. Activation of TGFβ signaling results in a Smad3-mediated enhancement of VDR-dependent transcription (Yanagi *et al.*, 1999; Yanagisawa *et al.*, 1999). This cooperative activity may have implications for bone growth and remodeling, because TGFβ and 1,25$(OH)_2D_3$ have been shown to synergistically increase alkaline phosphatase activity in cultured osteoblasts (Bonewald *et al.*, 1992; Wergedal *et al.*, 1992).

Abundant evidence, including the fact that cells lacking Smad4 can still respond to TGFβ, supports the existence and importance of Smad-independent TGFβ signaling pathway(s) (Sirard *et al.*, 1998; Dai *et al.*, 1999). In studies using a mutated TβRI that was defective in recruitment of Smads, activation of the MAPK pathway was observed in response to TGFβ (Yu *et al.*, 2002; Itoh *et al.*, 2003). In mammary epithelial cells, this mutant receptor was able to activate p38 and stimulate apoptosis but was not sufficient to induce epithelial to mesenchymal transition (Yu *et al.*, 2002). Further support for a role for the MAPK pathway comes from studies in which various TGFβ-induced responses were impaired by inhibiting components of the MAPK pathway, either using inhibitors or dominant negative expression constructs (reviewed in Janssens *et al.*, 2005). TGFβ activation of MAPK pathways has been shown to lead to ERK, c-Jun N-terminal kinase (JNK) and p38 MAPK activation and subsequent phosphorylation of components of the Jun, Fos and ATF family of transcription factors (reviewed in Mulder, 2000). TGFβ activated kinase (TAK1) is a member of the MAPK family that is also activated in response to TGFβ (Yamaguchi *et al.*, 1995). Mice null for its binding protein, TGF-beta activated kinase-1 binding protein-1 (TAB1) exhibit delayed ossification, bony malformations and reduced TGFβ responsiveness (Komatsu *et al.*, 2002). Other substrates for the type I receptor have been reported, including FKBP12, WD40, and farnesyl transferase (for review see Derynck and Zhang, 2003). However, the signaling function of these pathways is less clear than the Smad pathway.

In osteoblasts and osteoblast-like cell lines, TGFβ activation of MAPK signaling is required for several critical events, including; changes in cell morphology (Karsdal *et al.*, 2001), type I collagen and collagenase 3 expression (Palcy and Goltzman, 1999; Selvamurugan *et al.*, 2004), inhibition of osteocalcin, alkaline phosphatase and mineralization (Lai and Cheng, 2002; Sowa *et al.*, 2002), and osteoblast to osteocyte transition (Karsdal *et al.*, 2002). In osteoclasts, TGFβ-induced osteoclastogenesis (Karsdal *et al.*, 2003) and osteoclast chemotaxis (Pilkington *et al.*, 2001) have both been shown to be dependent on MAPK. Some of these events may be dependant on dual signaling through both the MAPK and Smad-dependent pathways.

Crosstalk occurs between the Smad and MAPK pathways, which increases the complexity of the signaling response. This may occur through physical interactions between molecular components of the two pathways (reviewed in Janssens *et al.*, 2005). For example, JNK has been shown to phosphorylate Smad3, enhancing its activation (Engel *et al.*, 1999). However, such crosstalk can also have antagonistic effects. For example, TGFβ-activated c-Jun enhanced the interaction of Smad2 with a co-repressor, thus inhibiting Smad2 signaling (Pessah *et al.*, 2001).

A major mechanism by which TGFβ regulates osteoblast function may be through its effects on the transcription factor, Runx2, which occur through both Smad-dependent and Smad-independent TGFβ signaling pathways (reviewed in Ito and Miyazono, 2003). Whereas BMP-activated Smads 1 and 5 bind and activate Runx2 in osteoblasts, the TGFβ responsive Smad3 represses Runx2 function (Zhang and Derynck, 2000; Zhang *et al.*, 2000). Specifically, Smad3 binds and represses Runx2 transactivation of the Runx2 and osteocalcin promoters (Alliston *et al.*, 2001). This occurs by recruitment of transcriptional co-repressors, HDACs 4 or 5, to the Smad3/Runx2 complex (Kang *et al.*, 2005). In contrast to Smad3, Smad2 does not repress Runx2 function, but may act to enhance it (Alliston *et al.*, 2001; Selvamurugan *et al.*, 2004). These interactions may be greatly facilitated by the targeting of Smads and Runx2 to the same intranuclear foci through Runx2 binding to the nuclear matrix (Zaidi *et al.*, 2002). Additionally, the transcriptional co-regulator, menin, further modulates the activity of BMP- and TGFβ-activated Smad/Runx2 complexes to help control the progression of osteoblast differentiation (Sowa *et al.*, 2003).

In addition to the Smad-dependent pathway discussed above, TGFβ also regulates Runx2 function by Smad-independent mechanisms. Thus, the balance between these two pathways may be critical for the progression of osteoblast differentiation. For example, whereas TGFβ-activated Smad3 represses Runx2 function, TGFβ activation of ERK 1 and 2 and p38 MAPK enhances Runx2 phosphorylation and function (Selvamurugan *et al.*, 2004). Additionally, TGFβ increases Runx2 longevity by inducing p300-dependent acetylation of Runx proteins to prevent their association with the Smurf ubiquitin ligases and their subsequent degradation (Jin *et al.*, 2004). Whether or not Smads are required for TGFβ-dependent Runx2 acetylation remains unknown.

Because TGFβ can regulate Runx2 function by several mechanisms, and because Runx2 is a key regulator of TβRI expression, TGFβ and Runx2 form a regulatory feedback loop that may control the progression of osteoblast differentiation. Both the TGFβ pathway and Runx2 integrate information from multiple signaling pathways to modulate their respective activities. For example, in mesenchymal stem cells, TGFβ crosstalks with the Wnt signaling pathway by inducing the translocation of β-catenin in a Smad3-dependent manner (Jian *et al.*, 2006). Smad3 is also implicated in the PTH-dependent increase in osteoblast β -catenin levels (Tobimatsu *et al.*, 2006).

Overall, the delicate balance of Smad and non-Smad-mediated signaling, together with the crosstalk of TGFβ with other signaling pathways, may explain the ability of TGFβ to promote some aspects of osteoblast differentiation while inhibiting others. Furthermore, the subtleties of this regulation may be difficult to replicate *in vitro,* which likely explains some differences across studies of TGFβ action in bone, as will be discussed in the following section. Genetically modified animal models are powerful tools that will likely be the major approach for the future elucidation of the physiologically relevant signaling pathways in bone and other tissues (see Section X).

IN VITRO EFFECTS OF TGFβ ON BONE CELLS

TGFβ is a potent multifunctional cytokine whose major effects in the body appear to be as a regulator of cell growth, a stimulator of matrix production, and an inhibitor of the immune system. TGFβ is also a key player in several human diseases, including fibrotic disorders (reviewed in Sheppard, 2006; Leask, 2007), aortic aneurisms (Loeys *et al.*, 2005; Pannu *et al.*, 2005), and various types of cancer, where TGFβ has been implicated as a tumor suppressor (Buck and Knabbe, 2006; Jakowlew, 2006). Clearly, the actions of TGFβ are widespread; however, here we will focus on the effects of TGFβ in bone cells.

The discovery of large amounts of TGFβ stored in bone matrix led to reports from several laboratories that TGFβ is a major player in bone remodeling. Numerous *in vitro* studies have examined the actions of TGFβ on bone cells, and the data appear to be somewhat contradictory. Upon reviewing these studies, it becomes apparent that the effects of TGFβ in different bone cell culture systems depend upon factors such as the differentiation stage of the cells, the cell density, culture conditions, and concentrations of TGFβ. However, it is clear that TGFβ has dramatic effects at every stage of the bone remodeling process, affecting both bone resorption and bone formation (reviewed in Janssens *et al.*, 2005). A general consensus from the literature is that TGFβ, released and activated by resorbing osteoclasts, stimulates subsequent bone formation by recruiting osteoblast precursors and stimulating them to proliferate, thereby expanding the pool of committed osteoblasts. Thus, TGFβ has been viewed as a “coupling factor” that links bone resorption to subsequent bone formation. TGFβ also stimulates osteoblasts to synthesize extracellular matrix proteins. However, if TGFβ is not downregulated during later phases, it will actually inhibit the later phases of osteoblast differentiation and mineralization. Some of the key studies providing evidence for the involvement of TGFβ at the various stages of bone remodeling are summarized in the following section.

In contrast to BMPs, TGFβ does not induce uncommitted mesenchymal cell lines to differentiate along the osteoblastic pathway (Katagiri *et al.*, 1994). However, once these cells have become committed to the osteoblastic lineage, TGFβ can act as a potent chemotactic factor to recruit osteoblast precursors to sites of bone formation (Lucas, 1989; Pfeilschifter *et al.*, 1990; Hughes *et al.*, 1992). A chemotactic epitope of TGFβ has been identified at residues 368 to 374 (Postlethwaite and Seyer, 1995), which induces the chemotactic migration of neutrophils, monocytes, and fibroblasts. However, this peptide has yet to be tested on osteoblast precursors. Many studies using cell lines, osteoblast-enriched cell cultures or bone organ cultures have reported that TGFβ has a mitogenic effect on osteoblast precursors (Centrella *et al.*, 1986; Robey *et al.*, 1987; Hock *et al.*, 1990; Chen and Bates, 1993). In contrast, other studies have suggested a growth inhibitory effect of TGFβ on osteoblasts (Noda and Rodan, 1986; Antosz *et al.*, 1989). The differences in results are probably due to biphasic effects of TGFβ at high versus low concentrations, as well as the culture conditions and differentiation state of the cells.

TGFβ is well known as a potent stimulator of ECM protein expression in numerous cell types and often also inhibits expression of proteases that degrade ECM proteins, leading to an overall accumulation of ECM (Roberts, 1998). However, published reports have shown both stimulatory and inhibitory effects of TGFβ on type I collagen production in osteoblasts (reviewed in Bonewald and Dallas, 1994; Janssens *et al.*, 2005). Similarly, conflicting data have been reported concerning the effects of TGFβ on fibronectin, osteopontin, osteonectin, and decorin expression as well as on expression of alkaline phosphatase (reviewed in Bonewald and Dallas, 1994; Janssens *et al.*, 2005). The stimulatory effects are generally seen in the earlier stages of osteoblast differentiation. In contrast, the effects of TGFβ in late osteoblasts and on the mineralization process are mostly inhibitory. It has been shown to suppress markers of the calcified matrix, such as osteocalcin, in fetal rat calvarial cells and ROS 17/2.8 osteoblast-like cells (Noda and Rodan, 1989; Harris *et al.*, 1994). Although TGFβ stimulates the formation of osteoid, it actually appears to suppress mineralization of the osteoid (reviewed in Bonewald and Dallas, 1994; Janssens *et al.*, 2005). For example, TGFβ suppresses the mineralization of fetal rat calvarial cells (Harris *et al.*, 1994). One potential reason for the differential effects of TGFβ on early versus late osteoblasts is that there appears to be a decrease in expression of TβRI and TβRII during the transition from pre-osteoblasts to maturing osteoblasts (Centrella *et al.*, 1995; Takeuchi *et al.*, 1996). This may be mediated by TGFβ downregulating expression of its own receptors (Centrella *et al.*, 1996; Gebken *et al.*, 1999). Additionally, many of the effects of TGFβ on bone formation are likely mediated via Runx2, and in later stages of differentiation, TGFβ antagonizes the effects of BMP2 on Runx2 (Spinella-Jaegle *et al.*, 2001). This may be mediated by physical interaction of TGFβ-activated Smad3 with Runx2 to inhibit its binding to Runx2-responsive elements, including one within the Runx2 promoter itself (Alliston *et al.*, 2001).

In vitro studies examining the effects of TGFβ on osteoclast activity are equally complex and many studies again appear to be contradictory. This is partly due to the variety of different culture models used, some of which examine formation of osteoclasts from mononuclear precursors, in either the presence or absence of supporting cells, and some of which examine bone resorption by mature osteoclasts. Spleen cells, bone marrow cells, and peripheral blood mononuclear cells from various species have all been used as sources of osteoclast precursors and/or mature osteoclasts for these studies. An overall consensus from the literature is that TGFβ regulates all the stages in osteoclast resorption, including recruitment of osteoclast precursors to the resorption site, differentiation into the mature osteoclast, bone resorption, and osteoclast apoptosis. Similar to other modulators of osteoclastic activity, these effects of TGFβ on osteoclasts either may represent direct actions of the growth factor on osteoclasts or their precursors or may be mediated indirectly via actions on osteoblasts and/or stromal cell populations.

In bone marrow cultures, which are heterogeneous populations containing both osteoclast precursors and osteogenic cells, TGFβ has biphasic effects. At concentrations in the picogram range, TGFβ stimulates the formation of osteoclast-like cells, but at nanogram concentrations TGFβ is inhibitory (reviewed in Fox and Lovibond, 2005; Janssens *et al.*, 2005). This appears to be mediated via modulation of expression of RANKL, OPG, and M-CSF in the stromal cell populations, because low concentrations of TGFβ increase the ratio of RANKL to its inhibitor, OPG, and increase expression of M-CSF, all of which act to enhance osteoclast formation (Takai *et al.*, 1998; Karst *et al.*, 2004). In contrast, at higher concentrations of TGFβ, expression of RANKL and M-CSF is reduced and OPG is increased (Karst *et al.*, 2004).

In cultures of isolated osteoclasts grown in the presence of RANKL and M-CSF (i.e., without supporting stromal cells), many investigators have reported stimulation of osteoclast-like cell formation by TGFβ (reviewed in Fox and Lovibond, 2005; Janssens *et al.*, 2005). These osteoclastogenic effects are likely due to direct actions of TGFβ on osteoclast precursors to upregulate their expression of RANK, the receptor for RANKL, as well as NFκB. Clearly, the situation *in vivo* is more complex than in these isolated cell culture systems, where the responses of stromal cells and immune cells to TGFβ also affect the process of osteoclastogenesis.

In studies that have examined the effects of TGFβ on bone resorption, again the outcome depends on the type of culture model used and the presence of other cell types. When the assay requires differentiation of osteoclasts from their mononuclear precursors, as in the fetal rat long bone resorption assay, TGFβ is usually found to be inhibitory, consistent with its inhibitory effects on osteoclastogenesis (Pfeilschifter *et al.*, 1988; Dieudonne *et al.*, 1991; Hattersley and Chambers, 1991). In contrast, when the assay starts with already formed osteoclasts, such as in the calvarial resorption assay, TGFβ has almost invariably been found to be stimulatory (Tashjian *et al.*, 1985; Lerner, 1996). These stimulatory effects may be mediated by the generation of prostaglandins by TGFβ (Tashjian *et al.*, 1985), possibly via induction of prostaglandin synthase II in osteoblasts (Pilbeam *et al.*, 1993).

Another important level at which TGFβs may control bone resorption is by inducing the apoptosis of mature osteoclasts (Hughes *et al.*, 1996). TGFβ regulates apoptosis or programmed cell death in a number of tissues, such as liver, uterine epithelial cells, and hemopoietic cell lines, and will cause cell death of various tumor cell lines, such as prostate, liver, and kidney tumors (reviewed in Bursch *et al.*, 1992). TGFβ also induces programmed cell death in osteoclasts and antagonizes the effects of cytokines such as IL-6 that promote osteoclast survival (Hughes *et al.*, 1996). Because active TGFβ is thought to be released from the bone matrix during osteoclastic resorption (Pfeilschifter and Mundy, 1987), an intriguing theory is that TGFβ promotes osteoclast apoptosis as a negative feedback mechanism to limit the extent of bone resorption within a healthy bone. Theoretically, this would be achieved via its actions on osteoclasts to induce apoptosis and on osteoblasts to reduce RANKL expression (reviewed in Fox and Lovibond, 2005). This negative feedback mechanism would then presumably be overridden in pathological states by a strong stimulus to promote osteoclast survival, such as IL-6 or IL-1.

In summary, the use of *in vitro* culture systems has been and will continue to be a powerful tool to identify the cellular and molecular pathways involved in mediating the effects of TGFβ on osteoblasts and osteoclasts. However, care must be taken when interpreting the results of these *in vitro* studies, and the strengths and limitations of the various assays should always be considered when making conclusions.

IN VIVO EFFECTS OF TGFβ ON BONE

TGFβ will induce new bone formation if injected in close proximity to bone. However, unlike the bone morphogenetic proteins, TGFβ does not stimulate bone formation when injected into ectopic sites. Early *in vivo* experiments in which TGFβ was injected over the calvaria of mice showed conclusively that TGFβ stimulates new mineralized bone formation (Noda and Camilliere, 1989; Mackie and Trechsel, 1990; Marcelli *et al.*, 1990). TGFβ has also been shown to initiate chondrogenesis and osteogenesis when applied to the rat femur (Joyce *et al.*, 1990). Soon after these studies were performed, it was shown that a single injection of TGFβ1 can induce bone closure of a nonhealing skull defect (Beck *et al.*, 1991). Several independent studies have confirmed the usefulness of TGFβ1 in repair of large bone defects (Kamakura *et al.*, 2001; Dean *et al.*, 2005; Srouji *et al.*, 2005) and in implant fixation (Lin *et al.*,2001; Sumner *et al.*, 2006). When TGFβ is combined with demineralized bone matrix, an acceleration of osteoinduction is observed, together with increased resorption of the demineralized bone matrix carrier (Kibblewhite *et al.*, 1993). Prostaglandins may be important for some of the effects of TGFβ on osteogenesis (Mackie and Trechsel, 1990; Marcelli *et al.*, 1990).

Systemic administration of TGFβ stimulates cancellous bone formation in both juvenile and adult rats (Rosen *et al.*, 1994), in the hindlimb unloaded rat (Machwate *et al.*, 1995), and in the aging mouse model (Gazit *et al.*, 1999). However, Kalu and co-workers (1993) did not find significant effects of TGFβ2 on cancellous bone loss after ovariectomy. Even though no significant effect was observed on bone loss, TGFβ2 did prevent the increase in TRAP(+)-multinucleated cells and caused a decrease in the number of trabecular osteoclasts. These studies suggest that bone loss in these different systems is mediated through different mechanisms and that TGFβ treatment may be more efficacious in some conditions of bone loss as compared to others. Perhaps in the ovariectomized rat model, resorption is accelerated greatly beyond the levels of formation so that TGFβ has little effect on bone loss. Interestingly, from *in vivo* models examining the effects of injections of TGFβ over murine calvaria (see earlier discussion), it was only after the injections of TGFβ had ceased that the osteoid became mineralized. Together these data suggest that TGFβ may be required to initiate new bone formation, but that it must be removed before mineralization can proceed.

Agents that regulate bone formation or resorption appear to alter the amount of TGFβ in bone. For example, bones from ovariectomized rats have less TGFβ than normal rats (Finkelman *et al.*, 1992), vitamin D-deficient rats have less TGFβ than vitamin-replete animals (Finkelman *et al.*, 1991), and studies using intermittent treatment with parathyroid hormone showing increased bone mass also show increased TGFβ (Pfeilschifter *et al.*, 1995). The only other factor that appears to share this characteristic with TGFβ is insulin-like growth factor.

BONE PHENOTYPES OF TRANSGENIC AND KNOCKOUT MICE WITH ALTERATIONS IN TGFβ ISOFORMS, LTBPs, AND COMPONENTS OF THE TGFβ SIGNALING PATHWAY

Loss-of-function and gain-of-function genetically modified mouse models have provided key insights into the role of

TGFβ signaling *in vivo*. Transgenic mice have been generated with targeted gene deletions of all three TGFβ isoforms, their binding proteins, LTBPs 3 and 4, and the type I and II TGFβ receptors, as well as their signaling effectors, Smads 2, 3, and 4. Except in the case of LTBP4, all of these knockout mouse models either are embryonic lethal prior to skeletogenesis or show bone defects ranging from moderate to severe, underscoring the critical importance of TGFβ signaling in both the embryonic and postnatal skeleton.

Mice with targeted gene deletions for each of the TGFβ isoforms have different phenotypes, suggesting that each isoform has specific nonoverlapping expression patterns and/or functions (i.e., deletion of a particular isoform of TGFβ in knockout animals cannot be compensated for by the other isoforms). Deletion of the TGFβ-1 gene results in premature death due to inflammatory disease (Shull *et al.*, 1992; Kulkarni and Karlsson, 1993; Boivin *et al.*, 1995). The mice appear normal until after weaning, when massive infiltration of lymphocytes and macrophages into multiple organs occurs, leading to organ failure and death. Interestingly, the reason for the appearance of this phenotype after weaning appears to be because TGFβ-1 can be transferred across the placenta and through the milk from the heterozygous mother to her offspring, leading to a maternal rescue prior to weaning (Letterio *et al.*, 1994).

To eliminate the effects of multifocal inflammation, TGFβ-1 null mice were generated on a severe combined immunodeficiency (SCID) background (Diebold *et al.*, 1995). Although inflammation is absent in these mice, due to the lack of T and B cells, and they do survive into adulthood, they are 50% to 80% of the size of their TGFβ-1-expressing littermates, show a lack of vigor, and do not thrive. Geiser and colleagues examined the skeletons of TGFβ1 deficient mice maintained on an immunosuppressive drug and showed that the width of the growth plate, their longitudinal bone growth, and total mineral content as well as the elastic properties of the bone were reduced (Geiser *et al.*, 1998). A subsequent study examined the properties of bones from TGFβ-1-null mice by histology and Fourier transformed infrared microscopy (Atti *et al.*, 2002). These authors showed a reduction in mineral content, mineral crystallinity, and collagen maturity in the cortical bone. There was also a dramatic depletion of osteoblasts on the trabecular bone surfaces, leading to an imbalance in modeling versus bone resorption. Together, the results of these studies are consistent with a potential role for TGFβ-1 in bone quality and in bone modeling.

Mice that lack the TGFβ-2 isoform show a perinatal lethal phenotype and exhibit a wide range of developmental defects, including cardiac, lung, eye, ear, and urogenital defects (Sanford *et al.*, 1997). The skeletal abnormalities include craniofacial defects as well as defects in the ribs, sternum, vertebrae, mandible, and long bones. The craniofacial defects include decreased size and reduced cranial ossification, agenesis of the alisphenoid and occipital bones, and mandibular abnormalities. Cleft palate was also observed but was not fully penetrant. The noncranial skeletal manifestations included limb laxity, bifurcation of the sternum, rib deformities, shortened radius and ulna, absence of specific muscle attachment sites, and vertebral defects resembling spina bifida occulta.

Mice lacking TGFβ-3 also show a perinatal lethal phenotype, probably due to delayed lung development (Kaartinen *et al.*, 1995). These mice show failure of the palatal shelves to fuse, resulting in cleft palate. No other craniofacial abnormalities were observed, suggesting that the effect of TGFβ-3 on palatal shelf fusion is not just a general defect in craniofacial development (Kaartinen *et al.*, 1995; Proetzel *et al.*, 1995). These studies corroborate those of Brunet *et al.* (1995), who found that antibody and antisense oligonucleotides to TGFβ-3 but not TGFβ-1 or TGFβ-2 prevented normal embryonic palate fusion in the mouse. Blavier *et al.* (2001) have further shown that TGFβ-3 deficient mice showed abnormalities in expression of MMPs and their inhibitor, TIMP-2, which may be responsible for the cleft palate phenotype. These studies implicate TGFβ-3 in the complex epithelial-mesenchymal interactions that facilitate correct positioning of the palatal shelves, remodeling of the extracellular matrix, and fusion of the palatal shelves.

Because a large proportion of TGFβ is found in association with LTBPs 1, 3, or 4, absence of or mutations in one of these proteins would also be expected to affect TGFβ processing, storage, and/or activation. Dabovic and co-workers have successfully deleted the gene for LTBP3 in mice (Dabovic *et al.*, 2002, 2005). Postnatally, these mice develop craniofacial skeletal abnormalities, including rounding of the cranial vault, overextension of the mandible, and ossification of the synchondroses. The animals also show reduced body size and kyphosis and develop osteosclerosis as well as severe osteoarthritis with age. Mice lacking LTBP4 exhibit lung emphysema, cardiovascular abnormalities, and colorectal cancer, but skeletal abnormalities have not been reported (Sterner-Kock *et al.*, 2002). As the phenotypes of these mice resemble those associated with perturbed TGFβ signaling, the abnormalities in both the LTBP3 and LTBP4 knockout mice have been interpreted as being primarily due to misregulation of TGFβ. Recent evidence supports this in the case of LTBP4 (Koli *et al.*, 2004), underscoring the importance of TGFβ interactions in the biology of these extracellular matrix proteins.

Deletion of TGFβ receptors results in embryonic lethality, precluding examination of their roles in skeletogenesis in conventional knockout models (reviewed in Chang *et al.*, 2002). However, the importance of TGFβ signaling in the skeleton has become apparent from humans and mice harboring mutations or tissue-specific deletions of TGFβ receptors. Humans with mutations in the TβRI and

TβRII genes exhibit craniosynostoses and cleft palate, as well as other soft-tissue abnormalities (Loeys *et al.*, 2005). Through these types of studies, the TGFβ pathway has emerged as a central regulator of palatogenesis, because targeted mutations in TGFβ-3, TβRI, TβRII, Smad2, or sometimes TGFβ-2 in mice all result in cleft palate (Proetzel *et al.*, 1995; Sanford *et al.*, 1997; Nomura and Li, 1998; Dudas *et al.*, 2004; Cui *et al.*, 2005). Conditional deletion of TβRII in Col2a-expressing cells using the Cre/lox recombinase system revealed that TβRII is also required for axial skeletal patterning (Baffi *et al.*, 2004, 2006). The size and spacing of the vertebrae were altered in these mice, and there were defects in closure of the neural arches. Intervertebral disks (IVDs) were either missing or incomplete. Mice that survived postnatally showed changes in the length of specific bones. The vertebral malformations in these mice are likely due to loss of TβRII expression in the sclerotome, which gives rise to the axial skeleton. Expression of a dominant negative TβRII later in osteoblast differentiation using the osteocalcin promoter showed that TβRII signaling is required for crosstalk between osteoblasts and osteoclasts (Filvaroff *et al.*, 1999). Loss of osteoblast TGFβ signaling in these mice resulted in decreased bone resorption and increased bone mass, showing that TGFβ participates in the homeostatic control of bone mass.

Gene deletions of the effectors of TGFβ signaling, the Smads, would be expected to produce phenotypes that overlap with knockouts of the ligands and their receptors. Because Smad4 is the only known co-Smad for all TGFβ superfamily members, it is not surprising that conventional knockout of Smad4 is early embryonic lethal (Sirard *et al.*, 1998; Yang *et al.*, 1998). However, mice with a conditional deletion of Smad4 in the chondrocytes showed dwarfism, associated with a disorganized growth plate in which the resting zone of chondrocytes was expanded and the proliferative zone was reduced (Zhang *et al.*, 2005). There was also increased hypertrophic differentiation and ectopic bone was observed in the perichondrium.

Homozygous or heterozygous deletion of Smad2 results in embryonic lethality, but Smad2 heterozygous embryos die later in development and exhibit abnormal mandibular development (Nomura and Li, 1998). Smad3-deficient mice are viable and exhibit an osteopenic phenotype (Borton *et al.*, 2001; Yang *et al.*, 2001). This is apparently because loss of Smad3 causes premature osteocyte apoptosis, which impairs production of bone matrix and alters its material properties (Borton *et al.*, 2001; Balooch *et al.*, 2005). Although Smads2 and 3 have 95% sequence homology, the differences in their knockout phenotypes suggest that they have nonoverlapping functions *in vivo*. They also appear to activate different sets of target genes and have different mechanisms of transcriptional activation, again suggesting the nonredundancy of their function (Yang *et al.*, 2003).

Transgenic mice overexpressing active TGFβ-2 driven by the osteocalcin promoter have been described as osteoporotic (Erlebacher and Derynck, 1996). These mice overproduce a constitutively active form of TGFβ2, which is therefore not regulated through the normal physiological control mechanisms of latency, matrix storage, and activation. Conversely, when the osteocalcin promoter is used to drive overexpression of the dominant-negative TGFβ type II receptor, the mice show increased bone mass (Filvaroff *et al.*, 1999). Together, these studies implicate TGFβ as a factor that couples bone resorption to bone formation. If excess amounts of TGFβ are present, then bone remodeling may become uncoupled. Thus, the osteoporotic and osteopetrotic phenotypes of these mouse models likely result from disruption of this coupling mechanism. Although the overall phenotypes appear to contradict many of the observations concerning TGFβ function outlined in the previous paragraphs, in part because of the pathological levels of TGFβ signaling in these transgenic models, such contradictions highlight the importance of the tight regulation of TGFβ expression and activation in bone. It must be expressed and activated at the appropriate time and location and then must be removed or inactivated. For example, excess amounts of TGFβ in mature osteoblasts, as occurs in the TGFβ2 overexpression model, actually appear to inhibit mineralization in the later stages of osteoblast maturation. Bone diseases can potentially result from an imbalance in TGFβ isoforms and their bioavailability, as will be discussed in the following section.

ROLE OF TGFβ IN HUMAN SKELETAL DISEASES

TGFβ has been implicated in a number of disorders of the skeletal system. A particularly exciting recent discovery was that mutations in the TGFβ-1 gene are responsible for Camurati-Engelmann disease (CED; reviewed in Janssens *et al.*, 2006). This is a rare autosomal progressive skeletal dysplasia characterized by hyperostosis and osteosclerosis that mainly affects the diaphyses of the long bones as well as the base of the skull (Fig. 2). Patients experience limb pain, muscular weakness, and a waddling gait and are easily fatigued. They may also have complications such as anemia, leukopenia, and hepatosplenomegaly. Several studies have shown that the genetic defect underlying this condition is mutation of the TGFβ1 gene. The majority of the mutations are in exon 4 in the region coding for the propeptide (LAP), and the Arg218 residue is a particular hot spot for mutations (reviewed in Janssens *et al.*, 2006). In general these mutations are close to cysteine residues 223 and 225 and are thought to cause premature activation of the latent TGFβ complex by destabilizing the disulfide bridging of the LAP homodimers, thereby reducing their ability to associate with mature TGFβ-1 (Saito *et al.*, 2001;

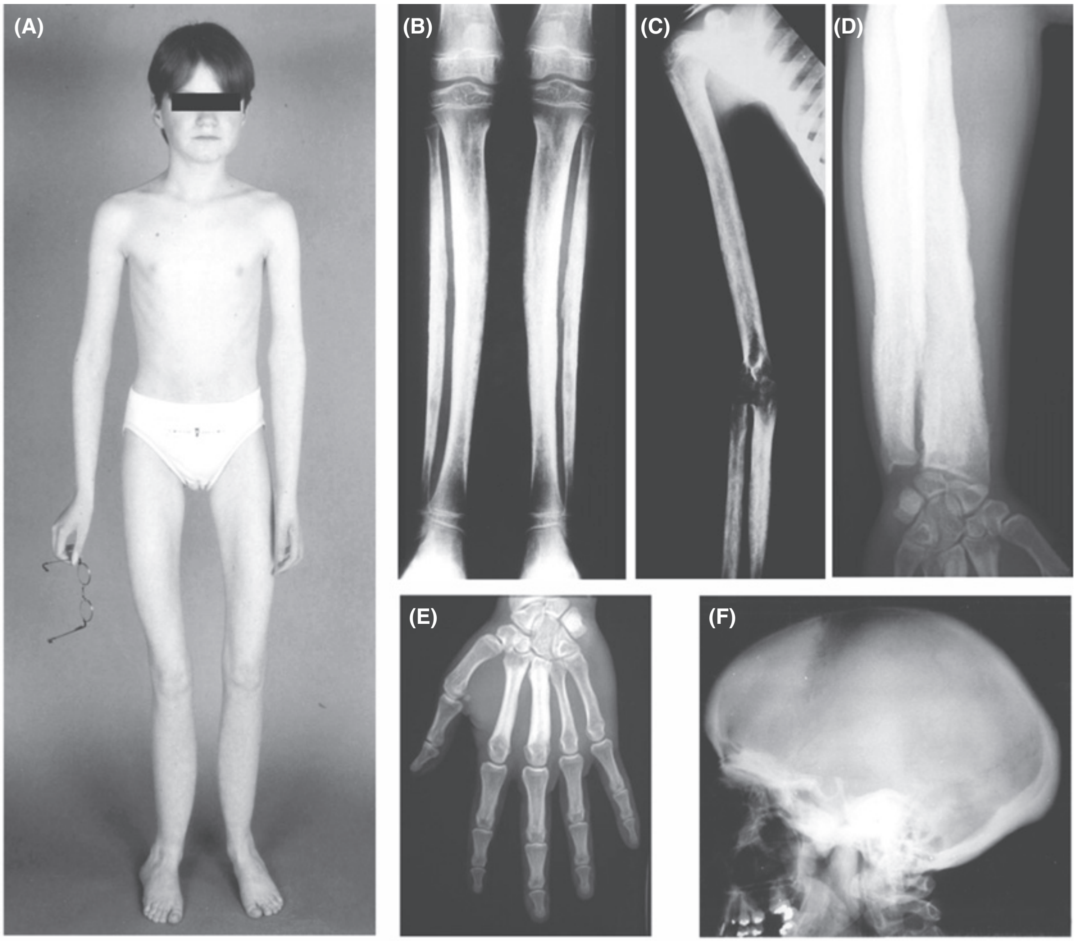

FIGURE 2 Clinical features of CED. (**A**) Clinical picture of a CED patient at the age of 15. Note the absence of subcutaneous fat (weight 27 kg), muscle hypotrophy, and valgus deformity of the knees and feet. Muscle weakness restricts her maximum walking distance to 20 to 50 m. Secondary sex characteristics (breast development, menstruation) were delayed. (**B**) AP radiographs of both lower legs of a CED patient. Note that there is cortical thickening and a severe modeling defect in the diaphyses of both tibiae and fibulae. (**C**) Radiograph of the right arm (AP view) of a CED patient, showing thickening of the cortex of the diaphyseal portion of the humerus, ulna, and radius, resulting in narrowing of the medullary canal. Note also the modeling defect of the long bones, which is most extensive at the diaphysis of the ulna. (**D**) Radiograph of the forearm of a CED patient. Marked cortical thickening at the diaphysis of the ulna and radius can be observed, causing obliteration of the medullary cavity and hypertrophy of the long bones. Note extension of the cortical sclerosis toward the distal metaphysis of the radius. (**E**) Radiograph of the left hand (AP view) of a CED patient, showing cortical sclerosis, cortical thickening, and medullary cavity obliteration at the diaphysis of metacarpals 2 and 3. (**F**) Radiograph of the skull (lateral view) of a CED patient. Sclerosis of the calvaria, the tympanic portion of the skull base, and the ascending ramus of the mandible is visible. Note relatively small frontal and sphenoidal sinuses resulting from adjacent sclerosis of the frontal bone and upper part of the face. The maxillary sinuses are spared. Images and figure legends reproduced with permission from Janssens *et al.* (2006), *Journal of Medical Genetics*, **43**, 1–11.

Janssens *et al.*, 2003). Other mutations in exon 1 impair secretion, leading to intracellular retention of the molecule. All the mutations investigated to date appear to increase the activity of TGFβ.

Because TGFβ1 and its receptors are widely expressed, it is surprising that the disease phenotype in CED is predominantly localized in the skeleton. Janssens and colleagues (2006) proposed that a potential reason for the skeletal predominance of the phenotype is that osteoblasts are one of the few cell types to secrete large amounts of their TGFβ as the small latent TGFβ complex, lacking LTBPs (Bonewald *et al.*, 1991; Dallas *et al.*, 1994). The presence of LTBP in the latent complexes of other cell types may have a protective effect so that stability of the complex is less affected by the CED mutations.

Recently, misregulation of TGFβ has been proposed to underlie many of the phenotypic features of Marfan syndrome, a disorder associated with mutations in the gene for fibrillin-1 reviewed in Judge and Dietz (2005) and Mizuguchi and Matsumoto (2007). The most serious clinical feature of this disease is the cardiovascular abnormalities that can lead to aortic dissection. However, these patients also exhibit skeletal abnormalities including tall stature due to overgrowth of the long bones, arachnodactyly, joint hypermobility, scoliosis, and pectus excavatum. Although fibrillin does not appear to directly bind TGFβ, LTBP1 is structurally related to fibrillins and colocalizes with fibrillin-1 in cultured osteoblasts and in the periosteum (Dallas *et al.*, 2000). Fibrillin-1 binds to the C terminus of LTBP1 and may therefore indirectly regulate the bioavailability of TGFβ (Isogai *et al.*, 2003). Chaudhry and co-workers have recently shown that application of a fibrillin-1 peptide encoded by exons 44–49 causes release of TGFβ1 from the extracellular matrix. This appears to occur via inhibition of the association of the C terminus of LTBP1 with the N terminus of fibrillin-1 (Chaudhry *et al.*, 2007). Neptune and co-workers showed misregulation of TGFβ in the lungs of fibrillin-1-deficient mice and showed dramatic improvement of the lung phenotype by treatment with TGFβ neutralizing antibodies (Neptune *et al.*, 2003). The pharmacological manipulation of TGFβ has therefore now become a major avenue of research for the treatment of Marfan-related disorders. Interestingly, women with Marfan syndrome have increased osteopenia (Kohlmeier *et al.*, 1995). A potential explanation for this may be due to altered TGFβ signaling.

In addition to these genetically inherited disorders, TGFβ has been implicated in joint degenerative diseases, such as osteoarthritis (OA) and rheumatoid arthritis (RA). TGFβ expression is upregulated in synovial tissue from patients with OA and RA (Lafyatis *et al.*, 1989; Taketazu *et al.*, 1994; Szekanecz *et al.*, 1995). Through its anti-inflammatory properties, TGFβ is thought to protect against the development of RA (Lafyatis *et al.*, 1989). In particular, interleukin-1 (IL-1) may be one of the major mediators of cartilage degeneration in RA. TGFβ appears to antagonize its proinflammatory effects in chondrocytes (Takahashi *et al.*, 2005) and in synovial fluid from diseased joints (Lotz *et al.*, 1990). Another mechanism by which TGFβ antagonizes the effects of IL-1 may be by inhibiting expression of its receptor in articular chondrocytes (Redini *et al.*, 1993). Although the anti-inflammatory properties of TGFβ are beneficial, TGFβ also appears to have detrimental effects by promoting fibrosis and/or the formation of osteophytes. For example, Scharstuhl and colleagues showed that inhibition of TGFβ in a mouse model of OA prevented osteophyte formation but impaired cartilage repair (Scharstuhl *et al.*, 2002). In order to develop more effective therapies to treat RA and OA, there is clearly a need for agents that stimulate cartilage repair while at the same time inhibiting breakdown of the cartilaginous matrix. A potential approach for separating out the beneficial effects of TGFβ on cartilage repair from its negative effects on fibrosis was recently reported (Blaney Davidson *et al.*, 2006). In an animal model of IL-1-induced OA, adenoviral overexpression of TGFβ and Smad7 was used to show that overexpression of Smad7 reduced the amount of synovial fibrosis induced by TGFβ but did not reduce the beneficial effects of TGFβ on cartilage repair. This type of approach may therefore provide new opportunities for treatment of these debilitating diseases, and TGFβ is being actively explored as a potential therapeutic agent for joint degenerative diseases (reviewed in Grimaud *et al.*, 2002).

Although increased expression of TGFβ appears to occur in OA as a protective response against inflammation, in some cases downregulation of TGFβ signaling may play a causal role in the development of joint degenerative diseases. Disruption of Smad3 in mice is associated with an OA phenotype (Yang *et al.*, 2001). Further support for a role for TGFβ in the development of OA comes from studies showing that mice lacking the gene for LTBP3 developed OA (Dabovic *et al.*, 2002). These investigators interpreted the phenotype as being due to decreased TGFβ signaling (Dabovic *et al.*, 2005).

In addition to its role in the diseases discussed earlier, TGFβ has been implicated in osteoporosis. Thus, polymorphisms in TGFβ1 genes have been associated with increased risk of osteoporosis and alterations in TGFβ responsiveness or amounts of bone matrix-stored TGFβ have been reported in animal models of osteoporosis (reviewed in Fromigue *et al.*, 2004). However, the literature in this field is somewhat controversial and it is beyond the scope of this chapter to provide an extensive review on this topic. Several good reviews on this subject have been recently published, and the reader is referred to Chapters 73, 74, and 80 of this book for further information on osteoporosis.

TGFβ has been implicated as a tumor suppressor and TGFβ signaling appears to play a major role in many other steps in the progression to malignancy (reviewed in Pardali and Moustakas, 2007). Of particular significance in relation to skeletal biology is the established role of TGFβ as a key mediator of cancer–bone cell interactions in cancers that metastasize to bone, such as breast and prostate cancers (reviewed in Guise *et al.*, 2005)). Again, an extensive discussion of this topic is beyond the scope of the current chapter, and the reader is referred to Chapter 61 of this book for further information on bone metastatic cancers.

SUMMARY

The drive and determination to discover osteoinductive factors were responsible for the initial discovery of the TGFβs and other members of this superfamily in bone. However, with their discovery has come the challenge of unraveling their function in bone and the challenge to control the therapeutic use and delivery of these factors. Nature has developed a surprisingly complex system of regulation from latency and activation of the TGFβ molecule to an intricate system of receptors and a vast array of regulatory molecules that either enhance or inhibit the activity of this factor. Although in the past decade great advances have been made toward our understanding of the function and regulation of this growth factor in the skeleton, there are still many unanswered questions: What are the physiological mechanisms for activation of TGFβ in skeletal tissues?

How is TGFβ regulated by ECM proteins, and what are the specific roles and signaling pathways of the different isoforms? A greater understanding of TGFβ, its effects, and its regulation will be the key to developing its potential for use as a therapeutic in disorders of skeletal metabolism.

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Chapter 54

Bone Morphogenetic Proteins and the Skeleton

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INTRODUCTION

BMPs influence all cell types that reside within the skeleton, but the information they provide to skeletal cells is highly context-specific. Here we discuss the current understanding of how BMPs work to regulate bone formation during skeletal development and postnatal skeletal growth, in maintaining bone mass, and during bone repair.

DISCOVERY OF BONE MORPHOGENETIC PROTEINS

The discovery of bone-inducing molecules, bone morphogenetic proteins (BMPs), came directly from the pioneering work of Dr. Marshall Urist, who was the first to report that protein extracts from demineralized bone matrix were able to induce bone formation at ectopic sites in rodents. Because the process initiated by the implantation of these extracts closely resembled the cellular events seen during endochondral bone formation and fracture healing, he characterized this activity as morphogenetic (Urist, 1965; Fig. 1). Dr. Urist's work led to the identification of the first BMP genes in 1988 and to the realization that BMP activity within bone is the sum of the activities of several BMP genes (Wozney *et al.,* 1988). We now know that at least five related BMPs (BMP2, BMP4, BMP5, BMP6, and BMP7; see Chapter 51, Fig. 1) are deposited into bone matrix and have osteoinductive activity (Celeste *et al.,* 1990; Kingsley, 1994a; Luyten *et al.,* 1989; Ozkaynak *et al.,* 1990, 1992; Sampath and Reddi, 1981; Sampath *et al.,* 1987; Urist *et al.,* 1973; Wang *et al.,* 1988, 1990; Wozney *et al.,* 1988).

BMP THERAPEUTICS AID BONE REPAIR

The success of preclinical studies evaluating the ability of bone-derived BMPs and recombinant human (rh) BMPs to induce *de novo* bone formation led the way for clinical trials in which the safety and efficacy of rhBMP2 or rhBMP7 (Osteogenic protein-1, OP-1) was examined. BMPs have been shown to be safe and effective alternatives to autogenous bone grafts, and rhBMP2 received FDA approval for use in spine fusion in 2002 (Valentin-Opran *et al.,* 2002; Carlisle and Fischgrund, 2005; Villavicencio *et al.,* 2005; Singh *et al.,* 2006; Slosar *et al.,* 2007), to augment tibial fracture repair in 2006 (Govender *et al.,* 2002; Jones *et al.,* 2006; Swiontkowski *et al.,* 2006), and most recently to aid in bone regeneration in the oral cavity (Boyne *et al.,* 2005; Fiorellini *et al.,* 2005). OP-1 (BMP7) has been granted a Humanitarian Device Exemption (intended to benefit patients with conditions that are manifested by fewer than 4000 individuals in the United States per year), allowing use in patients as an alternative to autograft for treatment of recalcitrant long-bone nonunions (Brown *et al.,* 2006; Ronga *et al.,* 2006). In these indications, BMPs are typically applied with a matrix or carrier that aids in defining the shape and volume of the new bone produced, and also helps to retain BMP at the site of application.

Although human clinical studies have proven that BMPs are safe and efficacious bone-inducing agents when added exogenously to sites undergoing repair, we know very little about the normal physiological roles endogenous BMPs play in skeletal tissues. Next we highlight our current understanding of the skeletal actions of endogenous

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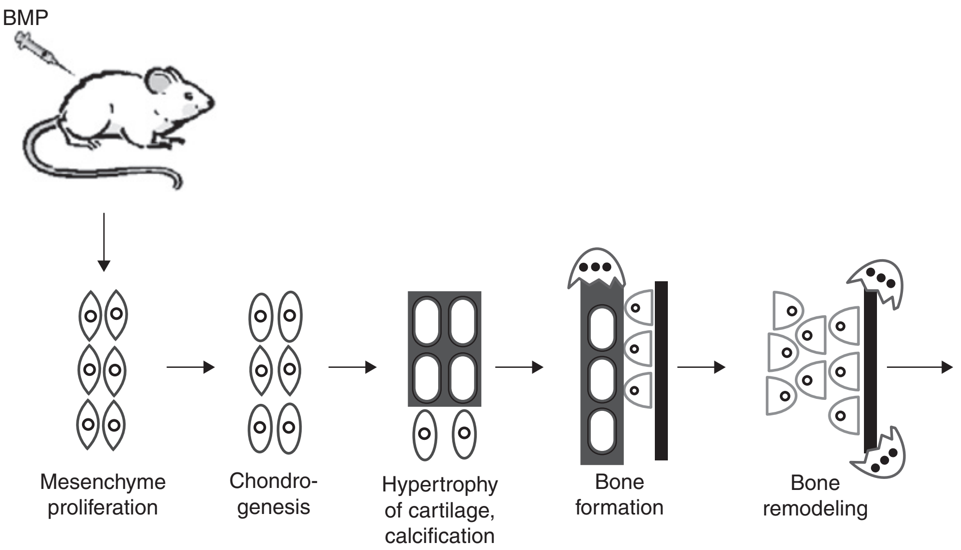

FIGURE 1 Implantation of BMP at ectopic sites in rodents induces *de novo* bone formation. Mesenchymal progenitors condense at the site of BMP implantation and differentiate into chondrocytes; chondrocyte hypertrophy occurs and a mineralized matrix is laid down; new vessels arrive at the site bringing bone marrow elements including osteoclasts that remove the calcified cartilage; and, osteoblast differentiation by mesenchymal cells at the site results in bone formation.

BMPs and discuss how this understanding will define new clinical roles for BMPs.

BMPS ARE PRODUCED BY AND ACT ON SKELETAL CELLS

Individual BMPs, like other members of the transforming growth factor-β (TGF-β) superfamily, are transcribed as large precursor molecules with amino-terminal signal sequences and prodomains of variable size and heterogeneity. These BMP precursors dimerize and are cleaved at RXXR consensus cleavage sites at the time of secretion, releasing a mature BMP dimer that consists of 110–140 amino acids with seven absolutely conserved cysteines. The three-dimensional conformation that results from the location of six of the seven cysteines makes BMPs highly resistant to heat, denaturants, and extremes of pH. Although these unique properties were of fundamental importance in the initial separation of BMPs from bone, we still do not know if native BMPs found in bone exist as homodimers, heterodimers, or combinations of both, because the methods used to purify BMPs destroy their native conformation (Aono *et al.,* 1995; Israel *et al.,* 1996).

In skeletal tissues, BMPs are made *de novo* by osteoblasts, chondrocytes, and their progenitors (Rosen and Wozney, 2002), and are also secreted by invading macrophages and vascular endothelial cells that participate in fracture repair (Csiszar *et al.,* 2005; Sipe *et al.,* 2004; Rosen and Wozney, 2002). When BMPs are secreted from cells, they have one of several fates: They should immediately exert their actions locally through interactions with BMP receptors on the surface of target cells; they may be bound up and prevented from acting by extracellular antagonists present at the site of BMP secretion; or, they may interact with extracellular matrix proteins that serve to sequester or enhance BMP activity by anchoring it to the matrix where it is more available for target cell interactions (Arteaga-Solis *et al.,* 2001; Ohikawara *et al.,* 2002; Lanain *et al.,* 2001). Within the skeleton, target cells for BMPs are located in the perichondrium, periosteum, growth plate, articular cartilage, newly formed bone, and bone marrow stroma (Abe *et al.,* 2000; Brunet *et al.,* 1998; Devlin *et al.,* 2003; Enomoto-Iwamoto *et al.,* 1998; Rountree *et al.,* 2004; Wu *et al.,* 2003; Kobayashi *et al.,* 2005). At each of these sites, changing BMP activity can alter cell proliferation, influence cell fate decisions by pluripotent progenitors, or change extracellular matrix production and its subsequent mineralization.

BMPs exert their effects through transmembrane serine/threonine kinases known as type I and type II receptors (see Chapter 51). These closely related proteins are composed of a short cysteine-rich extracellular domain, a single transmembrane-spanning domain, and an intracellular domain with serine and threonine kinase regions (Franzen *et al.,* 1993; Lin *et al.,* 1992). BMPs can bind to both type I and type II receptors alone with low affinity (Liu *et al.,* 1995). High-affinity binding of BMPs is achieved only when both receptors are present. Although some differences exist with regard to receptor preferences, osteogenic BMPs utilize the same type I (Alk2, Alk3, and Alk6) and type II receptors (BMP RII, ActRII, ActRIIb; Aoki *et al.,* 2001; Miyazono, 2002), and the signal transduced by each osteogenic BMP appears to be identical in skeletal target cells (Korchynskyi *et al.,* 2003; ten Dijke *et al.,* 2003; Peng *et al.,* 2003; Noth *et al.,* 2003). Comparison of results from many labs using a variety of cell and animal models of bone formation points to the conclusion that osteogenic BMPs are interchangeable in their ability to activate the osteoblast phenotype in osteoprogenitor cells, induce ectopic bone formation at nonskeletal sites (Wang *et al.,* 1990; Gitelman *et al.,* 1994; Franceschi *et al.,* 2000), enhance fracture repair (Yasko *et al.,* 1992; Cook *et al.,* 1994; Cho *et al.,* 2002), and activate osteoblast differentiation in primary populations of bone marrow stromal cells (Abe *et al.,* 2000; Locklin *et al.,* 2001), primary and clonal cell lines derived from bone (Yamaguchi *et al.,* 1991; Thies *et al.,* 1992; Hughes *et al.,* 1995; Roman-Roman *et al.,* 2003), and C2C12 muscle cells (Katagiri *et al.,* 1994; Ebisawa *et al.,* 1999; de Jong *et al.,* 2002; Yeh *et al.,* 2002). In addition to activation of the canonical Smads 1, 5, and 8, BMPs have also been shown to activate MAPK and AKT pathways, and based on current published information, each of the osteogenic BMPs appears to have the same capacity to activate these signaling pathways as well (Zuzarte-Luis *et al.,* 2004; Aubin *et al.,* 2004).

BMP ACTIVITY IS PRECISELY REGULATED

Once secreted, the availability of BMPs for receptor interactions is mediated by the presence of extracellular antagonists that bind to BMP proteins and prevent their subsequent interaction with BMP receptors (Dionne *et al.,* 2001). Developmental analysis of the function of BMP antagonists suggests that they guard against excessive BMP activity during formation of the skeleton. For example, inactivation of the gene for the potent BMP antagonist noggin results in hyperplasia of cartilage condensations and failure of joint formation due to increased BMP activity (Brunet *et al.,* 1998). This phenotype is reminiscent of that seen in humans with missense mutations in *Noggin*. Both proximal symphalangism and multiple synostosis syndromes, autosomal-dominant disorders, are characterized by joint fusion caused by the union of two individual bones (Gong *et al.,* 1999). Gain-of-function studies in the chick limb have shown that too much noggin, the opposite situation, blocks BMP activity and inhibits normal skeletal chondrogenesis (Capdevila and Johnson, 1998; Pathi *et al.,* 1999). Mice engineered to over-express noggin in the skeleton display osteopenia and severe bone fragility, highlighting the importance of precisely regulating BMP activity to normal skeletal function (Tsumaki *et al.,* 2002). Another potential regulator of BMP activity in the skeleton is chordin. Cysteine-rich (CR) domains in the chordin molecule provide high-affinity binding sites for BMPs, sequestering them and making them unavailable to their receptors (Holley *et al.,* 1993). Chordin expression patterns in the skeleton place it at sites of BMP activity (Piccolo *et al.,* 1996), and when chordin is overexpressed during chick limb development, there is a substantial delay in chondrocyte maturation and a negative effect on endochondral ossification (Zhang *et al.,* 2002). The control of skeletal BMP activity by chordin is likely to be affected by another BMP binding protein, Tsg, that interacts with both BMP-7 and chordin and enhances the ability of chordin to antagonize BMPs (Zakin *et al.,* 2005). Connective tissue growth factor (CTGF), a secreted protein found in ECM, can also antagonize BMP activity. CTGF contains CR domains similar to those found in chordin, and these domains provide binding sites for BMP-4 and TGF-β. Whereas CTGF binding to BMP-4 serves to antagonize BMP activity, CTGF binding to TGF-β enhances TGF-β receptor binding. Transgenic mice that overexpress CTGF under regulation of a Col-XI promoter show decreased bone density within a few months after birth, establishing CTGF as a negative regulator of bone formation (Abreu *et al.,* 2002). Whether this is due to the ability of CTGF to bind BMPs and inhibit their activities remains to be determined. Follistatin may also regulate the availability of BMP activity in bone by binding to BMPs or through modulation of activin signaling (Merino *et al.,* 1999). In addition to a role in antagonizing BMP activity, it is conceivable that at least some of these BMP-binding proteins regulate the diffusion of BMPs through the extracellular matrix. A similar activity has been identified for short gastrulation (sog), the *Drosophila* homologue of chordin (Ashe and Levine, 1999). Furthermore, these proteins may play direct roles in modulating BMP interactions with the extracellular matrix. For example, sog has been shown to physically interact with integrins (Araujo *et al.,* 2003).

Once the BMP signaling cascade is activated in skeletal cells, there appear to be many feedback loops whose purpose is to modulate the BMP effect. To date, BMP signaling has been shown to intersect with the mitogen-activated protein kinase (MAPK) pathway in osteoblasts, the Indian Hedgehog (Ihh)–parathyroid hormone related peptide (PTHrP) pathway in chondrocytes and osteoblasts, the Wnt pathway during limb development and bone formation, fibroblast growth factor (FGF) pathway during limb patterning, and with the TGF-β and activin pathways in bone cells (Centrella *et al.,* 1991; Chung *et al.,* 2001; Lanske *et al.,* 1996; Alvarez *et al.,* 2002; Chen *et al.,* 2007; Gori *et al.,* 2001; Rawadi *et al.,* 2003; Yoon *et al.,* 2006).

AMOUNT OF BMP ACTIVITY OR ACTION OF INDIVIDUAL BMPs?

Our ability to remove specific BMPs and observe skeletal development in mice has shown that in most instances, loss of any individual BMP can be compensated for by the other BMPs present. For example, lack of BMP5 (Green, 1958; Kingsley *et al.,* 1992; DiLeone *et al.,* 1998), BMP6 (Solloway *et al.,* 1998), or BMP7 (Dudley *et al.,* 1995; Luo *et al.,* 1995; Dudley and Robertson, 1997) has little consequence on the embryonic skeleton, and elimination of either BMP2 or BMP4 from the limb prior to the start of endochondral ossification does not affect skeletal development (Tsuji *et al.,* 2006; Bandyopadhyay *et al.,* 2006). However, mice in which combinations of osteogenic BMPs have been removed display a variety of phenotypes, ranging from normal bone formation (Kim *et al.,* 2001; Zhao, 2003) to absence of osteoblast differentiation (Bandyopadhyay *et al.,* 2006), raising the possibility that based on expression levels, some BMPs may be more important than others. BMP3 is one of the most abundant BMPs in demineralized bone matrix (Wozney and Rosen, 1993). Most surprisingly, however, examination of the phenotype of BMP-3 null mice revealed that the loss of BMP-3 during embryogenesis results in increased bone density, with a doubling of trabecular volume at 5 to 6 weeks of age (Daluiski *et al.,* 2001). Although the cellular basis for this phenotype is currently under investigation, preliminary data suggest that BMP-3 can antagonize osteogenic BMPs by binding to a subset of type II BMP receptors (Gamer *et al.,* 2005).

BMPs AND THE ADULT SKELETON

BMP activity is required to maintain normal bone function in the adult skeleton because generalized loss of BMPs through overexpression of BMP antagonists by osteoblasts leads to osteopenia, bone fragility, and spontaneous fracture (Gazzerro *et al.,* 1998, 2005; Wu *et al.,* 2003). How much BMP activity is required, how BMPs affect bone remodeling, and which specific BMPs are the endogenous mediators of bone formation in the postnatal skeleton remain open questions. BMP activity found in bone declines with age (Syftestad and Urist, 1982; Fleet *et al.,* 1996; Moerman *et al.,* 2004), and this decrease correlates with the observation that osteoprogenitors are less effective at replicating, differentiating into osteoblasts, and laying down bone matrix in older animals. It appears that BMPs 4, 5, and 6 are not required for bone remodeling, because mice lacking any one of these BMPs maintain normal bone mass (Kingsley *et al.,* 1992; DiLeone *et al.,* 1998; Solloway *et al.,* 1998; Tsuji and Rosen, unpublished observations). In contrast, mice lacking BMP2 are unable to maintain their bone mass as they age and undergo spontaneous fracture with 100% penetrance (Tsuji *et al.,* 2006). When BMP3, a negative regulator of bone formation is removed, mice have increased bone mass as they age (Daluiski *et al.,* 2001), another indication that BMP activity and postnatal bone mass are intimately linked.

Bone remodeling, the removal of existing bone and its replacement by new bone, is carried out in a highly controlled manner and requires the differentiation of both osteoblasts and osteoclasts from precursors located in the bone marrow environment (Manolagas and Jilka, 1995). The exact nature of the signals that control remodeling remains to be established, but it is likely that both osteoblast and osteoclast precursors are affected by systemic and local signals and by mechanical stimulation (Rodan, 1998). BMPs have been proposed to be among the local signals that induce commitment of mesenchymal stem cells resident in bone marrow into osteoprogenitors and osteoblasts (Abe *et al.,* 2000; Manolagas and Weinstein, 1999). Several lines of evidence make this hypothesis an attractive one. Many data exist to show that BMPs are present in bone matrix in a form that allows for their release or presentation to marrow stromal cells, cells that can differentiate into osteoblasts in response to BMPs (Rosen *et al.,* 1996). Osteoblasts have also been shown to synthesize and secrete BMPs both *in vitro* and *in vivo,* suggesting that once BMPs initiate MSC differentiation, a positive feedback loop is created, allowing for the production of additional BMP signals (Suzawa *et al.,* 1999). In addition, BMPs are thought to regulate the transcription of several osteoblast-specific transcription factors, which in turn may regulate transcription of RANK ligand, a signal important for the differentiation of hematopoietic progenitors into osteoclasts (Manolagas and Weinstein, 1999). These tantalizing links between BMPs and bone remodeling have become the focus of much research and may lead to new therapeutic approaches to osteopenia. In designing treatments that utilize site-specific activation of endogenous BMPs, it is fundamentally important to identify which BMPs are available to affect bone remodeling and which cell types at each step of the remodeling process are the targets of these signals. If BMPs are effectors of remodeling, it will be necessary to determine whether factors that are known to change the balance in remodeling toward formation or resorption do so through effects on these BMPs. *In vitro* studies have shown that estrogens and glucocorticoids, two agents known to affect remodeling, increase BMP-6 synthesis by osteoblasts (Boden *et al.,* 1997; Ricard *et al.,* 1998). However, mice lacking BMP6 have no apparent skeletal defects and do not develop osteopenia, suggesting that identifying the roles that BMPs play in remodeling will require an understanding of the influence of individual BMPs in this process (Solloway *et al.,* 1998).

It will also be of great interest to determine whether the decrease in MSC present in bone marrow that occurs with aging is related to the changes in levels of specific BMPs found in bone matrix in older animals (D'Ippolito *et al.,* 1999). If BMPs are required for MSC survival, as well as MSC differentiation, changing the BMP content of bone matrix and bone marrow may have important clinical benefits. A hint that this may be the case has been provided by the production of transgenic mice in which noggin, a potent BMP antagonist, is overexpressed using the bone-specific osteocalcin promoter. These mice have osteopenia and are prone to fracture, suggesting that a reduction in osteogenic BMPs available to the postnatal skeleton reduces overall bone formation and bone healing (Devlin *et al.,* 2003). The discovery that the genetic defect in fibro-ossificans dysplasia progressiva (FOP) is an activating mutation of Alk2, a type I BMP receptor (Shore *et al.,* 2006), and the report that conditional inactivation of another type I BMP receptor, Alk3, regulates the size of the hematopoietic stem cell niche in mice (Zhang *et al*., 2003) points to an important role for BMP signaling in mesenchymal stem cell differentiation.

New bone formation also occurs in the adult skeleton during the process of fracture repair, where osteoblast precursors resident in marrow and periosteum differentiate into osteoblasts in a highly regulated manner. BMPs have been shown to be present at fracture repair sites, and several lines of evidence support the role of BMPs in this process. First, all of the cell types that synthesize new bone during fracture healing have been shown to be targets for BMPs *in vitro* and to possess BMP receptors *in vivo* (Bostrom *et al.,* 1995). Recent studies using mice in which BMP2 is inactivated only in the limb skeleton have identified BMP2 as required for periosteal activation, a critical initiation step for fracture repair (Tsuji *et al.,* 2006). The availability of animal models in which specific BMPs, BMP receptors, and BMP antagonists have been removed should allow us to address this issue and to begin to understand what regulates endogenous BMP production during fracture healing.

Another circumstance in which the adult skeleton produces new bone is during distraction osteogenesis, a method of bone lengthening that takes advantage of the inherent capacity of bone to repair after breaking (Paley, 1988). We are just beginning to understand the molecular and cellular events that form the basis of distraction osteogenesis, and from these initial studies, it seems likely that BMPs are important mediators of bone formation at the distraction site. Data from a rat model of distraction osteogenesis link the mechanical stress/tension needed for successful distraction osteogenesis with BMP gene expression (Sato *et al.,* 1999). The precise link between mechanical stress and BMP gene expression remains to be discovered, as do the other signaling pathways that must interact with BMPs to produce the cascade of events that results in temporally delayed bone formation. Understanding what regulates the temporal differences in bone formation seen between distraction osteogenesis and normal fracture healing, which are thought to be BMP-mediated events, should provide insight into how BMPs affect the rate of repair.

Finally, new bone formation in adults is also found in several instances of chronic joint disease, of which osteoarthritis (OA) is by far the most common. BMPs have been localized in both normal and arthritic joints, and polymorphisms in the BMP2 gene and in asporin, an ECM protein that is able to regulate the activity of both BMP-2 and TGF-β, have been associated with arthritis (Kizawa *et al.,* 2005; Lories and Luyten, 2005; Yamada *et al.,* 2007). Mice lacking expression of the Bmpr1a gene specifically in developing joints show articular cartilage erosion after birth that resembles human OA (Rountree *et al.,* 2004). Greater understanding of the requirements for BMP signaling in the maintenance of synovial joints may allow for the design of novel OA therapies.

CONCLUSIONS

In the 20 years since the identification of the BMP genes, it has become clear that BMPs are involved in almost every aspect of bone biology. Our increasing understanding of how BMPs exert their effects on skeletal cells coupled to the realization that BMP activity is tightly regulated to restrict its action provide entrance points for investigating the physiological significance of BMP activity in the skeleton. Several important questions remain to be addressed, including why BMP therapeutics must be used in microgram amounts in the clinic whereas endogenous BMPs are active at far lower levels, and how BMP signals are integrated with other kinds of signals, in particular, Wnts and FGFs to maintain skeletal homeostasis.

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Chapter 55

Bone Morphogenetic Protein Receptors and Actions

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INTRODUCTION: BMPs

Bone morphogenetic proteins (BMPs) were originally identified as proteins that induce the formation of bone and cartilage tissues when implanted at ectopic sites in rats (Wozney *et al.,* 1988; Reddi, 1998). *In vitro,* BMPs have potent effects on the regulation of growth and differentiation of chondroblast and osteoblast lineage cells. Moreover, BMPs have been shown to be multifunctional cytokines with a wide range of biological activities in various cell types, including epithelial cells, mesenchymal cells, endothelial cells, monocytes, and neuronal cells (see Chapter 50).

BMPs belong to a larger family, known as the transforming growth factor-β (TGF-β) family, which includes TGF-βs, activins and inhibins, nodal, myostatin, and Müllerian inhibiting substance (MIS, also known as anti-Müllerian hormone). More than a dozen molecules have been identified in the BMP subfamily; these can be classified into several subgroups based on similarities in structure and function. BMP-2 and BMP-4 are highly similar and form one subgroup (BMP-2/4 group). BMP-5, BMP-6, BMP-7 (also termed osteogenic protein-1; OP-1), and BMP-8 (OP-2) form another subgroup (OP-1 group). Growth and differentiation factor-5 (GDF-5, also termed cartilage-derived morphogenetic protein-1, CDMP-1), GDF-6 (CDMP-2, also termed BMP-13), and GDF-7 (BMP-12) belong to a third subgroup (GDF-5 group), and BMP-9/GDF-2 and BMP-10 form a fourth subgroup (BMP-9 group; Fig. 1; Kawabata and Miyazono, 2000). Most of the members of the BMP-2/4, OP-1, and BMP-9 groups have been shown to induce formation of bone and cartilage tissues *in vivo,* whereas those of the GDF-5 group induce cartilage and tendon-like tissues. In contrast, certain other BMPs and GDFs, including BMP-3 and myostatin (also termed GDF-8), do not induce formation of bone and cartilage tissues *in vivo*. Evaluation of osteogenic activities of 14 different BMPs/GDFs by adenoviral gene transfer *in vitro* revealed that BMP-2, BMP-6, and BMP-9 were most potent in the induction of alkaline phosphatase activity and osteocalcin expression in C3H10T1/2 cells, and that they play critical roles in inducing differentiation of mesenchymal progenitor cells into osteoblasts (Cheng *et al.,* 2003).

In this chapter, mechanisms for the signal transduction by BMPs on mesenchymal progenitor cells, are described and compared to the TGF-β signaling system. Although the signaling mechanisms of serine-threonine kinase receptors and Smads have been intensively studied in invertebrates, i.e., in *Drosophila* and *Caenorhabditis elegans* (Zimmerman and Padgett, 2000; Raftery *et al.,* 2006), BMP signaling pathways in mammals will be the principal focus of this chapter.

BMP RECEPTORS

Serine-Threonine Kinase Receptors

Members of the TGF-β family bind to two distinct types of serine-threonine kinase receptors, termed type I and type II receptors (Heldin *et al.,* 1997; Shi and Massagué, 2003). Both type II and type I receptors are essential for signal transduction. TGF-β and activin ligands bind to type II receptors independently of type I receptors, whereas type I receptors can bind these ligands only in the presence of type II receptors. In contrast, BMPs bind to type I receptors in the absence of type II receptors, whereas they bind to type II receptors only weakly in the absence of

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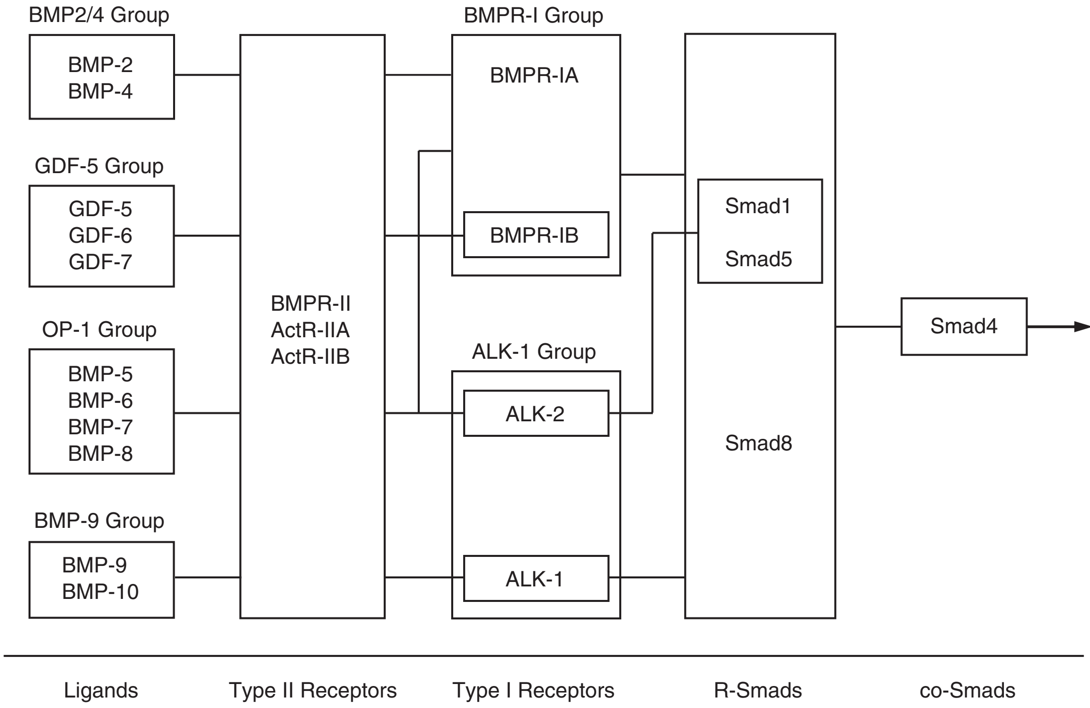

FIGURE 1 Relationships between BMPs, type II and type I receptors, and Smad proteins in signal transduction. Among the receptors of the ALK-1 group, ALK-2 serves as a receptor for the BMPs in the OP-1 group, whereas ALK-1 serves as a receptor for those in the BMP-9 group. Similarly, BMPR-IB, but not BMPR-IA, preferentially functions as a type I receptor for GDF-5. Of the BMP-R-Smads, Smad1, and Smad5 are activated by ALK-2, whereas all three BMP-R-Smads are activated by BMPR-IA and BMPR-IB.

type I receptors, and their binding affinity is dramatically increased when both types of receptors are present (see later discussion; Liu *et al.,* 1995; Rosenzweig *et al.,* 1995). The binding affinities of BMP-2 to BMP type I receptors are 1 to 11 n*M*, while those to type II receptors are 50 to 100 n*M* (see later discussion). The type II receptor kinase transphosphorylates the type I receptor, which transmits specific intracellular signals.

The structures of the type I and type II receptors are schematically shown in Figure 2A. They have a relatively short extracellular domain, a single membrane-spanning domain, and an intracellular domain containing a serine-threonine kinase domain. The extracellular domains have several conserved cysteines, which are important for the formation of characteristic three-dimensional structures (Greenwald *et al.,* 1999; Kirsch *et al.,* 2000a). In addition to the serine-threonine kinase domain, the intracellular domains of type I receptors, but not those of type II receptors, have a characteristic GS domain (glycine- and serine-rich domain) located N-terminal to the serine-threonine kinase domains. In the kinase domain, type I receptors have a characteristic structure, denoted L45 loop, between kinase subdomains IV and V. The C-terminal tails of type I receptors are very short (less than 9 amino acids), whereas those of type II receptors are more than 24 amino acids long. The C-terminal tail of BMPR-II is much longer (530 amino acids) than other type II receptors. Although the functional importance of the C-terminal tail of BMPR-II is not fully known, several molecules have been shown to interact with the C-terminal tail of BMPR-II, and mutations in this domain have been found in some patients with pulmonary arterial hypertension (see later discussion; IPPH Consortium, 2000; Deng *et al.,* 2000; Morrell, 2006).

BMP Type I Receptors

Seven type I receptors, termed activin receptor-like kinases (ALKs) 1 through 7, have been identified in mammals. ALKs can be classified into three subgroups, i.e., the BMPR-I group, the ALK-1 group (see Fig.1), and the TβR-I group (Kawabata and Miyazono, 2000). ALK-3 and ALK-6 are very similar to each other (BMPR-I group) and are also denoted BMP type IA and type IB receptors (BMPR-IA and BMPR-IB), respectively. The kinase domains of BMPR-IA and BMPR-IB have 85% amino acid sequence identity. Functional differences between the intracellular domains of BMPR-IA and BMPR-IB have not been fully determined. ALK-5, ALK-4, and ALK-7 are structurally similar to each other (TβR-I group), and ALK-5 and ALK-4 serve as TGF-β and activin/nodal type I receptors (TβR-I and ActR-IB), respectively. ALK-7 and ALK-4 have been reported to bind nodal (Reissmann *et al.,* 2001). ALK-1 and ALK-2 are structurally similar to each other

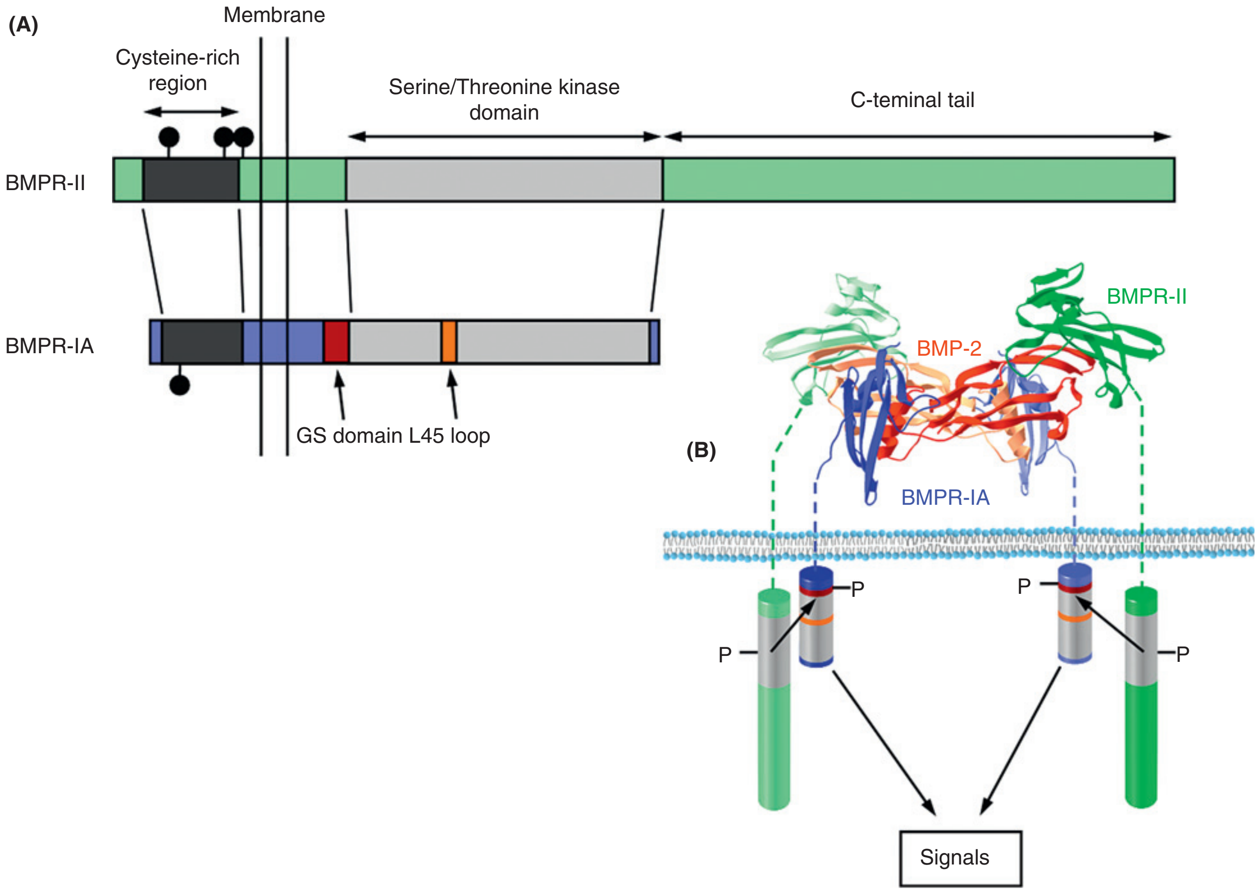

FIGURE 2 BMP type II and type I receptors. (**A**) Structure of BMPR-II and BMPR-IA. ActR-IIA and ActR-IIB have similar structures, but shorter C-terminal tails than BMPR-II. BMPR-IB and ALK-1/2 have overall structures similar to that of BMPR-IA. Closed circles indicate N-glycosylation sites. (**B**) BMP-2 has an elongated structure and binds to BMPR-IA and BMPR-II through wrist and knuckle epitopes, respectively. The ribbon diagram of hypothetical BMP-2-BMPRIA-BMPR-II ternary complex in the cell membrane is shown. The structure of BMPR-II was superposed onto that of ActR-II in the ternary complex containing BMP-2, BMPR-IA, and ActR-II (Protein Data Bank entry 2GOO). Subunits of the BMP-2 dimer are shown in red. The extracellular domains of BMPR-IA and BMPR-II are shown in blue and green, respectively. In the intracellular parts, BMPR-II transphosphorylates the GS domain of BMPR-IA, and BMPR-IA kinase transduces intracellular signals. (See plate section)

(ALK-1 group), and they are distantly related to the other type I receptors. Despite the low degree of similarity between the receptors of the ALK-1 group and those of the BMPR-I group, they transduce similar intracellular signals.

BMPR-IA is expressed in various types of cells, including human foreskin fibroblasts, MC3T3-E1 osteoblasts, C2C12 myoblasts, and ROB-C26 osteoprogenitor cells (ten Dijke *et al.,* 1994; Nishitoh *et al.,* 1996; Ebisawa *et al.,* 1999). ALK-2 is also widely expressed in various cells, including human foreskin fibroblasts, MC3T3-E1 cells, and C2C12 cells. In contrast, expression of BMPR-IB is restricted to certain cell types, including ROB-C26 cells and glioblastoma cell lines, and that of ALK-1 is limited to endothelial cells and certain other cells. In the postimplantation mouse embryos, BMPR-IA and ALK-2 are nearly ubiquitously expressed, whereas BMPR-IB shows a more restricted expression profile and is observed in mesenchymal precartilage condensations, premuscle masses, central nervous system, and some other tissues (Dewulf *et al.,* 1995; Verschueren *et al.,* 1995). ALK-1 is expressed in several different tissues and organs, but its expression is highest in blood vessels, mesenchyme of the lung, and submucosal layer of gastrointestinal tracts (Roelen *et al.,* 1997).

Members of the BMP subfamily bind with different affinities to BMPR-IA, BMPR-IB, ALK-1, and ALK-2, expressed in mammalian cells. Under physiological conditions, BMP-2 and BMP-4 bind to BMPR-IA and BMPR-IB (ten Dijke *et al.,* 1994). In contrast, BMP-6 and BMP-7 bind strongly to ALK-2 and weakly to BMPR-IB (Ebisawa *et al.,* 1999). GDF-5 preferentially binds to BMPR-IB, but not to other receptors (Nishitoh *et al.,* 1996). BMP-9 and BMP-10 bind to ALK-1 in the presence of BMPR-II (Brown *et al.*, 2005; David *et al.,* 2007; Scharpfenecker *et al.*, 2007). Moreover, TGF-β binds to TβR-I as well as ALK-1 in endothelial cells (Oh *et al.,* 2000; Goumans *et al.*, 2003). In addition to members of the BMP family, MIS binds to ALK-2, BMPR-IA and BMPR-IB in the presence of its specific type II receptor, MISR-II (Gouédard *et al.*, 2000; Zhan *et al.*, 2006). Thus, BMP type I receptors are shared by certain other members of the TGF-β family.

Truncated forms of BMPR-IA and BMPR-IB, lacking the intracellular domains, or kinase inactive forms with mutations in the ATP-binding sites, have been widely used to study the functions of BMPs. BMPs induce differentiation of C2C12 cells into osteoblast-like cells, but in the presence of the truncated form of BMPR-IA, cells fail to become osteoblasts, differentiating instead into mature myocytes (Namiki *et al.*, 1997).

The two unique structures in the intracellular domain, i.e., the GS domain and L45 loop, are observed only in type I receptors, but not in type II receptors. Mutations in the GS domains (see later discussion) lead to constitutive activation of type I receptors. The constitutively active BMP type I receptors, including those of the BMPR-I group and the ALK-2 group, stimulate osteoblast differentiation *in vitro* (Fujii *et al.*, 1999). The L45 loop located between kinase subdomains IV and V of type I receptors is most important for the determination of intracellular signaling.

BMP Type II Receptors

Three receptors, i.e., BMPR-II, ActR-II, and ActR-IIB, serve as type II receptors for BMPs. BMPR-II is specific for BMPs, whereas ActR-II and ActR-IIB are shared by activins and BMPs. In contrast to type I receptors, these type II receptors appear to bind most BMPs.

Two alternatively spliced variants of BMPR-II have been identified (Rosenzweig *et al.*, 1995; Liu *et al.*, 1995). The long form of BMPR-II is composed of 1038 amino acids, and includes the long C-terminal tail rich in serines and threonines. The short form lacks the long C-terminal tail, but its expression is rare in most tissues. Although the long and short versions have been reported to be functionally indistinguishable in *Xenopus* assays (Ishikawa *et al.*, 1995), nonsense mutations in the C-terminal tail have been found in some patients with familial pulmonary arterial hypertension, indicating that this region plays an important role in signaling activity (IPPH Consortium, 2000; Deng *et al.*, 2000).

BMPR-II binds ligands only weakly by itself, but its ligand-binding affinity is increased in the presence of type I receptors. ActR-II and ActR-IIB bind activins and BMPs, but the binding affinities of ActR-II and -IIB to BMPs are lower than those to activins (Yamashita *et al.*, 1995). ActR-II and -IIB bind activins with high affinity in the absence of type I receptors. Similar to BMPR-II, however, ActR-II and -IIB bind BMPs with high affinity only in the presence of type I receptors (Rosenzweig *et al.*, 1995; Liu *et al.*, 1995). Moreover, type II receptors affect the binding preferences of BMPs for type I receptors (Yu *et al.*, 2005).

BMPR-II, ActR-II, and ActR-IIB are widely expressed in various tissues. BMPR-II is expressed in skeletal muscle, heart, brain, and lung, and plays an important role in mesoderm formation during early embryogenesis (Beppu *et al.*, 2000).

Co-receptors for BMPs

Although type II and type I receptors are sufficient for transduction of intracellular signaling by TGF-β family proteins, signaling activity of certain members is regulated by co-receptors, including Cripto for nodal signaling, and βglycan and endoglin for TGF-β signaling. Glycosylphosphatidylinositol (GPI)-anchored proteins of the repulsive guidance molecule (RGM) family, including RGMa, DRAGON/RGMb, and hemojuvelin/RGMc, serve as co-receptors for BMP-2 and BMP-4, and enhance BMP signaling (Samad *et al.*, 2005; Babitt *et al.*, 2005, 2006). They interact with BMP type I receptors and bind selectively to BMP-2 and BMP-4, but not to BMP-7 or TGF-β1 .

BMPs play a key role in metabolism in hepatocytes, in which hemojuvelin and hepcidin function as downstream components in the BMP signaling pathway and regulate iron homeostasis. Acting as a BMP co-receptor, hemojuvelin induces the expression of hepcidin in hepatocytes upon BMP stimulation, and hepcidin in turn decreases iron absorption by the intestine and iron release from macrophages (Babitt *et al.*, 2006). Mutations in the *hemojuvelin* gene as well as in the *hepcidin* gene cause juvenile hemochromatosis, which is characterized by accumulation of iron in various organs.

Molecular Mechanism of BMP Receptor Activation

BMP Binding to Receptor Extracellular Domains

The extracellular domains of BMP type I receptors interact with ligands even in the absence of type II receptors. Because a soluble form of BMPR-IA lacking the transmembrane and intracellular domains binds BMPs in the absence of type II receptors, it may be useful as an antagonist of BMPs (Natsume *et al.*, 1997). The binding affinity of BMP-2 for soluble BMPR-IA is about 1 n*M*, and that for BMPR-IB is about 11 n*M* (Kirsch *et al.*, 2000b). In contrast, binding affinities for the extracellular domains of BMPR-II and ActR-II are only 100 and 50 n*M*, respectively.

Several studies solved the structures of binary complexes containing ligands and receptor extracellular domains, i.e. BMP-2-BMPR-IA (Kirsch *et al.*, 2000a), BMP-7-ActR-II (Greenwald *et al.*, 2003), and activin-ActR-IIB (Thompson et al., 2003). A ternary structure has been reported for BMP-2 in the complex with BMPR-IA and ActR-II (Allendorph et al., 2006). The structure of the BMPR-II extracellular domain has also been determined (Mace et al., 2006).

Two receptor-binding motifs have been identified in BMP-2 (Kirsch et al., 2000b; see Fig. 2B). A large epitope 1 (termed *wrist epitope*) is a high-affinity binding site for BMPR-IA, and a smaller epitope 2 (termed *knuckle epitope*) is a low-affinity binding site for BMPR-II. The BMP-2/BMPR-IA interface of the complex structure consists of one BMPR-IA molecule containing both BMP-2 monomers, and the wrist epitopes from both monomers contribute to binding to BMPR-IA, whereas the knuckle epitope from only one monomer is involved in the binding to BMPR-II. The wrist and knuckle epitopes are very closely located to each other. Certain BMP-2 mutants in the knuckle epitope (e.g., Ala-34 and Leu-90), but not in the wrist epitope, function as antagonists of BMP-2, because these mutants bind to BMPR-IA with the wrist epitope, but fail to interact with BMPR-II through the mutated knuckle epitope.

The extracellular domain of ActR-II has a structure termed the *three-finger toxin fold*, composed of three pairs of β-strands projecting from the palm domain with a conserved scaffold of disulfide bridges (Greenwald et al., 1999). The extracellular domains of ActR-II and BMPR-IA have very similar three-dimensional configurations but with some differences (Kirsch et al., 2000a). The BMPR-IA extracellular domain consists of two β-sheets and one α-helix (helix α1); the latter is missing in ActR-II and may serve as a key element in type I receptors for specific ligand-binding.

Activation of Serine-Threonine Kinases

Small fractions of type II and type I receptors are present as preexisting homodimers as well as heterodimers on the cell surface in the absence of ligand stimulation (Gilboa et al., 2000). All BMP receptors form oligomers, including type II/type I heteromers (BMPR-II/BMPR-IA and BMPR-II/BMPR-IA) and type II/type II and type I/type I oligomers (BMPR-II/BMPR-II, BMPR-IA/BMPR-IA, BMPR-IB/BMPR-IB, and BMPR-IA/BMPR-IB). Ligand-binding increases hetero- and homo-oligomerization of the receptors, except for BMPR-II homodimers. Receptor oligomerization may also induce conformational alterations of these receptor molecules.

The type II receptor kinase is constitutively active in the absence of ligand. Upon ligand binding, the type II receptor kinase transphosphorylates the GS domain of type I receptor, resulting in the activation of type I receptor kinases (see Fig. 2B). The intracellular substrates are then activated by the type I kinase (Wrana et al., 1994).

Phosphorylation of the GS domain is a critical event in signal transduction by the serine-threonine kinase receptors. Crystallographic analysis of the intracellular domain of TβR-I revealed that the inactive conformation of the TβR-I kinase is maintained by physical interaction between the GS domain, the N-terminal lobe, and the activation loop of the kinase (Huse et al., 1999). Upon phosphorylation of the GS domain by TβR-II, the TβR-I kinase is converted to an active conformation. Mutations of Thr-204 in TβR-I and the corresponding Gln in BMP type I receptors to acidic amino acids (Asp or Glu) lead to constitutive activation of the type I receptors (Wieser et al., 1995). The constitutively active type I receptors transmit intracellular signals in the absence of ligands or type II receptors, indicating that type I receptors are a downstream component of the type II receptor signaling pathways and that they determine the specificity of the intracellular signals. However, truncation of the C-terminal tail of BMPR-II was found in some familial pulmonary arterial hypertension patients, suggesting that certain non-Smad signals may be transduced through BMPR-II (IPPH Consortium, 2000; Deng et al., 2000).

In a yeast two-hybrid screening using TβR-I as a bait, FKBP12, a binding protein for immunosuppressants such as FK506 and rapamycin, was obtained (Wang et al., 1994). FKBP12 binds to the Leu-Pro sequence in the GS domain of type I receptors, and negatively regulates the activity of type I receptors (Wang et al., 1996; Chen et al., 1997). FKBP12 thus prevents spontaneous activation of type I receptors by type II receptors.

The L45 loop of the N lobe in the type I receptor protrudes from the kinase domain (Huse et al., 1999) and specifically interacts with intracellular substrates, e.g., Smads (Feng and Derynck, 1997). Amino acid sequences of the L45 loop are conserved in each type I receptor subgroup, but diverge between different subgroups. The L45 loop of the BMPR-I group is more similar to that of the TβR-I group than that of the ALK-1 group; however, receptors of the BMPR-I and ALK-1 groups activate a similar set of Smads, whereas those of the TβR-I group activate a distinct set of Smads.

Pseudoreceptor: BAMBI

Xenopus BAMBI is a pseudoreceptor for serine-threonine kinase receptors with a high degree of sequence similarity to the human *nma* gene product (Onichtchouk et al., 1999). BAMBI/Nma is structurally related to type I serine-threonine kinase receptors, but the former does not have the intracellular domain. BAMBI stably interacts with various type I and type II receptors and inhibits signaling by these receptors. Since the expression of BAMBI and *nma* is induced by BMP and TGF-β (Onichtchouk et al., 1999; Akiyoshi et al., 2001), BAMBI/Nma may regulate BMP and TGF-β signaling via a negative feedback loop. Expression of BAMBI is also induced by Wnt-β-catenin signaling (Sekiya et al., 2004).

Mutations of BMP Receptors *in Vivo*

Phenotypes of BMP Receptor-Deficient Mice

BMPs play essential roles during embryogenesis, and mice lacking BMP-2 or BMP-4 exhibit defects in mesoderm formation during early embryonic stages. Similarly, gene targeting of *Bmpr1a* or *Bmpr2* leads to defects in mesoderm formation. Homozygous *Bmpr1a* mutant mice die by E9.5 due to defects in mesoderm formation during gastrulation (Mishina *et al.*, 1995). *Bmpr2* null mice also die by E9.5, and their phenotype is very similar to that of *Bmpr1a*$^{-/-}$ mice (Beppu *et al.*, 2000). BMP signaling mediated by ALK-3/BMPR-IA is essential for hair follicle renewal in adult skin (Andl *et al.*, 2004; Yuhki *et al.*, 2004).

BMPR-IB-deficient mice are viable, but exhibit short limbs and abnormal digit cartilage, similar to *Gdf5* null mice (Baur *et al.*, 2000; Yi *et al.*, 2000). Analysis of *Gdf5*; *Bmpr1b* double mutant mice revealed that GDF-5 is a physiological ligand for BMPR-IB *in vivo,* although other receptors may also be involved in the GDF-5 signaling pathway. In addition, analysis of *Bmp7*; *Bmpr1b* double knockout mice revealed that BMP-7 and BMPR-IB may act in part in overlapping pathways. BMPR-IB is also essential for female fertility; *Bmpr1b*-null mice exhibit defects in cumulus cell expansion (Yi *et al.*, 2001).

Some, but not all, ActR-II-deficient mice exhibit hypoplasia of the mandible and other skeletal abnormalities (Matzuk *et al.*, 1995). Defects in the reproductive system and decrease in the secretion of follicle stimulating hormone (FSH) are also observed in these mice. ActR-IIB-deficient mice exhibit complicated cardiac defects, and abnormalities in lateral asymmetry and vertebral patterning (Oh and Li, 1997). ActR-II and ActR-IIB also play important roles in the formation of the endocrine pancreas (Kim *et al.*, 2000).

Human Diseases Induced by Mutations of BMP Receptors

Primary pulmonary arterial hypertension is characterized by stenosis of precapillary pulmonary arteries. Proliferation of endothelial cells and smooth muscle cells is observed in the pulmonary arteries of pulmonary arterial hypertension and leads to high pulmonary artery pressure. Familial pulmonary arterial hypertension occurs as an autosomal dominant disorder with reduced penetrance. Mutations in the human *BMPR2* gene are found in some familial pulmonary arterial hypertension patients (IPPH Consortium, 2000; Deng *et al.*, 2000). Mutations can be observed in various regions of this gene, including the extracellular domain, transmembrane domain, intracellular domain, and even the C-terminal tail. BMP signaling may thus play an important role in the maintenance of vascular integrity in the pulmonary arteries.

Mutations in human *ALK3/BMPRIA* as well as those in *SMAD4/MADH4* have been found in some patients with an autosomal dominant syndrome juvenile polyposis (Howe *et al.*, 2001; Zhou *et al.*, 2001). Although perturbations of TGF-β signaling are known to lead to development of gastrointestinal cancers, BMP signaling also plays a key role in the control of growth of gastrointestinal epithelium and development of cancers.

Mutations in human *ALK1* gene as well as those in *ENG* gene (encoding endoglin) cause hereditary hemorrhagic telangiectasia (HHT, also known as Osler-Weber-Rendu disease), an autosomal dominant vascular disorder characterized by skin and mucosal telangiectasia, pulmonary, cerebral, and hepatic malformations, and bleeding due to these vascular abnormalities (McAllister *et al.*, 1994; Johnson *et al.*, 1996).

A mutation in *ALK2/ACVR1* causes fibrodysplasia ossificans progressiva (Shore *et al.*, 2006), an autosomal dominant disorder of skeletal malformations and progressive ossification in muscular tissues. A heterozygous single nucleotide mutation (617G-> A; Arg-206 to His) was found in individuals with this disorder. Since Arg-206 is located in the GS domain of ALK-2, the mutation may induce destabilization of the GS domain, leading to hyperactivation of the ALK-2 kinase.

Brachydactyly (BD) type A2 is an autosomal dominant malformation characterized by short and laterally deviated index fingers and shortening and deviation of the first and second toes. Mutations in the GS domain or kinase domain of the *BMPR1B/ALK6* gene, which act in a dominant-negative manner, are responsible for the malformation (Lehmann *et al.*, 2003).

GDF-5 preferentially binds to BMPR-IB, whereas it binds to other type I receptors only weakly (Nishitoh *et al.*, 1996; Nickel *et al.*, 2005). A mutation of Arg-438 to Leu in GDF-5 (R438L mutant) is responsible for proximal symphalangism, characterized by fusion of carpal and tarsal bones and ankylosis of the proximal interphalangeal joints. The R438L mutant of GDF-5 acquires an ability to bind to BMPR-IA with a high binding affinity, indicating that the GDF-5 mutant exhibits biological activity similar to BMP-2 (Seemann *et al.*, 2005; Nickel *et al.*, 2005).

As described earlier, mutations in the human *Hemojuvelin* gene, encoding a BMP co-receptor hemojuvelin/RGMc, were identified in patients with juvenile hemochromatosis, which is characterized by accumulation of iron in various organs (Babitt *et al.*, 2006).

INTRACELLULAR SIGNALING

Signaling by Smad proteins

Structure of Smads

Three Classes of Smads

Smads are the major signal transducers for the serine-threonine kinase receptors (Heldin *et al.*, 1997; Miyazono *et al.*, 2000; Shi and Massagué, 2003). Activated type I receptor kinases phosphorylate receptor-regulated Smads (R-Smads). R-Smads then form a complex with common-partner Smads (co-Smads) and translocate to the nucleus. The oligomeric Smad complexes regulate the transcription of target genes through interaction with various transcription factors and transcriptional coactivators or co-repressors. Inhibitory Smads (I-Smads) negatively regulate the action of R-Smads and/or co-Smads.

Eight different Smads have been identified in mammals. They can be classified into three subclasses as described earlier, i.e., R-Smad, co-Smad, and I-Smad. R-Smads are subdivided into those activated in BMP signaling pathways (BMP-specific R-Smads) and those in TGF-β/activin signaling pathways (TGF-β/activin-specific R-Smads). BMP-specific R-Smads include Smad1, Smad5, and Smad8, whereas Smad2 and Smad3 are TGF-β-specific R-Smads. Smad4 is the only co-Smad in mammals, shared by both BMP and TGF-β/activin signaling pathways. Smad6 and Smad7 are I-Smads. Smad1 and Smad5 have been shown to induce the differentiation of C2C12 myoblasts into osteoblasts, a process facilitated by the addition of Smad4 (Fujii *et al.,* 1999). Moreover, osteoblast differentiation by Smad1/5 requires nuclear translocation induced by receptor activation.

Smads have highly conserved N- and C-terminal regions termed Mad homology (MH) 1 and MH2 domains, respectively, which are bridged by a linker region of variable length and amino acid sequence (Fig. 3). MH2 domains are found in all three classes of Smads, whereas MH1 domains are conserved only in R-Smads and co-Smads. The N-terminal regions of I-Smads highly diverge from those of the other Smads. R-Smads have a characteristic Ser-Ser-X-Ser sequence (SSXS motif) at the C-terminal ends. The SSXS motif is phosphorylated by the type I receptors, resulting in the activation of R-Smads for signaling.

Smad1, Smad5, and Smad8 are structurally highly similar to each other, and the functional differences between them are unknown. However, Smad1 and Smad5, but not Smad8, are activated by BMP-6 and BMP-7 (Ebisawa *et al.,* 1999), whereas all three BMP-specific R-Smads are activated by BMP-2 (Aoki *et al.,* 2001). Smad1 and Smad5 are activated by TGF-β in endothelial cells and some other cells through activation of ALK-1 (Goumans *et al.,* 2003).

MH2 Domain

The MH2 domain, composed of about 200 amino acids, has various important functions. It is responsible for (1) interaction with receptors, (2) binding to SARA (Smad anchor for receptor activation), (3) oligomer formation with other Smads, (4) interaction with various DNA-binding proteins, and (5) transcriptional activation. In the absence of receptor activation, the MH2 and MH1 domains physically interact with each other and repress each other's function. Upon phosphorylation of the SSXS motif by receptors, this interaction is eliminated, and R-Smads become activated.

A region composed of 17 amino acids, termed the *L3 loop*, protrudes from the surface of the molecule and interacts with the L45 loop of type I receptors (Chen *et al.,* 1998; Shi *et al.,* 1997; Wu *et al.,* 2000). The amino acid sequences of the L3 loop are conserved in BMP-specific R-Smads and in TGF-β/activin-specific R-Smads, but diverge between these two groups. In addition to the L3 loop, the α-helix H1 in the MH2 domain, composed of eight amino acids, is also required for the interaction of BMP-specific R-Smads with type I receptors of the ALK-1 group (Chen and Massagué, 1999; see Fig. 3).

MH1 Domain

The MH1 domains, composed of approximately 130 amino acids, are responsible for (1) binding to specific DNA sequences, (2) interaction with certain DNA-binding proteins, (3) nuclear translocation, and (4) repression of the function of MH2 domains. The direct-DNA binding of Smads occurs through an 11-amino-acid "β-hairpin loop," which protrudes from the surface of the molecule (Shi *et al.,* 1998). The structure of the β-hairpin loop is conserved in R-Smads and co-Smad in mammals, and Smad4 and Smad3 bind to the characteristic Smad-binding elements (SBEs; AGAC or GTCT sequence) through this domain (Zawel *et al.,* 1998). BMP-specific R-Smads bind

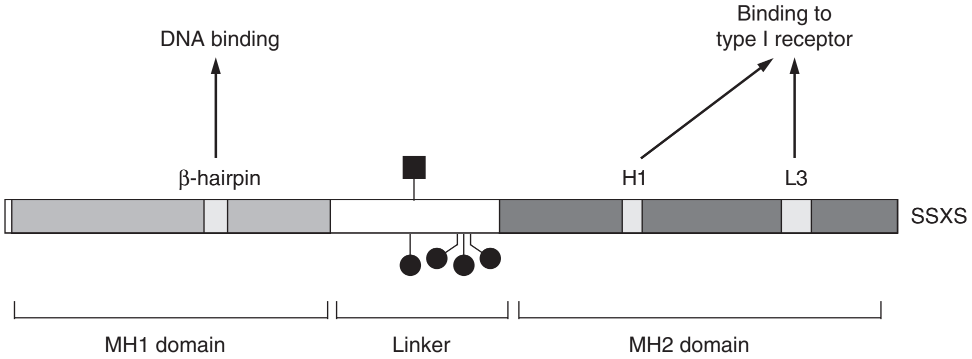

FIGURE 3 Structure of R-Smads. Circles in the linker region indicate the PXS/TP (or S/TP) motif, and the square indicates the PY motif.

to the AGAC/GTCT sequence only weakly, whereas they bind to GC-rich sequences with relatively high affinity (Kim *et al.,* 1997; Kusanagi *et al.,* 2000).

Linker Region

The linker regions of R-Smads contain four copies of PXS/TP (or S/TP) motifs that are phosphorylated by MAP (mitogen-activated protein) kinases (Kretzschmar *et al.,* 1997). When these motifs are phosphorylated, R-Smads become unable to translocate into the nucleus.

The PPXY sequence (known as the PY motif) interacts with proteins containing WW domains. A PY motif is found in all Smads except Smad4 and Smad8. Certain members of the HECT family E3 ubiquitin ligases, including Smurf1 and Smurf2, interact with Smad1 and Smad5 through the PY motif and degrade them in a ligand-independent manner (Zhu *et al.,* 1999).

An alternatively spliced variant of Smad8, lacking 37 amino acids in the linker region, has been observed (Watanabe *et al.,* 1997). Whether there is a functional difference between the long and short versions of Smad8 remains to be determined.

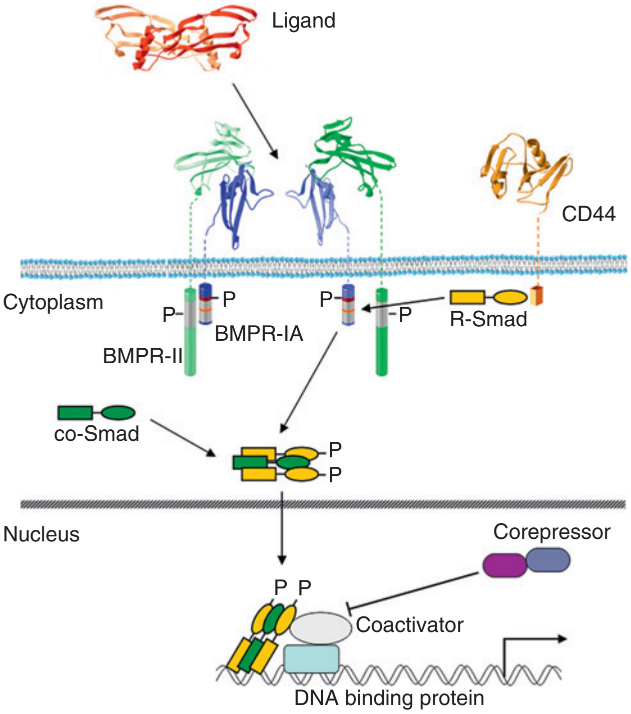

FIGURE 4 Mechanism of activation of Smad signaling pathway. R-Smads exist in the cytoplasm through interaction with membrane anchoring proteins, e.g., CD44 and endofin. Upon phosphorylation by type I receptors, R-Smads form complexes with co-Smad (Smad4), translocate into the nucleus, and regulate transcription of target genes through interaction with transcription factors (DNA binding proteins) and transcriptional coactivators. Transcriptional co-repressors inhibit the transcription induced by transcriptional coactivators. (See plate section)

Smad Signaling in the Cytoplasm

R-Smads are anchored at the cell membrane by interacting with various cytoplasmic proteins, including SARA and other molecules (Fig. 4). SARA binds to Smad2/3, but not to BMP-specific R-Smads (Tsukazaki *et al.,* 1998), whereas endofin interacts with Smad1 as well as Smad4 and enhances BMP signaling (Shi *et al.,* 2007; Chen *et al.,* 2007). dSARA in *Drosophila* was shown to interact with Mad, the *Drosophila* homologue of Smad1/5/8 (Bennett and Alphey, 2002).

SARA and endofin contain a phospholipid interaction domain, termed the FYVE domain, which targets them to the plasma membrane. SARA presents R-Smads to type I receptors and facilitates their activation (Tsukazaki *et al.,* 1998). R-Smads transiently interact with and become phosphorylated by activated type I receptors. In addition to SARA and endofin, Smad1 was shown to interact with the cytoplasmic domain of CD44, a receptor for hyaluronan (Peterson *et al.,* 2004). In chondrocytes, Smad1 is anchored at the plasma membrane by CD44 and presented to the BMP receptors for activation. Disruption of interaction between hyaluronan and CD44 leads to inhibition of Smad1 phosphorylation induced by BMPs.

The interaction between type I receptors and R-Smads is determined by the L45 loop of type I receptors and L3 loop and α-helix HI of the MH2 domains of R-Smads (see Fig. 3; Wu *et al.,* 2000). Phosphorylation occurs at the last two serine residues in the SSXS motif of the R-Smads. R-Smads then form heteromeric complexes with co-Smads, possibly composed of two or one molecule(s) of R-Smads and one molecule of co-Smad (Kawabata *et al.,* 1998; Shi, 2006).

Smad Signaling in the Nucleus

In the nucleus, Smads regulate transcription of target genes through (1) direct binding to DNA, (2) interaction with other DNA-binding proteins, and (3) recruitment of transcriptional coactivators and/or co-repressors (see Fig. 4).

In addition to the characteristic GTCT/AGAC sequence specific for Smad3 and Smad4, BMP-specific R-Smads bind to GC-rich sequences (GCCGnCGC motif) found in the promoter region of *Smad6* (Ishida *et al.,* 2000). BMP-specific R-Smads bind to other GC-rich sequences, e.g., bre7 (TGGCGCC sequence) in *Bambi*, *Vent2*, and *Smad7*. In the promoter of *Id1* gene, both SBEs and GC-rich boxes are most important for efficient transcriptional activation (Lopez-Rovira *et al.*, 2002; Korchynskyi and ten Dijke, 2002).

Smads interact with various DNA-binding proteins, and this interaction may be a critical event in the exhibition of specific effects of TGF-β family proteins in different types of cells. In certain BMP target genes, Smads may regulate transcription through indirectly binding to DNA with other transcriptional factors. Many DNA-binding proteins, including Runx, Menin, OAZ, MyoD, Vent2, Hoxc-8, and Msx1, interact with BMP-specific R-Smads (Miyazono *et al.*, 2005).

Runx is a family of transcription factors that regulate various biological events, including hematopoiesis and osteogenesis (Ito and Miyazono, 2003). Three mammalian isoforms of Runx, Runx1 through 3, interact with R-Smads. Mice lacking the *Runx2* gene (also termed *Cbfa1* or polyomavirus enhancer binding protein-αA; *PEBP2αA*) exhibit complete loss of bone formation. Haploinsufficiency of *RUNX2* in human is responsible for the development of the autosomal dominant bone disease cleidocranial dysplasia. Expression levels of Runx2 are low in undifferentiated mesenchymal cells. BMP facilitates expression of Dlx5 (Miyama *et al.*, 1999; Lee *et al.*, 2003), which in turn induces expression of Runx2 in osteoprogenitor cells. BMP-specific R-Smads physically interact with Runx2 upon activation by BMP receptors (Hanai *et al.*, 1999; Zhang *et al.*, 2000), and they cooperatively activate the transcription of target genes, leading to facilitation of the osteoblast differentiation.

Menin, the product of the *MEN1 (multiple endocrine neoplasia 1)* gene, may be required for mesenchymal stem cells to differentiate into the osteoblast lineage. Menin physically interacts with BMP-specific R-Smads and Runx2 in mesenchymal stem cells and facilitates transcriptional activity induced by BMP-specific R-Smads and Runx2 in the early stage of osteoblast differentiation (Sowa *et al.*, 2004).

In addition to the transcription factors that positively regulate the transcription of target genes, certain DNA-binding proteins act as transcriptional repressors in the absence of BMP/TGF-β stimulation, and this repression may be relieved after interaction with Smads upon ligand stimulation (Miyazono *et al.*, 2000). A homeodomain protein Hoxc-8 binds to the promoter region of the *osteopontin* gene and represses its transcription. Upon BMP stimulation, Smad1 interacts with Hoxc-8, which then dissociates from the DNA-binding element (Shi *et al.*, 1999).

p300 and CBP (CREB binding protein) have histone acetyltransferase (HAT) domains, which upregulate gene transcription by loosening nucleosomal structure and by increasing the accessibility to the general transcription machinery. p300 and CBP interact with various transcription factors; they interact with Smad1, -2, and -3 upon ligand stimulation and enhance Smad-dependent transcription of target genes (Miyazono *et al.*, 2000; Massagué and Wotton, 2000). Transcriptional complexes containing Smad1, 5, and 4 and CBP have been shown to bind to the promoter of colony-stimulating factor-1 (CSF-1) and increase the expression of CSF-1 in C2C12 cells (Ghosh-Choudhury *et al.*, 2006). In addition to Smads, p300 interacts with Runx proteins and stimulates Runx-dependent transcription. Runx3 is acetylated on lysine residues by p300 upon stimulation by TGF-β, leading to prevention of ubiquitin-dependent degradation of Runx by Smurf and stabilization of the transcriptional complex containing Runx3 and p300 (Jin *et al.*, 2004; see later discussion). GCN5 and P/CAF are transcriptional coactivators, which belong to the GNAT (GCN5-related N-acetyltransferase) superfamily. Similar to p300 and CBP, GCN5 interacts with BMP-specific R-Smads and enhances BMP-induced transcriptional activity (Kahata *et al.*, 2004).

Transcriptional co-repressors, including c-Ski, SnoN, and Evi-1, recruit histone deacetylases (HDACs) to Smad complexes (Miyazono, 2000). In contrast to p300 and CBP, they induce nucleosomal condensation, and consequently repress the transcription of target genes (see later discussion).

Target Genes for BMPs

Through DNA microarray analyses, many BMP target genes have been identified in various cells (Korchynskyi *et al.*, 2003; Clancy *et al.*, 2003; Peng *et al.*, 2003; Kowanetz *et al.*, 2004; de Jong *et al.*, 2004). Early mesenchymal cells, e.g., C2C12 cells, have been widely used for identification of BMP target genes during osteoblastic differentiation, and numerous BMP-early response genes have been identified in these cells. BMP-early response genes can be subdivided into immediate early (2 hours after BMP stimulation), intermediate early (6 hours after BMP-2 stimulation), and late early response genes (24 hours after BMP stimulation; de Jong *et al.*, 2004). Genes that are involved in signal transduction have mainly been identified as immediate early genes, including *Id1-3, Smad6, Smad7, OASIS, Prx2, TIEG,* and *Snail*. The intermediate and late early response genes are involved in osteoblast differentiation processes, and transcription factors involved in Notch and Wnt signaling, e.g., *Hey1* (also known as *HesR1* and *Herp2*) and *Tcf7,* respectively, are induced by BMP-2 treatment. In addition, some negative regulators of BMP signaling, including Smad6 and Smad7, are induced by BMPs in most cell types.

Synexpression groups are sets of genes that show similar expression profiles in certain biological processes. The BMP-4 synexpression group in the early stages of *Xenopus* embryos includes eight members, *BMP4* and *BMP7, BMPR-II, BAMBI, Smad6, Smad7, Tsg,* and *Vent2*, which encode components of the BMP signaling cascade (Karaulanov *et al.*, 2004).

Among the various BMP target genes, Id (inhibitor of differentiation or inhibitor of DNA binding) genes are induced by BMP in various types of cells and are one of the most important targets of BMPs (Ogata *et al.*, 1993). Id proteins inhibit cell differentiation and stimulate cell proliferation (Miyazono and Miyazawa, 2002). The four isoforms of Id proteins (Id1 through 4) exhibit similar, but not identical, biological activities. The ubiquitously expressed basic helix-loop-helix (bHLH) transcription factors, including E2A gene products, associate with tissue-specific bHLH transcription factors, including MyoD and myogenin in muscle, and activate transcription of genes containing E-box sequences in their promoters. Id proteins have an HLH dimerization domain, but lack the basic region responsible for DNA binding. Through interacting with and sequestering ubiquitously expressed bHLH transcription factors, they antagonize the transcription induced by bHLH transcription factors. Id proteins induced by BMP repress transcription induced by bHLH heterodimers containing MyoD/myogenin, resulting in the inhibition of myogenesis.

Regulation of Smad Signaling

I-Smads

I-Smads, i.e., Smad6 and Smad7, function as antagonists of R-Smad/co-Smad signaling (Fig. 5). I-Smads interact with type I receptors activated by type II receptors through their MH2 domains. Unlike R-Smads, however, they do not dissociate from type I receptors, and thus prevent the activation of R-Smads (reviewed in Miyazono *et al.*, 2000). Smad6 has also been reported to form a complex with Smad1 and compete with Smad4 for oligomer formation. As a third mechanism, Smad6 has been reported to interact with a transcription factor Hoxc-8, and thereby inhibit the interaction between Smad1 and Hoxc-8 and repress the transcription of the *osteopontin* gene induced by Smad1 (Bai *et al.*, 2000). Smad7 inhibits both TGF-β and BMP signaling, whereas Smad6 preferentially represses BMP, but not TGF-β or activin, signaling.

In mammalian cells, Smad7 is located in the nucleus, and exported to the cytoplasm upon TGF-β stimulation (Itoh *et al.*, 1998). I-Smads are also exported to the cytoplasm by Smurf proteins, leading to association of I-Smads with type I receptors (see later discussion).

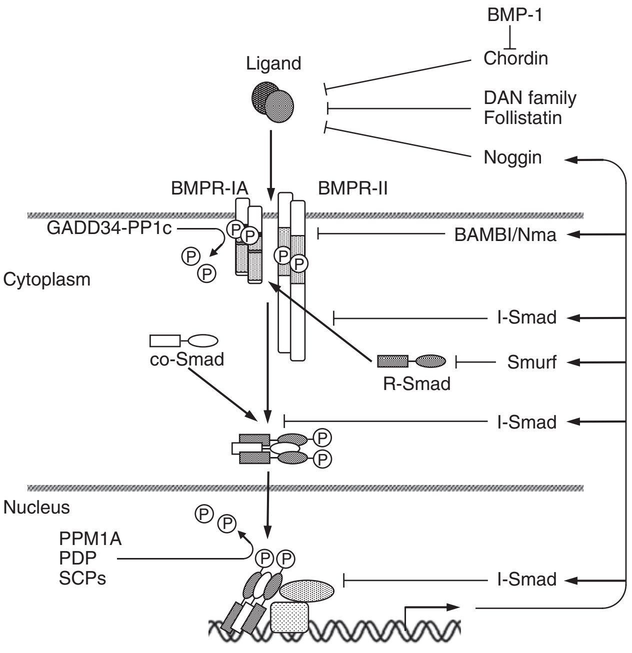

FIGURE 5 Regulation of BMP signaling pathways. BMP signaling is negatively regulated by extracellular antagonists, pseudoreceptor BAMBI/Nma, E3 ubiquitin ligase Smurf, I-Smads, and phosphatases, including PP1c, PPM1A, PDP, and SCPs. Expression of some of these regulatory molecules is induced by BMP or TGF-β signaling.

Expression of I-Smads is regulated by various stimuli, including growth factors, mechanical stress, interferon-γ, and NF-κB signaling (reviewed in Miyazono, 2000). Expression of I-Smads is strongly induced by TGF-β/activins and BMPs; transcription of Smad7 and Smad6 has been shown to be induced by direct effects of TGF-β-specific R-Smads and BMP-specific R-Smads (Nagarajan *et al.*, 1999; Ishida *et al.*, 2000). R-Smad/co-Smad and I-Smad thus form a negative feedback loop for regulation of TGF-β family signaling. I-Smads are strongly induced by BMP during osteoblastic differentiation in a biphasic fashion. I-Smads are transiently induced by BMP within 2 hours, followed by gradual, sustained induction after 2 days during the osteoblastic maturation phase (Maeda *et al.*, 2004). Endogenous TGF-β is activated during this period and induces the expression of I-Smads. Osteoblastic differentiation of mesenchymal progenitor cells at the maturation phase can thus be enhanced by a small molecule TGF-β type I receptor kinase inhibitor SB431542 (Maeda *et al.*, 2004).

In addition, expression levels of I-Smads are decreased by some E3 ubiquitin ligases, including Arkadia (Koinuma *et al.*, 2003) and Jab1/CSN5 (Kim *et al.*, 2004). AMSH (associated molecule with the SH3 domain of STAM) binds to Smad6 upon BMP stimulation and antagonizes Smad6 by interfering with the association of Smad6 with BMP type I receptors and with Smad1 (Itoh *et al.*, 2001).

Proteasome-Dependent Degradation of Smads

R-Smads are degraded by the ubiquitin–proteasome pathway in ligand-independent and ligand-dependent fashions (Miyazono, 2000). Protein ubiquitylation is triggered by E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin-ligases. Of these, E3 ligases are most important for specific recognition and degradation of target proteins. HECT type E3 ligases Smurf1 and Smurf2 physically associate with R-Smads (Zhu *et al.*, 1999; Zhang *et al.*, 2001; Lin *et al.*, 2000). This interaction occurs between the WW motif in Smurfs and the PY motif in the linker region of R-Smads. Smurfs degrade BMP-specific R-Smads in a ligand-independent fashion. Smurfs thus regulate the intracellular pool of R-Smads and inhibit TGF-β family signaling.

Smurfs also interact with I-Smads (Kavsak *et al.*, 2000; Ebisawa *et al.*, 2001). Smurfs induce nuclear export of I-Smads and facilitate the interaction of I-Smads with type I receptors. The enhanced interaction of I-Smads with type I receptors results in suppression of TGF-β family signaling. Moreover, Smurfs induce degradation of the receptors, leading to a decrease in the number of cell surface receptors. Smurf1 also induces the degradation of Runx2 and reduces alkaline phosphatase activity and production of osteocalcin in osteoprogenitor cells (Zhao *et al.*, 2003). Transgenic mice overexpressing Smurf1 in osteoblasts thus show reduction in bone formation (Zhao *et al.*, 2004).

After nuclear translocation and transcriptional activation, R-Smads may be degraded by the ubiquitin-proteasome pathway (Lo and Massagué, 1999; Fukuchi *et al.*, 2000). This ligand-dependent degradation of R-Smads may be important for termination of TGF-β family signaling.

Dephosphorylation of Receptors by Protein Phosphatase 1 (PP1)

In addition to Smurfs, Smad7 interacts with GADD (growth arrest and DNA damage protein) 34, a regulatory subunit of the PP1 holoenzyme (Shi *et al.*, 2004). Smad7 recruits the catalytic subunit of PP1 (PP1c) through GADD34 to TβR-I and induces its dephosphorylation. SARA also interacts with PP1c. dSARA recruits PP1c to *Drosophila* BMP type II receptor PUNT, resulting in suppression of Dpp signaling by dephosphorylating type I receptors (Bennett and Alphey, 2002).

Smad Phosphatases

Several phosphatases have been shown to dephosphorylate the SSXS motif of R-Smads. PPM1A/PP2C, the prototype of PPM (metal ion-dependent protein phosphatase) family phosphatases, interacts with Smad1 and Smad2/3 and dephosphorylates the phospho-SSXS motif (Duan *et al.*, 2006; Lin *et al.*, 2006). PPM1A dephosphorylates the C-terminal SSXS motif, but not the linker region. Other phosphatases, including PDP (pyruvate dehydrogenase phosphatase; Chen *et al.*, 2006) and the SCPs (small C-terminal domain phosphatases; Knockaert *et al.*, 2006), decrease the level of phospho-Smad1 and suppress BMP signaling in mammals, *Xenopus*, and *Drosophila*.

Transcriptional Co-repressors

Transcriptional co-repressors, including c-Ski, SnoN, and Evi-1, repress Smad signaling (Miyazono, 2000). Ski was originally identified as the oncogene of the avian Sloan-Kettering retrovirus. SnoN is structurally closely related to c-Ski. c-Ski and SnoN interact with Smads as well as with N-CoR and mSin3A and recruit HDACs to the Smad complexes (Liu *et al.*, 2001). c-Ski and SnoN interact with Smad2/3 at the N-terminal regions, whereas they interact with Smad4 through the middle portions (Mizuide *et al.*, 2003; Wu *et al.*, 2002; Qin *et al.*, 2002). c-Ski and SnoN repress BMP signaling both *in vitro* and *in vivo* through interaction with Smad4 (Wang *et al.*, 2000; Takeda *et al.*, 2004).

Evi-1 is a sequence-specific transcriptional repressor, which is highly expressed in some human myeloid leukemias and myelodysplastic syndromes. Evi-1 binds to Smad2, Smad3, and Smad1 and represses TGF-β as well as BMP signaling (Kurokawa *et al.*, 1998; Alliston *et al.*, 2005). Evi-1 interacts with a transcriptional co-repressor CtBP, and decreases acetylation of histones upon ligand stimulation (Palmer *et al.*, 2001; Alliston *et al.*, 2005).

Other BMP Signaling Regulators

Some other molecules, including Tob, SANE (Raju *et al.*, 2003), and CIZ (Shen *et al.*, 2002), interact with BMP-specific R-Smads and regulate BMP signaling through various mechanisms.

Tob (transducer of ErbB2) is a member of the antiproliferative gene family, which inhibits growth of NIH3T3 cells and T lymphocytes. Tob-deficient mice show osteosclerosis with an increased bone mass later in their lives (Yoshida *et al.*, 2000). Tob inhibits BMP signaling in osteoblasts through interaction with BMP-specific R-Smads. In addition, Tob associates with I-Smads, and inhibits BMP signaling through interaction with BMP type I receptors (Yoshida *et al.*, 2003).

Crosstalk with Other Signaling Pathways

BMP signaling is regulated negatively and positively by various signaling pathways (Herpin and Cunningham, 2007). The Erk MAP kinase pathway is activated by peptide growth factors. Erk phosphorylates the serines and threonines in the PXT/SP (or T/SP) motif, four copies of which are present in the linker regions of R-Smads. R-Smads phosphorylated by Erk are unable to translocate into the nucleus, and thus signaling by BMPs and TGF-β is inhibited by peptide growth factors through the action of Erk MAP kinase (Kretzschmar *et al.*, 1997). Phosphorylation of the linker region of BMP-specific R-Smads also facilitates the interaction with and degradation by Smurf1 (Sapkota *et al.*, 2007). In contrast to the antagonistic effects, MAP kinase signaling enhances BMP signaling under certain conditions, e.g., activation of Erk MAP kinase pathway by FAK (focal adhesion kinase; Tamura *et al.*, 2001).

Leukemia inhibitory factor (LIF) and BMP-2 synergistically induce astrocytic differentiation of neuroepithelial cells (Nakashima *et al.*, 1999). LIF activates STAT3 through gp130 and JAKs, and BMP-2 activates BMP-specific R-Smads. Although direct interaction between STAT3 and BMP-specific R-Smads is not observed, they are indirectly associated through binding to p300, which results in cooperative transcriptional activation of target genes.

Notch encodes a transmembrane receptor that is proteolytically cleaved upon binding of ligands. The cleaved product, the Notch intracellular domain (NICD), translocates into the nucleus, where it associates with the transcription factor CSL/RBP-Jκ/CBF-1. The NICD-CSL complexes induce transcription of target genes, including *Hey* and *Hes,* which encode bHLH transcriptional regulators. Both synergy and antagonism have been observed between the Notch and BMP signaling pathways. Expression of Hey1 and Hes1 is induced by BMP in various cells, including osteoprogenitor cells, and it is required for inhibition of myogenic differentiation induced by BMP-4 (Dahlqvist *et al.*, 2003). Smad1 physically interacts with NICD and activates the transcription of *Hey1*. In contrast, Hey1 induced by BMP-2 has been reported to negatively regulate osteoblast maturation by suppressing the transcriptional activity of Runx2 (Zamurovic *et al.*, 2004).

BMP and Wnt signaling pathways act synergistically on various biological processes. Wnt signaling induces the stabilization and accumulation of β-catenin, resulting in the interaction with the Lef1/Tcf family transcription factors and transcription of target genes. BMP-dependent activation of Msx2 is mediated by cooperative binding of Smad4 and Lef1 to the Msx2 promoter (Hussein *et al.*, 2003). Tcf7, a downstream effector of Wnt signaling, is induced by BMP-2 and inhibits myogenic differentiation through suppression of the expression of myogenin and MyoD (de Jong *et al.*, 2004).

In Vivo Abnormalities Associated with Smad Genes

Mutations of the human *SMAD4/DPC4* gene are frequently found in pancreatic cancer, biliary tract cancer, metastatic colon cancer, and juvenile polyposis (reviewed in Miyazono *et al.*, 2000). *Smad4* null mice die before E7.5 with gastrulation defects and an abnormal visceral mesoderm, which are similar to the phenotypes of BMP-4- and BMPR-IA-deficient mice (Sirard *et al.*, 1998). Homozygotic loss of the *Smad4* gene together with *Apc* mutations results in progression of intestinal tumors (Takaku *et al.*, 1998). *Smad4* heterozygous mice develop gastric polyposis 6 to 12 months after birth, which eventually progresses to gastric cancer as a result of loss of heterozygosity (Takaku *et al.*, 1999; Xu *et al.*, 2000).

Smad5-deficient mice exhibit abnormalities in angiogenesis with dilated vessels and decreased number of smooth muscle cells surrounding vessels (Yang *et al.*, 1999; Chang *et al.*, 1999). The phenotype of *Smad5*-null mice is similar to that of the *Alk1*-null mice as well as those of TGF-β-, TβR-II-, or endoglin-deficient mice. Because ALK-1 binds TGF-β in endothelial cells and activates BMP-specific R-Smads (Oh *et al.*, 2000; Goumans *et al.*, 2003), Smad5 might be an important signal transducer for TGF-β in endothelium. Smad5 is also required for development of left–right asymmetry (Chang *et al.*, 2000). Targeted disruption of the mouse *Smad1* gene demonstrates an essential role for Smad1 in chorioallantoic fusion and primordial germ cell formation (Lechleider *et al.*, 2001; Tremblay *et al.*, 2001). Smad8-deficient mice do not exhibit overt abnormalities during embryogenic or postnatal development (Hester *et al.*, 2005; Arnold *et al.*, 2006).

Smad6-null mice exhibit cardiac defects with abnormal valve formation and outflow tract septation (Galvin *et al.*, 2000). Some *Smad6*-null mice survive through adulthood, but exhibit aortic ossification and elevated blood pressure, indicating that Smad6 is required for development and homeostasis of the cardiovascular system.

Transgenic mice expressing Smad6 in chondrocytes exhibit dwarfism with osteopenia and delayed chondrocyte hypertrophy (Horiki *et al.*, 2004). Transgenic mice expressing Smurf1 in chondrocytes do not show significant abnormalities, but double transgenic mice expressing Smad6 and Smurf1 in chondrocytes show more delayed ossification than the Smad6 transgenic mice (Horiki *et al.*, 2004). Smurf1-deficient mice exhibit age-dependent increase of bone mass, due to enhanced osteoblast activity induced by activation of MEKK2 and the JNK signaling cascade (Yamashita *et al.*, 2005).

Non-Smad Pathways

In addition to the Smad pathways, BMP-2 and GDF-5 have been shown to activate the p38 MAP kinase pathway (Iwasaki *et al.*, 1999; Nakamura *et al.*, 1999; Kimura *et al.*, 2000). The p38 MAP kinase activated by BMPs induces neurite outgrowth from PC12 pheochromocytoma cells, chondrocyte differentiation of ATDC5 mouse teratocarcinoma cells, and apoptosis in mouse hybridoma MH60 cells. Erk MAP kinases have also been shown to be activated by BMP-2 in certain cells. These non-Smad pathways activated by BMPs may play important roles in modulation of effects of Smads on cellular proliferation and differentiation.

TAK1 has been reported to act as a downstream component of BMP and TGF-β and activate the SAPK/JNK and p38 MAP kinase pathways. BMPR-IA directly interacts with XIAP, which activates TAB1-TAK1. However, activation of TAK1 by TGF-β or BMPs may occur only under certain conditions; under physiological conditions, TAK1 may serve as a downstream target of interleukin-1 (Ninomiya-Tsuji *et al.*, 1999).

The C-terminal tail of BMPR-II interacts with LIM kinase 1 (LMK1), Tctex1, Src, and Tribbles-like protein 3 (Trb3). LMK1 regulates actin dynamics through phosphorylation and inactivation of cofilin. BMPR-II has been reported to inhibit the ability of LIMK1 to phosphorylate cofilin through interaction with its C-terminal tail (Foletta *et al.*, 2003). LIMK1 has also been reported to be activated by BMPR-II binding and Cdc42, and to modulate BMP-dependent regulation of neurite formation (Lee-Hoeflich *et al.*, 2004). Tctex1 is a light chain of the motor complex dynein (Machado *et al.*, 2003). BMPR-II induces phosphorylation of Tctex, resulting in movement of Tctex along the microtubules and the efficient activation of downstream signal mediators. The C-terminal tail of BMPR-II also interacts with c-Src and decreases c-Src-activating phosphorylation at Tyr-418 in pulmonary smooth muscle cells upon ligand stimulation (Wong *et al.*, 2005). In addition to regulating the non-Smad pathways, the C-terminal tail may also regulate the Smad signaling pathway. Trb3 is associated with the BMPR-II C-terminal tail and is released from BMPR-II upon ligand binding. Trb3 then induces degradation of Smurf1, leading to stabilization of BMP-specific R-Smads and facilitation of the Smad signaling pathway (Chan *et al.*, 2007).

EXTRACELLULAR REGULATORS OF BMPs

BMP Antagonists

The biological activities of BMPs are tightly regulated by extracellular antagonists (see Fig. 5). A wide variety of extracellular antagonists of the TGF-β family exist and are divided into two different types: those that directly bind ligands (ligand-binding antagonists) and others that compete with ligands for binding to specific receptors (pseudoligand-type antagonists) (Miyazono, 2000). Lefty-1, Lefty-2, and zebrafish Antivin are typical pseudoligand-type antagonists, which interfere with binding of nodal and activin-like factors to activin type II receptors.

Most of the antagonists of BMPs are ligand-binding type antagonists, including Noggin, Chordin, Cerberus and its related proteins, Sclerostin, ectodin/uterine sensitization-associated gene-1 (USAG-1), Follistatin, and nephroblastoma overexpressed (Nov; Canalis *et al.*, 2003). Cerberus and its related proteins, including Gremlin, Caronte, and DAN, contain a conserved cystine-knot motif, and are collectively referred to as the DAN family. Sclerostin and USAG-1 are small secreted proteins with a cystine-knot structure similar to those in DAN family proteins. Nov is a member of the CCN family of cysteine-rich secreted proteins including connective tissue growth factor (CTGF) and cysteine-rich 61 (Cyr 61; Rydziel *et al.*, 2007).

These BMP antagonists have distinct expression profiles and different affinities with various BMP isoforms. Noggin associates with BMPs, including BMP-2, BMP-4, GDF-5, GDF-6, BMP-5, and BMP-6. Caronte functions as an antagonist of BMP-4, BMP-7, and nodal. Noggin competes with Cerberus, Gremlin, and DAN for binding to BMP-2, indicating that the binding sites in BMPs are shared by these antagonists (Hsu *et al.*, 1998). Cerberus binds to several cytokines, including BMPs, nodal-like factors, and Wnt, through independent binding sites in its molecule (Piccolo *et al.*, 1999). Follistatin is a potent antagonist of activins, but also antagonizes certain BMPs both *in vitro* and *in vivo.*

Limb development is regulated by various BMP antagonists, including Noggin, Chordin, Gremlin, and Follistatin (McMahon *et al.*, 1998; Merino *et al.*, 1999). Noggin is abundantly present in joint-forming spaces, and mutations in the human Noggin *(NOG)* gene are associated with skeletal dysplasia syndromes, such as proximal symphalangism (SYM1; Gong *et al.*, 1999). Noggin is also expressed in the follicular mesenchyme and involved in hair-follicle induction (Botchkarev *et al.*, 1999). In *Xenopus* embryos, Noggin and Chordin are expressed in Spemann's organizer and induce neural tissues and dorsalize ventral mesoderm. These BMP antagonists may thus play a central role in the formation of morphogen gradients during early embryogenesis.

Mutations of human Sclerostin *(SOST)* gene are responsible for sclerosteosis, a recessive autosomal sclerosing bone dysplasia characterized by progressive skeletal overgrowth (Balemans *et al.*, 2001; Brunkow *et al.*, 2001). Sclerostin was reported to interact with BMPs, interfere with the binding of BMPs to their receptors, and inhibits BMP-stimulated bone differentiation (Kusu *et al.*, 2003; Winkler *et al.*, 2003). However, recent data suggested that primary function of sclerostin in suppression of bone morphogenesis is induced by modulation of Wnt signaling (van Bezooijen *et al.*, 2004) through interaction with Wnt receptor LRP5/6, rather than BMP signaling (Li *et al.*, 2005; Semenov *et al.*, 2005).

Certain BMP antagonists may exert their effects through a negative feedback loop. Expression of Noggin is induced by BMPs in osteoprogenitor cells (Gazzero *et al.*, 1998). Other cytokines, including TGF-β, also induce expression of Noggin, indicating that expression of BMP antagonists is regulated by BMP signaling itself, as well as through crosstalk of signals.

BMP-1 Is a BMP Activator

BMP-1 does not belong to the TGF-β family but is a procollagen C-proteinase that cleaves procollagens and induces accumulation of extracellular matrix (Kessler *et al.*, 1996). BMP-1 homologues are present in *Drosophila (Tolloid)* and in *Xenopus (Xolloid);* studies of Tolloid and Xolloid suggest that BMP-1 may be an activator of BMPs, because it releases active BMPs from inactive complexes by cleavage of Chordin (Piccolo *et al.*, 1996; Marques *et al.*, 1997). The proteinase activity of BMP-1 is suppressed by Frizzled-related proteins, including Ogon/Sizzled, which results in stabilization of Chordin (Lee *et al.*, 2006). BMP-1 also cleaves latent TGF-β binding protein, leading to consequent activation of latent TGF-β by metalloproteinases, e.g. MMP2 (Ge and Greenspan, 2006). In addition, BMP-1-like proteinases activate the latent form of myostatin by cleavage of its prodomain (Wolfman *et al.*, 2003).

CONCLUSION

BMPs play multiple roles in various tissues including bone, cartilage, neurons, heart, kidney, liver, and lung. Signaling by BMPs is transduced by serine-threonine kinase receptors and Smad proteins. BMP signaling is tightly regulated by various mechanisms including extracellular antagonists, pseudoreceptors, and I-Smads. BMPs thus exhibit a diverse array of biological activities in various tissues. Further understanding of the BMP signaling pathways can aid the pharmacological regulation of biological activities of BMPs in various tissues.

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Chapter 56

Colony-Stimulating Factor-1

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INTRODUCTION

Bone and bone marrow exist in a close anatomical relationship, and several lines of evidence suggest a reciprocal interaction between the two tissues. Bone provides the microenvironment that is critical for the development of the hemopoietic stem cells, from which all the cells of the mammalian immune system derive (Arron and Choi, 2000; Walsh *et al.,* 2006). In the bone marrow reside the precursors of the cell lineages accounting for the cellular base of bone remodeling. Osteoclasts, the cells dissolving bone, originate from hemopoietic progenitors, the colony-forming unit granulocyte/macrophage (CFU-GM; Kurihara *et al.*, 1990; Hagenaars *et al.,* 1991; Hattersley *et al.,* 1991a, 1991b). Osteoblast lineage cells, the cells elaborating the extracellular matrix of bone and regulating its mineralization, are derived from mesenchymal progenitors of the marrow stroma, the colony-forming unit fibroblasts (CFU-Fs; Owen, 1985; Owen and Friedenstein, 1988).

The interactions between hemopoietic and bone cells are complex and occur at different levels. Immunoregulatory cytokines of hemopoietic origin modulate the development and activity of bone cells. In developing bone, osteoclasts resorb and invade the calcified cartilage rudiment. As a result, the primitive marrow cavity is formed and hemopoiesis is initiated. Bone cells take part in hemopoiesis by synthesizing growth factors essential for the proliferation and differentiation of hemopoietic cells. In the bone marrow, hemopoietic stem cells proliferate and differentiate into lineage-restricted progenitors that eventually give rise to the terminally differentiated cells of the lymphoid and myeloid series. This differentiation process is directed by a group of lineage-specific growth factors, the colony-stimulating factors (CSFs; Metcalf and Nicola, 1984). The hemopoietic CSFs are glycoproteins that were initially characterized by their ability to stimulate *in vitro* the clonal proliferation of hemopoietic multipotential stem cells and/or mono- or bipotential progenitors in semisolid (agar or methylcellulose) medium. Furthermore, CSFs, which include interleukin-3 (IL-3, also named multi-CSF), granulocyte-macrophage-CSF (GM-CSF), granulocyte-CSF (G-CSF), and colony-stimulating factor-1 (CSF-1; also named macrophage-CSF, M-CSF) will induce the activity of mature, specialized myeloid cells and are required for their survival.

CSFs are ideal candidate factors for being synthesized by osteogenic cells to modulate at the local level osteoclast recruitment and marrow hemopoiesis. In this chapter, the role of CSF-1 is described in modulating osteoclast development and activity. Functions outside of bone are only mentioned, without detailed elaboration. Other cytokines and interleukins besides CSF-1 do have intrinsic and/or synergistic effects on hemopoiesis and osteoclast development. Their respective roles in the local control of bone cell physiology are described in detail in other chapters of this book.

CSF-1 is the lineage-specific growth factor for cells of the mononuclear phagocyte system (MNPS) and for osteoclasts. The cytokine binds to a single class of high-affinity cell-surface receptors that are encoded by the proto-oncogene c-*fms* and belong to the receptor tyrosine kinase family. Expression of c-*fms* is considered a marker for the cells of the MNPS (Felix *et al.,* 1994; Stanley, 1994). Within this chapter, the effects of the cytokine on the cells of this lineage as well as the involved mechanisms are discussed.

MOLECULAR BIOLOGY AND BIOCHEMISTRY OF CSF-1

CSF-1 is the product of a single-copy gene, size and structure are well conserved between different species. The gene spans approx. 120 kb and is composed of 10 exons, 8 of which encode the translated sequence. Several transcripts,

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derived from a common RNA precursor by alternative splicing, encode either a secreted or a membrane-bound form of the cytokine (for review, see Sherr and Stanley, 1990).

All translation products contain an amino-terminal signal peptide of 32 amino acids. A hydrophobic 23-amino-acid region is followed by a charged "stop transfer" sequence, characteristic for many membrane-spanning proteins. The CSF-1 peptide is cotranslationally glycosylated and remains integrated in the membrane of the endoplasmic reticulum, the amino-terminal end facing the lumen. In the Golgi, the protein is O-glycosylated, the N-linked carbohydrate molecules are further processed, and eventually the cytokine can be modified by the attachment of a glycosaminoglycan side chain (Price *et al.*, 1992; Suzu *et al.*, 1992; Felix *et al.*, 1996). The secreted cytokine is synthesized as a 554-amino-acid precursor, which is processed to yield a disulfide-linked homodimer of 223 amino acids per chain, whereas the membrane-bound form is synthesized as a precursor protein of 256 amino acids, which subsequently is processed to a homodimer of 150 amino acids per chain.

THE ROLES OF SECRETED AND MEMBRANE-BOUND CSF-1

The osteopetrotic mouse strain *op* was the first animal model found to be deficient in a hemopoietic growth factor (Felix *et al.*, 1990b; Wiktor-Jedrzejczak *et al.*, 1990). The phenotype of these mice was characterized by a strong reduction in tissue macrophages and a virtual absence of osteoclasts (Marks and Lane, 1976). Thus, CSF-1 was described as the first obligate growth factor for osteoclasts, its activity being a prerequisite for functional bone resorption. The contributions of the molecular forms of CSF-1 to bone resorption and their regulation were the subject of intense study. The cytokine could be a constitutive part of the hemopoietic microenvironment in bone, essential but continuously present and not exerting regulatory functions. As a second possibility, CSF-1 may be involved in the acute regulation of bone resorption and as a consequence will be modulated by osteotropic hormones and cytokines. The aforementioned fact that CSF-1 is synthesized either as a secreted or as a membrane-bound molecule further complicated the elucidation of the cytokine's role in bone biology. In cell culture experiments, the membrane-bound form of CSF-1 was found to be more efficient than soluble CSF-1 in promoting osteoclast formation (Takahashi *et al.*, 1991). At that time, this was attributed to the proximal relationship of other membrane-associated factors regulating osteoclastogenesis, a hypothesis that was subsequently confirmed with the characterization of the osteoclastogenic growth factor RANKL (receptor activator of NF-κB ligand; Kong *et al.*, 1999).

The dependence of osteoclastogenesis and bone development on the biological activity of CSF-1 was further confirmed in animals lacking *c-Fms* (Dai *et al.*, 2002). The phenotype of the *c-fms*$^{-/-}$ mice closely resembled the phenotype of the *op* animals, including the osteopetrotic, hemopoietic, tissue macrophage and reproductive phenotypes. Furthermore, the osteopetrotic phenotype is resolved in the *c-fms*$^{-/-}$ animals with aging, as was previously reported for *op* animals. Further information on the spatial and temporal roles of *c-Fms* will be derived from the experimental use of conditional *c-fms* knockouts (Li *et al.*, 2006).

CSF-1 has been shown to be expressed by numerous cell types, such as activated macrophages and T cells, epithelial and mesenchymal cells, but the major source of circulating CSF-1 is endothelial (Metcalf *et al.*, 2001; Pixley and Stanley, 2004). *In vitro*, CSF-1 secretion by osteoblast lineage cells is stimulated by parathyroid hormone (PTH), interleukin-1 (IL-1), and tumor necrosis factor-α (TNFα) (Sato *et al.*, 1986; Felix *et al.*, 1988). In contrast to the rather ubiquitous expression *in vitro*, expression of the cytokine is tightly controlled *in vivo*. Levels of CSF-1 are dramatically increased in mice during early pregnancy (Bartocci *et al.*, 1986), the cytokine being induced by 17β-estradiol and progesterone in uterine glandular epithelial cells (Pollard *et al.*, 1987; Daiter *et al.*, 1992). In bone, expression of CSF-1 is correlated temporally and spatially with osteoclast formation and bone resorption (Hofstetter *et al.*, 1995). Although a considerable body of data has been collected, little is known about the transcriptional and translational regulation of the expression of CSF-1 *in vivo*. Deficiency in estrogen will cause a release of TNFα by bone marrow (Kimble *et al.*, 1996) and T cells (Roggia *et al.*, 2001, 2004) which in turn will affect osteoclastogenesis by direct or indirect actions on osteoblast and osteoclast lineage cells (Bertolini *et al.*, 1986; Fuller *et al.*, 2002; Nanes, 2003) or which will cause expansion of a subpopulation of stromal cells high in CSF-1 production (Kimble *et al.*, 1996).

The osteopetrotic mouse strain *op*, described earlier as deficient in active CSF-1, represents an ideal model to investigate the biological roles of the cytokine (Felix *et al.*, 1990b; Wiktor-Jedrzejczak *et al.*, 1990). The lack of biologically active CSF-1 was found to be caused by a point mutation within the coding region of the CSF-1 gene (Yoshida *et al.*, 1990). Treatment of newborn *op/op* animals with daily injections of recombinant human CSF-1 induced osteoclast formation and bone resorption, proving the deficiency in CSF-1 to be the cause of the osteopetrosis in this strain (Felix *et al.*, 1990a; Kodama *et al.*, 1991). Transgenic animals, in which CSF-1 expression was reconstituted within the *op* background, were instrumental in the elucidation of the specific roles of the molecular forms of CSF-1. Expression of the full-length cytokine, under control of a 3.13-kb fragment of the CSF-1 promoter, fully reversed the *op* phenotype (Ryan *et al.*, 2001). The correction of essentially all the defects of the *op* strain demonstrates that in these animals all the observed alterations are due to CSF-1 and not due to an unknown secondary defect. In contrast to the restoration of a normal phenotype in *op* animals expressing the soluble CSF-1, exclusive expression of the membrane bound molecule was sufficient for a partial phenotypic rescue only (Dai *et al.*, 2004), although low levels of circulating CSF-1, shed from the membrane, could be detected (Ovadia *et al.*, 2006). Despite the restoration of tooth eruption, reproductive functions, and F4/80$^+$ tissue macrophages of some tissues, macrophages of other tissues failed to develop, and some residual osteopetrosis and abnormal hemopoietic parameters were found in these mice. The two models demonstrated that soluble CSF-1 is sufficient for a complete reversal of the phenotype, whereas the membrane-bound molecule enables partial recovery. Whether this deficiency is due to specific effects of the molecular forms or whether it is caused by limited availability at specific sites remains an open question.

Because osteopetrosis is a major phenotype in *op* mice, it could be hypothesized that osteoblast-derived CSF-1 might affect the bone disease. Indeed, forced expression of soluble CSF-1 in osteoblasts in transgenic *op* mice carrying the CSF-1 gene under the control of the osteocalcin promoter restored osteoclastogenesis and as a consequence resolved the osteopetrotic phenotype (Abboud *et al.*, 2002).

The analysis of these animal models supports the hypothesis that the different molecular forms of CSF-1 may indeed induce different responses in the target cells, be it in concert with other ligands and receptors (i.e., RANKL/RANK) or by inducing different signal transduction cascades in their respective target cells. The availability of the soluble cytokine at sufficient levels, however, seems to allow for virtually full function and for the mediation of the full array of biological effects.

REGULATION OF CSF-1 EXPRESSION

Different functions of the secreted and the membrane-bound forms of CSF-1 suggest differential regulation. Indeed, various research groups described hormones, e.g., PTH, 1,25(OH)$_2$ vitamin D$_3$, and dexamethasone, cytokines such as TNFα, or mechanical stimuli to stimulate expression either of both molecular forms of CSF-1 or of the membrane-bound form of cytokine only (Rubin *et al.*, 1996, 1997, 1998; Yao *et al.*, 1998; Saunders *et al.*, 2006). Because the transcripts encoding CSF-1 are derived from a common hnRNA precursor by differential splicing, the regulation of the expression of membrane-bound and secreted CSF-1 occurs at the post-transcriptional level. The mechanisms that allow for this regulation, however, are not well understood.

A considerable effort was devoted to the elucidation of the transcriptional control of CSF-1 expression. The promoter of the murine CSF-1 gene was found to contain consensus sequences for numerous transcription factors (Harrington *et al.*, 1991). *In vitro*, TNFα was most effective in regulating CSF-1 expression through activation of the NF-κB signaling pathway, in cells of the osteoblast lineage. The relevance of the members of the NF-κB family of signaling molecules in bone metabolism was evident when double knockout mice, deficient in the NF-κBs p50 and p52, expressed an osteopetrotic phenotype caused by the absence of osteoclasts (Iotsova *et al.*, 1997). The dependence of CSF-1 expression on NF-κB was also demonstrated with osteoblastic cells derived from mice deficient in p50. In contrast to cells from wild-type controls, CSF-1 levels were not increased in p50-deficient osteoblasts upon treatment with TNFα (Yao *et al.*, 2000). Somewhat contradictory, however, was the finding that the NF-κB consensus sequence of the CSF-1 promoter did not affect the stimulation of CSF-1 expression by TNFα (Isaacs *et al.*, 1999; Rubin *et al.*, 2000) or that TNFα may (Kimble *et al.*, 1996) or may not (Balga *et al.*, 2006) increase CSF-1 transcript levels in primary osteoblasts. A further transcription factor shown to take part in the transcriptional regulation of CSF-1 is AP-1 (Konicek *et al.*, 1998).

In conclusion, the expression of the cytokine in different cell types is governed by general and by cell-type-specific *trans*-acting factors, allowing for a tight temporal and spatial regulation. As mentioned, however, it is still not clear whether CSF-1 is involved in the acute regulation of bone resorption or whether the growth factor acts as a constitutive and essential component of the hemopoietic microenvironment. Support for the latter comes from studies in which IL1, IL3, IL6, IL7, IL10, GM-CSF, G-CSF, bFGF, and TGFβ did not effect CSF-1 expression by cultured human bone marrow stromal cells (Besse *et al.*, 2000).

CSF-1 IN POSTMENOPAUSAL OSTEOPOROSIS

Peripheral blood monocytes from osteoporotic women (Pacifici *et al.*, 1987) or from women after oophorectomy (Pacifici *et al.*, 1991) were found to secrete elevated levels of IL-1 and TNFα. An increase in the production of these cytokines, and their critical role in increased bone loss, was confirmed in various animal studies (Ammann *et al.*, 1997; Kimble *et al.*, 1997; Roggia *et al.*, 2001). CSF-1 was first implicated in bone loss after estrogen deficiency, when it was found that after ovariectomy, under the influence of IL1 and TNFα, a subpopulation of stromal cells developed that synthesized increased levels of CSF-1 (Kimble *et al.*, 1996). Further studies on the mechanism of action of IL1 and TNFα on the expression of CSF-1 demonstrated the involvement of the early response gene *egr-1*. Under estrogen deficiency, *Egr-1* becomes phosphorylated.

In its phosphorylated state, *Egr-1* cannot interact with the transcription factor SP-1, causing an excess of free SP-1 to stimulate CSF-1 production via the SP-1 site in the CSF-1 promoter (Srivastava *et al.,* 1998). Consequently, in mice deficient in *Egr-1,* bone turnover is high, but estrogen deficiency fails to stimulate bone resorption any further (Cenci *et al.,* 2000). Furthermore, evidence has been presented suggesting a differential expression of the molecular forms of CSF-1 under estrogen deprivation. In cultures of human bone marrow cells (Sarma *et al.,* 1998) and in ovariectomized rats (Lea *et al.,* 1999), the expression of the membrane-bound form of CSF-1 was increased under estrogen deficiency, an effect that was reversed by the treatment with the hormone.

CSF-1 AND OSTEOIMMUNOLOGY

In the recent past, the crosstalk between cells of the immune defense and bone has attracted increased interest, and the term *osteoimmunology* has been coined (Arron and Choi, 2000; Walsh *et al.,* 2006). Immunomodulatory cytokines affect both cells of bone and of the immune system in physiological and pathophysiological conditions, and bone active diseases such as postmenopausal osteoporosis, inflammatory joint diseases such as rheumatoid arthritis, or clinical complications such as loosening of orthopedic implants are caused by identical sets of cytokines and cells and thus are governed by similar mechanisms. Activated T cells were supporting osteoclast formation by secreting the essential growth factor RANKL *in vitro* (Horwood *et al.,* 1998) and *in vivo* (Kong *et al.,* 1999). Most interesting, osteoclastogenesis induced by T cells is counteracted by interferon-γ, another T-cell product (Takayanagi *et al.,* 2000). The T cell–bone connection, however, not only is important in inflammatory and immunologic situations, but has been shown to be relevant in estrogen deficiency as well. Thus, not only may cells of the monocyte/macrophage lineage mediate the effect of estrogen deficiency on bone by the production of TNFα and IL1, but T lymphocytes contribute to bone loss through the synthesis of TNFα as well (Cenci *et al.,* 2000). The critical role of T cells in this process, however, is somewhat controversial. Athymic nude mice (Cenci *et al.,* 2000) and *Rag1*$^{-/-}$ mice that are deficient in both T and B lymphocytes (Medina *et al.,* 2000) were demonstrated to be protected from bone wasting after OVX, whereas others reported a failure of protection in T cell-deficient mice (Lee *et al.,* 2006). In all these cases, CSF-1 is postulated to represent a component of the hemopoietic environment, expression levels being increased by TNFα (Kimble *et al.,* 1996) and by estrogen deficiency (Srivastava *et al.,* 1998), and thus contributing to the stimulation of osteoclast development and activation. Furthermore, levels of CSF-1, among other inflammatory cytokines, were suggested to be increased in conditions of aseptic loosening of orthopedic implants caused by wear particles or metal ions (Pioletti and Kottelat, 2004; Jost-Albrecht and Hofstetter, 2006).

THE ROLE OF CSF-1 IN THE FORMATION OF OSTEOCLASTS

The role of CSF-1 in the development of osteoclasts *in vivo* and in physiological bone resorption has been extensively investigated and was described in detail earlier. For the elucidation of the detailed mechanisms of CSF-1 action in osteoclastogenesis, investigations in cell and organ culture systems were most rewarding, and the dependence of osteoclast formation on CSF-1 was confirmed and could be further elucidated. In cultures of ^{45}Ca-prelabeled fetal metatarsals, CSF-1 stimulated osteoclastogenesis and the release of ^{45}Ca. This effect was blocked by irradiation of the bone rudiments, indicating an action of CSF-1 on proliferating osteoclast precursors. The cytokine was not found to exert an effect on bone resorption in cultured fetal radii, a system in which bone resorption depends on the activation of mature osteoclasts (Corboz *et al.,* 1992). These data suggested an effect of CSF-1 on recruitment of osteoclasts rather than on the activation of mature cells. In cultures of ^{45}Ca-prelabeled fetal metatarsals from *op/op* mice, CSF-1 alone exerted only a slight effect on the release of ^{45}Ca. Bone resorption was stimulated, however, when the rudiments were cultured in the presence of CSF-1 plus PTH and $1,25(OH)_2D_3$ (Morohashi *et al.,* 1994). In this system, CSF-1 was required but not sufficient for the formation of osteoclasts. Although it was not clear at that time, with present knowledge it can be concluded that the process of osteoclast formation is initiated by CSF-1 through the induction of the proliferation of osteoclast precursors, whereas PTH and $1,25(OH)_2D_3$ induce osteoblastic and stromal cells to express RANKL supporting the late steps of osteoclastogenesis.

In vitro, osteoclasts are formed in cocultures of osteoblasts or stromal cells and hemopoietic precursor cells in the presence of $1,25(OH)_2D_3$ (Takahashi *et al.,* 1988). The coculture system can be divided into an early proliferative phase and a late differentiation and fusion phase. Whereas $1,25(OH)_2D_3$ is essential only during the second phase of the culture, CSF-1 was found to be required during both the proliferation and differentiation/fusion phases (Tanaka *et al.,* 1993). Neutralizing antibodies against GM-CSF did not affect osteoclast formation. Precursor cells grown in the presence of GM-CSF or interleukin-3, however, are capable of forming osteoclasts in coculture with osteoblasts (Takahashi *et al.,* 1991; Lari *et al.,* 2007). These cytokines may therefore support proliferation of early precursors, but are not able to replace CSF-1 at later stages of development. Somewhat contradictory was the finding that fusion of osteoclast precursors in long-term bone marrow cultures is not blocked by anti-CSF-1 antibodies, demonstrating that this step in osteoclast formation does not depend on the cytokine (Biskobing *et al.,* 1995). The notion that the late stages of osteoclast formation and of bone resorption are independent of the presence of CSF-1 was supported by some *in vivo* data. To induce osteoclast formation in osteopetrotic *op* mice, a single injection of the growth factor proved to be sufficient (Kodama *et al.,* 1993). In these experiments, however, the actual temporal availability of the cytokine cannot be assessed accurately, and conclusions on the cellular differentiation stages affected by CSF-1 should be drawn carefully.

The actions of hemopoietic growth factors are not restricted to the differentiation and activation of the target cells, but they are required for survival as well. Accordingly, CSF-1 is no exception to the rule and provides support for survival of osteoclast lineage cells (Fuller *et al.,* 1993). Indeed, enforced expression of Bcl-2, the protein blocking programmed cell death, in *op* mice restored macrophage development and resolved the osteopetrotic phenotype (Lagasse and Weissman, 1997). In mature osteoclasts, CSF-1 was found to cause an increase in the intracellular pH under involvement of the electroneutral Na/HCO_3 cotransporter NBCn1, intracellular acidification frequently being linked to apoptosis (Bouyer *et al.,* 2007). Other investigators reported a concomitant decrease in bone resorptive activity and increase of osteoclast life spans upon prevention of acidification (Karsdal *et al.,* 2005).

Data similar to those obtained with the *op* mouse were obtained with the osteopetrotic rat *tl* mutant. Phenotypically, *tl* rats and *op* mice are similar; in both cases the osteopetrotic phenotype is caused by the virtual absence of osteoclasts. Because the *tl* rat was not cured by marrow transplantation, and based on the phenotypic characteristics, the osteopetrosis was proposed to be caused by a deficiency in CSF-1, and indeed a frame-shift mutation in the CSF-1 gene could be demonstrated in this strain (Van Wesenbeeck *et al.,* 2002). Upon injection of CSF-1 into *tl/tl* rats, osteoclastogenesis was induced and bone resorption was restored (Marks *et al.,* 1992). Furthermore, osteoclast formation was induced in neonatal metatarsals from *tl/tl* rats when cultured in the presence of CSF-1, PTH, and 1,25$(OH)_2D_3$ (Peura and Marks, 1995), as was described previously for the murine *op/op* mutant (Morohashi *et al.,* 1994).

EXPRESSION OF THE CSF-1 RECEPTOR IN BONE

Osteoclast precursors and mature osteoclasts are among the target cells for CSF-1, because these cells were shown to express transcripts encoding the receptor (Hofstetter *et al.,* 1992; Weir *et al.,* 1993), as well as to bind the cytokine (Hofstetter *et al.,* 1995). Expression of *c-Fms* is reliant on the transcription factor PU.1 (DeKoter *et al.,* 1998). PU.1 controls the development of granulocytes, macrophages, and B and T lymphocytes. Granulocytic precursors deficient in PU.1 cannot differentiate along the macrophage/monocyte lineages with CSF-1 because of the lack of *c-Fms* expression; hence the osteopetrotic phenotype in PU.1 null mice due to osteoclast deficiency (Tondravi *et al.,* 1997).

RESOLUTION OF THE OSTEOPETROTIC PHENOTYPE IN *op* MICE

Osteopetrotic *op/op* mice (Marks and Lane 1976; Wink *et al.,* 1991; Begg *et al.,* 1993) as well as mice deficient in *c-Fms* (Dai *et al.,* 2002) undergo an age-dependent hemopoietic recovery, the marrow cavity and cellularity being comparable to normal at 22 weeks of age. Because both mutant mice will not recover genetically during life, osteoclasts must develop via a pathway independent of CSF-1. However, based on the complex post-transcriptional processing of CSF-1 transcripts, an incomplete penetration of the phenotype, due to a leaky mutation causing a delay in normal development, could not be excluded (Hume and Favot, 1995). The recovery of the *c-Fms* null mice, however, renders this possibility rather unlikely. If, on the other hand, CSF-1 would be partially redundant, a candidate growth factor to replace CSF-1 may be GM-CSF. Injections of GM-CSF alone did not reverse the osteopetrotic phenotype in *op/op* animals (Wiktor-Jedrzejczak *et al.,* 1994), but in combination with IL-3, GM-CSF corrected the osteopetrotic phenotype (Myint *et al.,* 1999). Furthermore, bone resorption and hemopoietic recovery proceeded with the same kinetics in *op/op* mice and in GM-CSF/CSF-1 double-knockout animals (Nilsson *et al.,* 1995), negating an essential role of GM-CSF in the recovery process, and nonadherent osteoclast precursors that were grown in the presence of GM-CSF could differentiate into osteoclasts only when the cells were subsequently cultured in the presence of CSF-1 and RANKL (Lari *et al.,* 2007). Thus, the formation of osteoclasts and the reconstitution of bone resorption in growing *op/op* animals require proliferation, differentiation, and activation of macrophages and osteoclasts by alternative pathways, independent of CSF-1 and GM-CSF.

A step toward the elucidation of the mechanisms leading to the resolution of the osteopetrotic phenotype in *op/op* mice was achieved when a single injection of vascular endothelial growth factor (VEGF) was found to resolve the osteopetrotic phenotype (Niida *et al.,* 1999). The authors suggest that VEGF is produced in *op/op* mice at levels sufficient for the survival and functioning of mature osteoclasts, but not for the recruitment of these cells at maximal levels, resulting in an osteopetrotic phenotype that is resolved with time. Consequently, mice with an *op* background containing a tyrosine-kinase defective VEGFR-1 (Flt-1) fail to recover, the osteopetrosis persists, and the animals are characterized by fibrotic bone marrow and extramedullary hemopoiesis (Niida *et al.,* 2005). The potential role of VEGF in the regulation of bone resorption was further strengthened by the finding that the factor acts chemotactically on osteoclasts and their precursors (Engsig *et al.,* 2000). Because VEGF is synthesized by hypertrophic chondrocytes (Gerber *et al.,* 1999), is integrated in the extracellular matrix of bone and cartilage, and can be released by metalloproteinases (Bergers *et al.,* 2000), location, availability, and release of the factor seem to be suited to act as a growth factor for the cells of the osteoclastic lineage.

EFFECTS OF CSF-1 ON MATURE OSTEOCLASTS AND SIGNAL TRANSDUCTION

Upon ligand binding, the tyrosine kinase receptor *c-Fms* dimerizes and autophosphorylates on specific tyrosine residues, which act as binding sites for SH2 and PTB domain-containing proteins that start specific signaling cascades (Hamilton, 1997). Subsequently, the receptor becomes internalized, dephosphorylated, multiubiquinylated, and degraded (Lee *et al.,* 1999). Depending on the differentiation and cell cycle states of the target cells, the CSF-1 dependent response may differ and may result in cell proliferation, survival, differentiation and cytoskeletal reorganization (Ross and Teitelbaum, 2005).

The effects exerted by CSF-1 on macrophages have been extensively studied, as have the mechanisms involved in signal transduction in these cells (Feng *et al.,* 2002). Ligand activation of the receptor kinase serves as the proximal signal that eventually elicits the mitogenic response. CSF-1 is required by mononuclear cells during the G1 phase of the cell cycle to enter the S phase, but the growth factor is not necessary during S, G2, and M phases. Withdrawal of the growth factor induces the cells to enter a quiescent state and leads to cell death. In osteoclasts, CSF-1 induces, as in macrophages, cell spreading. The cytokine increases the migration of osteoclasts, in this way decreasing the number of resorbing cells and as a consequence decreasing total resorptive activity (Fuller *et al.,* 1993). In addition to inducing migration of osteoclasts, CSF-1 induces the fusion of mature cells (Amano *et al.,* 1998). In summary, CSF-1 supports proliferation, differentiation, fusion, and survival (Karsdal *et al.,* 2005) of progenitors and mature osteoclasts. The cytokine, however, does not activate the bone-resorptive activity of the mature cells (Jimi *et al.,* 1999).

As mentioned above, binding of CSF-1 induces cell spreading both in macrophages and in osteoclasts. This effect is brought about by cytoskeletal rearrangements, involving c-*Src*-dependent tyrosine phosphorylation (Insogna *et al.,* 1997) and translocation of phosphatidylinositol-3-kinase (PI-3 K) from the cytoplasm to the cell membrane (Grey *et al.,* 2000; Palacio and Felix, 2001; Golden and Insogna, 2004). Although the cell spreading can be blocked by the specific PI-3 K inhibitor wortmannin (Amano *et al.,* 1998), deficiency in the monocyte-specific PI-3 K isoform PI-3Kβ does not affect the bone phenotype (Hirsch *et al.,* 2000). In c-*Src*-deficient mice, which are characterized by an osteopetrotic phenotype due to an osteoclastic failure to form a ruffled border (Boyce *et al.,* 1992), the autophosphorylation of *c-Fms* is not affected, whereas the phosphorylation of an as yet unidentified protein of 85 to 90 kDa is significantly reduced, suggesting an abnormal phosphorylation of downstream targets (Insogna *et al.,* 1997).

Other signal transduction pathways induced by CSF-1 involve *Cbl* (Lee *et al.,* 1999; Ota *et al.,* 2000), which is important for degradation of c-*Fms*. Furthermore, mitogen-activated protein kinases and phosphatases (Valledor *et al.,* 1999; Lee and States, 2000) are activated, finally leading to regulation of cell cycle progression by cyclins (Dey *et al.,* 2000).

The profound effects of CSF-1 on cytoskeletal rearrangement, which is required for motility, fusion, and cellular activity in osteoclasts, drew the attention to the combined actions of CSF-1 with integrin receptors binding to components of the extracellular matrix. The vitronectin receptor, $\alpha_v\beta_3$ integrin (Athanasou *et al.,* 1990), not only serves as an attachment site, but the integrin's capability to transmit matrix-derived signals to the cell interior is equally important. The failures in cytoskeletal rearrangements in mice deficient in *c-Src* (Soriano *et al.,* 1991), the non-receptor tyrosine kinase *Syk* (Mocsai *et al.,* 2004), or *Vav3* (Faccio *et al.,* 2005), a member of the family of guanine nucleotide exchange factors, result from impaired signaling through *c-Fms* and $\alpha_v\beta_3$ integrin.

This short summary of signal transduction pathways induced by CSF-1 upon binding to c-*Fms* indicates the complexity of signaling that is required to enable the multiple roles the growth factor exerts on the cells of the osteoclast lineage.

CSF-1 IN TUMOR METASTASIS

Certain cancers such as breast and prostate cancer metastasize preferentially to bone (Mundy, 1997). The response in bone toward the two primary cancers differs dramatically, prostate cancer generally inducing an osteoblast response, resulting in osteosclerotic lesions, whereas mammary cancer triggers preferentially an osteoclastic response with the consequence of osteolytic lesions. In mouse models of tumor metastasis, osteolytic tumors were found to express PTHrP (PTH-related protein) and CSF-1, both potent stimulators of bone resorption (van der Pluijm *et al.,* 2001). These studies were corroborated by the finding that only cell lines inducing osteolytic lesions in bone express osteoclastogenic cytokines such as PTHrP, CSF-1, and IL-8 (Schwaninger *et al.,* 2007). The data suggest an ability of metastasizing tumor cell lines to generate their own niche in bone, CSF-1 being one of the factors stimulating the accompanying bone resorption and contributing to the development of a suitable microenvironment for the tumors.

CONCLUDING REMARKS

From the original description of CSF-1 as an essential growth factor for osteoclast-lineage cells to the present state of knowledge, a huge body of information has been accumulated, describing many detailed aspects of the function and the mechanisms of the growth factor. CSF-1 and RANKL are the only growth factors that under physiological conditions are indispensable for functional osteoclastogenesis. Deficiency in these factors or their receptors leads to osteopetrosis due to the absence of osteoclasts. The dependence of osteoclast development on CSF-1, however, is not absolute, because an age-dependent resolution of the osteopetrotic phenotype was observed, and VEGF was found to restore osteoclastogenesis in CSF-1 deficient animals. In recent years our understanding on the complex processes regulating development and activation of osteoclasts has become more complete. In particular, the analysis of the interactions of signaling pathways in osteoclasts allowed for a dissection of the various effects exerted by the secreted and membrane-bound forms of CSF-1 on the respective target cells. Also, the understanding of the functional proximity of bone and the innate and specific immune systems opened up the exciting field of osteoimmunology, CSF-1 being a relevant player on this area.

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Chapter 57

Local Regulators of Bone

IL-1, TNF, Lymphotoxin, Interferon-γ, the LIF/IL-6 Family, and Additional Cytokines

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The bone remodeling cycle is highly regulated by a variety of factors (i.e., hormones, cytokines, growth factors, and physical force). These are produced both locally and systemically and act in concert to direct local rates of bone turnover. Many studies of the mechanisms regulating bone remodeling have concentrated on the role of cytokines because these factors appear to have crucial roles in both normal and pathological bone cell function. A variety of cytokines that were originally identified by their ability to regulate immune and hematopoietic cells are believed to be produced in the bone microenvironment either spontaneously or in response to specific stimuli. It has become apparent that during both health and disease, the production of cytokines by cells in the bone microenvironment and the responses of bone cells to these cytokines are regulated in a highly ordered manner. It is hypothesized that the spectrum of cytokines that are produced in bone defines the responses of bone cells to a particular state and predicts the subsequent development of normal or pathological bone remodeling. Diseases of bone where cytokines are believed to play an important role include osteoporosis, Paget's disease, periodontal disease, and the effects of malignancy on bone. Studies of the production of cytokines in bone and the responses of bone cells to these cytokines provide insights into the mechanisms that regulate the development of these diseases and could lead to new therapies for these conditions. The following is a broad overview of the actions of a number of cytokines on bone and the mechanisms by which bone cells respond to these cytokines.

EFFECTS OF INTERLEUKIN-1 ON BONE

Interleukin-1 (IL-1) is a multifunctional cytokine with a wide variety of activities. It is a family of two active peptides (IL-1α and IL-1β) that are encoded by two separate gene products. Both forms of IL-1 have identical activities and potencies (Dinarello, 1991). IL-1 is the first polypeptide mediator of immune cell function that was shown to regulate bone resorption (Gowen *et al.*, 1983; Lorenzo *et al.*, 1987) and formation (Canalis, 1986), and it is the major activity that had been identified as osteoclast-activating factor (OAF) (Dewhirst *et al.*, 1985). IL-1 is the most potent stimulator of bone resorption yet identified (Lorenzo *et al.*, 1987). It also increases prostaglandin synthesis in bone (Lorenzo *et al.*, 1987), an effect that may account for some of its resorptive activity because prostaglandins themselves are potent resorption stimuli (Klein and Raisz, 1970). The ability of IL-1 to stimulate bone resorption *in vivo* and *in vitro* requires that inducible nitric oxide synthase be present (Van't Hof *et al.*, 2000). Like a variety of other resorption stimuli, IL-1 increases receptor activator of nuclear factor κB ligand (RANKL) production in stromal/osteoblastic cells (Hofbauer *et al.*, 2001) by a mechanism that may require STAT3 activation (O'Brien *et al.* 1999). It also paradoxically stimulates osteoprotegerin (OPG) production in a human osteosarcoma cell line (Vidal *et al.*, 1998) by a mechanism that depends on activation of p38 and ERK MAP kinases (Lambert *et al.*, 2007). However, it is reported to decrease OPG production in primary rat osteoblasts (Tanabe *et al.*, 2005). IL-1 directly enhances the resorptive activity of mature osteoclasts (Jimi *et al.*, 1999) by a mechanism that may involve activation of nuclear factor κB (NF-κB) (Miyazaki *et al.*, 2000).

Direct stimulation of osteoclastogenesis by IL-1 in mixed murine stromal and hematopoietic cell cultures

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depends on RANKL expression in the stromal/osteoblastic cells, but not tumor necrosis factor (Ma *et al.*, 2004). Expression of myeloid differentiation factor 88 (MyD88), but not Toll/interleukin-1 receptor domain-containing adaptor inducing interferon-beta (TRIF) was necessary for IL-1 to stimulate RANKL production in osteoblasts and prolong the survival of osteoclasts (Sato *et al.*, 2004). Survival of osteoclasts by treatment with IL-1 appears to require PI3-kinase/AKT and ERK (Lee *et al.*, 2002). IL-1 receptor-associated kinase M (IRAK-M) is an inhibitor of IL-1 signaling, which, if deleted, leads to enhanced osteoclastogenic activity (LI *et al.*, 2005).

IL-1 stimulates osteoclast-like multinucleated giant-cell formation in cultured bone marrow through a prostaglandin-dependent mechanism (Akatsu *et al.*, 1991). Hence, IL-1 may be involved in the differentiation of osteoclasts from hematopoietic progenitor cells, and this appears to be an additional mechanism by which it regulates bone resorption. IL-1 is a potent bone resorption stimulus *in vivo* (Sabatini *et al.*, 1988). Its effects on bone formation appear to be mainly inhibitory (Canalis, 1986), although it does stimulate DNA synthesis in both bone organ cultures and primary cultures of human bone cells (Canalis, 1986; Gowen *et al.*, 1985).

IL-1 is produced by bone organ cultures. However, the cells responsible for this production are not clearly identified (Lorenzo *et al.*, 1990b). It appears that both hematopoietic and mesenchymal/osteoblastic cells can produce IL-1 and its production is enhanced when both cell types are cocultured together (Haynes *et al.*, 1999). Macrophages are a likely source of IL-1 in bone marrow (Horowitz *et al.*, 1989), and osteoblast-like cells from human adult bone can also produce IL-1β *in vitro* (Keeting *et al.*, 1991).

A natural inhibitor to IL-1 has been identified (Arend *et al.*, 1990). This peptide, IL-1 receptor antagonist (IL-1ra), is an analogue of IL-1 that binds but does not activate IL-1 receptors. IL-1ra blocks the ability of IL-1 to stimulate resorption and prostaglandin E2 (PGE_2) production in bone organ cultures (Seckinger *et al.*, 1990). Increased release of IL-1ra has recently been shown in the conditioned medium of cultured peripheral blood monocytes (PBMs) from either normal or osteoporotic postmenopausal women when levels are compared with cultures of PBM from premenopausal women or postmenopausal women who are treated with estrogen (Pacifici *et al.*, 1993).

There are two known receptors for IL-1, type I and type II (Dinarello, 1993a, b). All known biological responses to IL-1 appear to be mediated exclusively through the type I receptor (Sims *et al.*, 1993). Postreceptor signaling through the type I receptor involves sphingomyelin breakdown and production of ceramide (Kolesnick and Golde, 1994) in addition to activation of NF-κB (Jimi *et al.*, 1996). The type II IL-1 receptor appears to have little or no agonist activity, rather it functions as a decoy receptor that prevents the activation of IL-1 type I receptor (Colotta *et al.*, 1993). In addition, type II IL-1 receptor can be released and circulate in serum as a soluble binding protein that inhibits IL-1 interactions with the type I receptor (Dinarello, 1993a, b). Type II IL-1 receptor may also synergize with IL-1ra to inhibit activation of the type I IL-1 receptor by IL-1 (Burger *et al.*, 1995). One recent report found a decrease in the bone mass of mice that were deficient in the bioactive type I IL-1 receptor (Bajayo *et al.*, 2005); however, this has not been our experience (Vargas *et al.*, 1996).

Production of IL-1 appears to be involved in the development of osteoporosis. Increased IL-1 bioactivity has been found in the conditioned medium of PBM from some patients with a high-turnover form of this disease (Pacifici *et al.*, 1987). One group has found that *in vivo* estrogen treatment reduced the amount of IL-1 that was released from cultured PBM (Pacifici *et al.*, 1989). However, not all studies have confirmed this finding (Hustmeyer *et al.*, 1993; Stock *et al.*, 1989). In a related study, greater bone-resorbing activity was found in lipopolysaccharide (LPS)-stimulated, PBM-conditioned medium from ovariectomized women than in PBM-conditioned medium from premenopausal or estrogen-treated postmenopausal women (Cohen-Solal *et al.*, 1993). Neutralization studies showed that this activity results from both IL-1 and tumor necrosis factor (TNF)-α production *in vitro*. Measurements of IL-1 in the serum of pre- and postmenopausal women have produced conflicting results. One study showed that IL-1 levels were increased 30 days after ovariectomy (Fiore *et al.*, 1994), but others failed to find a correlation between serum IL-1α, IL-1β, or IL-1ra levels and indices of bone turnover in either pre- or postmenopausal women (McKane *et al.*, 1994) or between osteoporotic women and normal subjects (Khosla *et al.*, 1994). Because modulation of cytokine production and/or responses in the bone marrow microenvironment may be the key mechanism by which estrogens modulate bone cell function, recent studies have focused on the ability of estrogen withdrawal to regulate IL-1 production in bone marrow. Increased production of a number of cytokines including IL-1α has also been identified in the conditioned medium of bone marrow cultures from postmenopausal women who had discontinued estrogen replacement within 1 month compared with similar studies of premenopausal controls (Bismar *et al.*, 1995). Two groups have demonstrated in mice that IL-1α biological activity in bone marrow serum increases after ovariectomy but levels of IL-1α protein do not (Miyaura *et al.*, 1995; Kawaguchi *et al.*, 1995).

In vivo administration of IL-1ra inhibited the bone loss that occurred in ovariectomized rats (Kimble *et al.*, 1994). This effect was most pronounced after 4 weeks and was much less at earlier times. A trend demonstrating similar effects in the excretion of urinary type 1 collagen breakdown products (a measure of bone resorption) was also seen in postmenopausal women who were withdrawn from estrogen therapy and administered IL-1α

(Charatcharoenwitthaya *et al.*, 2007). In addition, treatment of mice for 2 weeks after ovariectomy with IL-1ra decreased the ability of marrow cell cultures to form osteoclasts *in vitro* and inhibited the excretion of pyridinoline cross-links (a marker of *in vivo* bone resorption) (Kitazawa *et al.*, 1994).

In humans polymorphisms in the gene for IL-1α were not associated with an increased risk of fractures (Knudsen *et al.*, 2007). However, a polymorphism in the IL-1β gene was associated with low bone mass (Moreno *et al.*, 2005) by one group. Interestingly, other groups did not find an association of bone mass with polymorphisms in the IL-1β gene but did with the gene for IL-1ra (Kim *et al.*, 2006; Langdahl *et al.*, 2000).

IL-1 has also been implicated as one mediator of the hypercalcemia that accompanies some forms of cancer. Production of IL-1 occurs in myeloma cells that are cultured *in vitro* (Carter *et al.*, 1990) and correlates with their ability to stimulate bone resorption and hypercalcemia *in vivo*. In addition, the uncoupling of bone resorption and bone formation, which is characteristic of the lytic bone lesions in myeloma, is reproduced by local *in vivo* infusions of IL-1 (Boyce *et al.*, 1989).

EFFECTS OF TUMOR NECROSIS FACTOR ON BONE

Like IL-1, TNF is a family of two related polypeptides (α and β) that are products of separate genes (Beutler and Cerami, 1986; Paul and Ruddle, 1988). TNF-α and -β have similar biological activities and are both potent stimulators of bone resorption (Bertolini *et al.*, 1986; Lorenzo *et al.*, 1987) and inhibitors of bone collagen synthesis (Bertolini *et al.*, 1986; Canalis, 1987).

As with IL-1, TNF binds to two cell surface receptors, the TNF-receptor 1 or p55, and the TNF-receptor 2 or p75 (Fiers, 1993). In contrast to IL-1, both receptors transmit biological responses. There appear to be interactions between the TNF-receptor 1 and TNF-receptor 2 (Tartaglia *et al.*, 1993), and for many responses, activation of both receptors is necessary to produce a full biological effect (Vandenabeele *et al.*, 1995). However, some effects can be induced by selective activation of either receptor (Sheehan *et al.*, 1995). Mice deficient in the TNF-receptor 1 and TNF-receptor 2 have been made (Rothe *et al.*, 1993; Erickson *et al.*, 1994). These animals appear healthy and breed normally, but lack normal immune responses and apoptotic mechanisms.

In vivo, TNF-α injections increased the serum calcium of mice (Tashjian *et al.*, 1987), and similar effects were seen with TNF-β (Garrett *et al.*, 1987). In a more detailed study, Chinese hamster ovary (CHO) cells that were genetically engineered to release large amounts of active TNF-α peptide were injected into nude mice (Johnson *et al.*, 1989). These animals became hypercalcemic within 2 weeks. Bone histomorphometry demonstrated a 10-fold increase in the number of osteoclasts in their bones compared with controls (animals injected with CHO cells that contained an empty vector). In addition, the percentage of the bone surface undergoing active resorption was similarly increased in animals receiving the TNF-producing cells.

The effects of TNF on resorption appear to be mediated by its effects on osteoclasts because osteoclast number increased after TNF treatment of bones (Johnson *et al.*, 1989) and because resorption stimulated by TNF was inhibited by calcitonin (Stashenko *et al.*, 1987). Like IL-1, TNF-stimulated induction of osteoclast-like cells formation in bone marrow culture (Pfeilschifter *et al.*, 1989) is mediated by increases in RANKL expression (Hofbauer *et al.*, 1999). However, in addition to increasing RANKL expression TNF also stimulates OPG in osteoblastic cell models (Hofbauer *et al.*, 1998).

TNF, either alone or in combination with IL-1 and transforming growth factor (TGF)-β, was shown by a variety of investigators to directly stimulate osteoclast formation in an *in vitro* culture system. This response appears to be direct and independent of RANK, because, in combination with IL-1 and TGF-β, it occurred in cells from RANK-deficient mice (Azuma *et al.*, 2000; Kim *et al.*, 2005; Kobayashi *et al.*, 2000). However, the significance of these *in vitro* findings is questionable because administration of TNF to RANK-deficient mice produced only an occasional osteoclast in one study and no osteoclasts in another (Li *et al.*, 2000, 2004). The ability of TNF to stimulate osteoclast formation in mixed stromal cell/osteoclast precursor cell cultures was dependent on the production of IL-1 (Wei *et al.*, 2005) as is the ability of TNF to induce joint damage in mice (Zwerina *et al.*, 2007). TNF-induced osteolysis in induced arthritic lesions in mice was also found to be dependent on macrophage colony-stimulating factor (M-CSF) production (Kitaura *et al.*, 2005). CD44 also appears involved in TNF-mediated arthritis in mice because arthritis produced by TNF was more severe in CD44-deficient mice (Hayer *et al.*, 2005).

TNF inhibits bisphosphonate-induced osteoclast apoptosis by upregulating the antiapoptotic molecules Ets-2 and Bcl-xL in osteoclasts (Zhang *et al.*, 2005). It also directly affects putative osteoclast precursor cells. It increased expression of the osteoclastogenic cofactor paired Ig-like receptor A (PIR-A), which enhances responses of these cells to RANKL (Ochi *et al.*, 2007) and it increases the circulating pool of CD11b$^{(high)}$ peripheral blood cells that can differentiate into osteoclasts (Li *et al.*, 2004). The latter effect appears to depend on upregulation of c-FMS expression, which is the receptor for M-CSF (Yao *et al.*, 2006).

The synergistic effects of TNF on RANKL-stimulated osteoclastogenesis are mediated by activation of the TNF-receptor 1 (p55) (Zhang *et al.*, 2000). TNF-receptor 1 also mediates the ability of TNF to stimulate RANKL expression in stromal cells (Abu-Amer *et al.*, 2004). RANKL, in turn, can induce TNF production in the osteoclast precursor cell model, RAW 264.7 and primary cultures, through its ability to enhance the transcriptional activity of the TNF gene (Nakao *et al.*, 2007; Zou *et al.*, 2005). TNF-induced osteoclastogenesis is associated with induction of the mitogen-activated protein kinases, p38, ERK, and JNK, as well as p21 (WAF1/Cip1), an inhibitor of cyclin-dependent kinase (Kwak *et al.*, 2005).

In bone organ cultures, TNF stimulates DNA synthesis (Canalis, 1987). However, in an osteoblast-like osteosarcoma cell line, ROS 17/2.8, TNF did not stimulate DNA synthesis but did inhibit collagen synthesis (Nanes *et al.*, 1989). Furthermore, addition of hydroxyurea, an inhibitor of DNA synthesis, to primary rat osteoblast-enriched cultures did not alter the inhibitory effects of TNF on collagen synthesis (Centrella *et al.*, 1988). Hence, the effects that TNF has on cell replication do not appear to be linked to its effects on collagen synthesis. *In vitro*, TNF directly inhibits the differentiation of osteoblast precursor cells into mature osteoblasts (Gilbert *et al.*, 2000). This effect appears to involve production of nitric oxide and peroxynitrite (Hikiji *et al.*, 1997, 2000). Osteoblast apoptosis (programmed cell death) can also be stimulated by TNF (Jilka *et al.*, 1998) and this response also may be mediated by nitric oxide production (Damoulis and Hauschka, 1997). TNF-receptor 1 mediates the ability of TNF to inhibit osteoblast differentiation (Gilbert *et al.*, 2005) and this effect depends, at least in part, on the ability of TNF to activate SAPK/JNK signaling (Mukai *et al.*, 2007).

In contrast to the inhibitory effects that continuous treatment with TNF has on collagen synthesis in bone, transient treatment with TNF for 24 hours causes a rebound increase in collagen synthesis in primary cultures of osteoblast-like cells from rat calvaria (Centrella *et al.*, 1988). Post-transcriptional regulation of message may also be involved in the effects that TNF has on collagen synthesis (Centrella *et al.*, 1988). TNF-α is produced by human osteoblast-like cell cultures (Gowen *et al.*, 1990) and its production is stimulated by IL-1, granulocyte-macrophage colony-stimulating factor (GM-CSF), and lipopolysaccharide but not by parathyroid hormone (PTH), 1,25(OH)$_2$ vitamin D$_3$, or calcitonin. Like RANKL, effects of TNF on osteoblastic cells are mediated by stimulation of NF-κB activity (Ali *et al.*, 1999; Yao *et al.*, 2000).

TNF production in bone may be involved in the development of postmenopausal osteoporosis. Estrogens are reported to modulate TNF production *in vitro* in human osteoblast cultures by one group (Rickard *et al.*, 1992). However, another group failed to find an effect of estrogen treatment on TNF protein production in human osteoblast-like cell cultures (Chaudhary *et al.*, 1992). Spontaneous production of TNF-α in cultured peripheral monocytes from women who had recently undergone ovariectomy was increased compared with levels from cells that were assayed preovariectomy (Pacifici *et al.*, 1991). Bone-resorbing activity in LPS-stimulated PBM-conditioned medium (CM) is increased in cultures from postmenopausal women when compared with CM from premenopausal or postmenopausal women that were treated with estrogen and this effect relies on TNF in the CM to some degree (Cohen-Solal *et al.*, 1993). Kimble *et al.* (1994) found that, like the effect of *in vivo* administration of IL-1ra, treatment of mice with soluble TNF receptor, an inhibitor of TNF action, reduced the ability of ovariectomy to decrease bone mass (Kimble *et al.*, 1995a, b). Interestingly, the most potent inhibition of the effects of estrogen withdrawal on bone mass was seen in rats that were treated with both IL-1ra and soluble TNF receptor. In a related study, it was demonstrated that mice overexpressing soluble TNF receptor 1, which binds TNF and inhibits its action, did not have increased bone loss after ovariectomy (Ammann *et al.*, 1995). Most recently it was shown that administration of soluble type 1 TNF receptor (an inhibitor of TNF action) significantly reduced the increase in urinary type 1 collagen breakdown products (a measure of bone resorption) that occurred in postmenopausal women who were withdrawn from estrogen therapy (Charatcharoenwitthaya *et al.*, 2007).

Recently, mice lacking mature T lymphocytes were found to not lose bone mass after ovariectomy and this effect appeared to be related to T lymphocyte-mediated production of TNF (Cenci *et al.*, 2000). However, this hypothesis is controversial because similar experiments using nude rats (Sass *et al.*, 1997) and nude, RAG2- or TCR-α-deficient mice (all of which lack functional T lymphocytes) demonstrated that OVX-induced trabecular bone loss in these models was equivalent to that seen in wild-type mice (Lee *et al.*, 2006). Curiously, loss of cortical bone with OVX was different between T-cell-deficient and wild-type models and dependent on the bone that was examined (Lee *et al.*, 2006). These results suggest that there may be compartmental and bone-specific effects of T-cell depletion on OVX-induced bone loss. Additional experiments will be required to determine how T cells are involved in this response of bone.

Estrogen can also directly suppress transcriptional activity of the TNF gene (Srivastava *et al.*, 1999). Increased follicle-stimulating hormone (FSH) production, which occurs after sex steroid withdrawal, can also stimulate TNF-α production in immune cells, which may enhance the ability of sex steroids withdrawal during the menopause to cause osteoporosis (Iqbal *et al.*, 2006).

Production or response to TNF has been linked to a number of human diseases. Polymorphisms in the gene for TNF-receptor 2 (p75) was associated with differences in bone density in women and may represent a risk factor for the development of osteoporosis (Mullin *et al.*, 2007; Tasker *et al.*, 2004). Production of TNF and RANKL by mononuclear cells at sites of failing hip implants was positively associated with bone loss, suggesting that these events are related (Holding *et al.*, 2006). Cherubism in children results from a gain-of-function mutation in the SH3BP2 gene, which, when expressed in a mouse model, produces inflammation, increased bone turnover, very large osteoclasts, and macrophages that express high levels of TNF-α (Novack *et al.*, 2007; Ueki *et al.*, 2007).

TNF-β has been implicated as one cause of the effects that hematological malignancies have on bone (Garrett *et al.*, 1987). A tumor cell line was established from a patient with multiple myeloma who had developed hypercalcemia and osteolytic bone lesions. This cell line was found to contain detectable steady-state levels of TNF-β mRNA and to produce TNF-β *in vitro*. Furthermore, an antibody to TNF-β blocked some but not all of the *in vitro* bone-resorbing activity that was present in the conditioned medium from these cells and from other established myeloma cell lines. Like IL-1, TNF stimulates bone resorption while inhibiting bone formation and mimics the *in vivo* responses of bone to hematological malignancy (Bertolini *et al.*, 1986).

TNF has also been linked to the resorptive effects that nonhematological malignancies have on bone. In one report, a squamous carcinoma cell line was shown to release a factor that stimulated monocyte cultures to produce TNF-α. This tumor did not itself produce TNF. However, implantation of the tumor into nude mice stimulated bone resorption, hypercalcemia, and leukocytosis that were reversed by a specific anti-TNF-α antibody (Yoneda *et al.*, 1991).

EFFECTS OF INTERFERONS ON BONE

Interferons are generally divided into two types. Type I interferons include interferon-α and -β (IFN-α and IFN-β). Interferon-γ (IFN-γ) is the only type II interferon. All interferons are regulatory cytokines with a wide variety of biological activities.

INF-α and INF-β are typically produced in response to invading pathogens (Takayanagi *et al.*, 2005). Mice deficient in the INF α/β receptor component IFNAR1 have a reduction in trabecular bone mass and an increase in osteoclasts (Takayanagi *et al.*, 2002). RANKL induces INF-β in osteoclasts, and INF-β, in turn, inhibits RANKL-mediated osteoclastogenesis by decreasing c-fos expression (Takayanagi *et al.*, 2002). INF-α has also been shown to inhibit bone resorption although its mechanism of action is not as well studied as that of INF-γ and -β (Avnet *et al.*, 2007). *In vivo*, INF-α had no effect on bone turnover (Goodman *et al.*, 1999).

Activated T lymphocytes and NK cells typically produce IFN-γ. *In vitro* it inhibits resorption (Gowen and Mundy, 1986; Peterlik *et al.*, 1985). This effect appears to be more specific for the response to IL-1 and TNF because lower concentrations of IFN-γ inhibit the maximum activity of these factors compared with resorption stimulated by PTH or 1,25(OH)$_2$ vitamin D$_3$ (Gowen *et al.*, 1986). The actions of IFN-γ on *in vitro* resorption appear to be mediated by its effects on osteoclast progenitor cells. IFN-γ inhibits the ability of 1,25(OH)$_2$ vitamin D$_3$, PTH, IL-1, and RANKL to stimulate the formation of osteoclast-like cells in cultures of human bone marrow (Takahashi *et al.*, 1986; Fox and Chambers, 2000). This effect is mediated at least in part by the ability of IFN to stimulate degradation of TRAF-6 (Takayanagi *et al.*, 2000), an intermediate in RANK signaling (Wong *et al.*, 1999). INF-γ is also reported to have stimulatory effects on resorption through its ability to stimulate RANKL and TNF-α production in T lymphocytes (Gao *et al.*, 2007).

IFN-γ is an inhibitor of other hematopoietic functions including erythropoiesis (Mamus *et al.*, 1985). However, it does not affect the resorptive activity of mature osteoclasts (Hattersley *et al.*, 1988). IFN-γ can synergistically augment IL-1- or TNF-stimulated nitric oxide (NO) production by cultured osteoblasts (Ralston *et al.*, 1995). NO appears to be a biphasic regulator of osteoclast-mediated bone resorption that stimulates at low concentrations and inhibits at high concentrations (Ralston *et al.*, 1995). It is possible that part of the inhibitory effects of IFN-γ on IL-1- and TNF-mediated resorption results from effects on NO synthesis. IFN-γ together with IL-1 or TNF synergistically stimulates NO production to high levels in bone, which inhibits osteoclast-mediated resorption by inducing apoptosis of osteoclast progenitors and inhibiting osteoclast activity (Van't Hof and Ralston, 1997).

The *in vitro* effects of IFN-γ on collagen synthesis are also inhibitory. In bone organ culture, IFN-γ decreases both collagen and noncollagen protein synthesis (Smith *et al.*, 1987). This effect was not dependent on prostaglandin synthesis (Smith *et al.*, 1987). The effects of IFN-γ on collagen synthesis in the osteoblast-like ROS 17/2.8 cell line are similar to those seen in organ cultures (Nanes *et al.*, 1989).

IFN-γ also inhibits DNA synthesis in bone organ cultures (Smith *et al.*, 1987), in human cultured bone cells with osteoblast characteristics, and in the ROS 17/2.8 cells (Nanes *et al.*, 1989). It also inhibits the stimulatory effects that TNF and IL-1 have on cell replication in bone cultures (Smith *et al.*, 1987) and that TNF has in human osteoblast-like cells (Gowen *et al.*, 1988) and the ROS 17/2.8 osteosarcoma cell line (Nanes *et al.*, 1989). Effects of IFN-γ on osteoblast function appear to involve nitric oxide production (MacPherson *et al.*, 1999).

The effects of IFN-γ on bone *in vivo* are markedly different from its actions *in vitro*. In rats, i.p. injection of IFN-γ for 8 days induced osteopenia (Mann *et al.*, 1994). In patients who have osteopetrosis and produce defective osteoclasts, administration of IFN-γ stimulates bone resorption and appears to partially reverse the pathology of the disease. These effects are possibly because of the ability of IFN-γ to stimulate osteoclast superoxide synthesis (Key *et al.*, 1992, 1995) or osteoclast formation *in vivo* (Vignery *et al.*, 1990).

ADDITIONAL CYTOKINES THAT ARE PRODUCED IN BONE OR HAVE EFFECTS ON BONE CELL FUNCTION

Interleukin-7

IL-7 is a cytokine that has diverse effects on the hematopoietic and immunological systems and is best known for its nonredundant role in supporting B and T lymphopoiesis. Studies have demonstrated that IL-7 also plays an important role in the regulation of bone homeostasis (Miyaura *et al.*, 1997; Weitzmann *et al.*, 2002); however, the precise nature of how IL-7 affects osteoclasts and osteoblasts is controversial, because it has a variety of actions in different target cells. Systemic administration of IL-7 upregulated osteoclast formation in human peripheral blood cells by increasing osteoclastogenic cytokine production in T cells (Weitzmann *et al.*, 2000), and IL-7 did not induce bone resorption and bone loss in T-cell-deficient nude mice *in vivo* (Toraldo *et al.*, 2003). In addition, treatment of mice with a neutralizing anti-IL-7 antibody inhibited ovariectomy-induced proliferation of early T-cell precursors in the thymus, demonstrating that ovariectomy upregulates T-cell development through IL-7. This latter effect may be a mechanism by which IL-7 regulates ovariectomy-induced bone loss (Ryan *et al.*, 2005). The interpretation of results from *in vivo* IL-7 treatment studies, however, are complicated by secondary effects of IL-7, which result from the production of bone-resorbing cytokines by T cells (Weitzmann *et al.*, 2000; Toraldo *et al.*, 2003).

IL-7 directly inhibited osteoclast formation in murine bone marrow cells that were cultured for 5 days with M-CSF and RANKL (Lee *et al.*, 2003). In IL-7-deficient mice, osteoclast number was markedly increased and trabecular bone mass was decreased compared with wild-type controls (Lee *et al.*, 2006). In addition, trabecular bone loss after ovariectomy was similar in wild-type and IL-7-deficient mice (Lee *et al.*, 2006). Curiously, IL-7 mRNA levels in bone increase with ovariectomy and this effect may be linked to alterations in osteoblast function with estrogen withdrawal (Weitzmann *et al.*, 2002; Sato *et al.*, 2007). Addition of IL-7 to the medium of newborn murine calvaria cultures inhibited bone formation, as did injection of IL-7 above the calvaria of mice *in vivo* (Weitzmann *et al.*, 2002).

IL-8 and MCP-1

A variety of additional cytokines have been shown to either be produced by bone cells or to have effects on bone cell function. Interleukin-8 (IL-8) and monocyte chemotactic peptide-1 (MCP-1) are members of the chemokine family of cytokines, which is named for the ability of its members to direct the migration of cells to sites of inflammation. Both are produced in bone cells in a regulated manner (Chaudhary and Avioli, 1995; Takeshita *et al.*, 1993; Zhu *et al.*, 1994). More than 20 different chemokines have been described. In addition to IL-8 and MCP-1, these include macrophage inflammatory proteins 1α, 1β, and 2 (MIP-1α, MIP-1β, and MIP-2) and RANTES (Regulated on Activation Normal T-cell Expressed and Secreted) (Horuk, 1994). All contain four conserved cysteine residues and are classified into two categories depending on whether the first two conserved cysteine residues are separated by an intervening amino acid (CXC) or not (CC). IL-8 is a member of the CXC chemokine group whereas MCP-1 belongs to the CC group. IL-8 has specific neutrophil chemotactic activity. It is produced by human osteoblast-like cells, the human osteosarcoma cell line MG-63, human bone marrow stromal cells, and human osteoclasts (Chaudary and Avioli, 1995; Chaudary *et al.*, 1992; Rothe *et al.*, 1998). Production of IL-8 in these cells was augmented by treatment with either IL-1 or TNF and was synergistically increased by their combination. Dexamethasone decreased the production of IL-8 in the cells in response to treatment with IL-1 or TNF. However, *in vitro* estrogen treatment had no effect.

MCP-1, which was originally called JE in mice, is a potent monocyte chemoattractant that is produced by stimulated osteoblasts (Zhu *et al.*, 1994). Like IL-8, IL-1 and TNF strongly stimulated its production in human and murine osteoblastic cells (Takeshita *et al.*, 1993; Zhu *et al.*, 1994; Graves *et al.*, 1999).

The function of IL-8 and MCP-1 in bone remains to be defined. However, MCP-1 does not appear to be involved in regulating the development or activity of osteoclasts because it did not stimulate the maturation of osteoclast-like cells in bone marrow cultures (Zhu *et al.*, 1994) nor did it affect bone resorption rates in organ cultures (Williams *et al.*, 1992) or in isolated osteoclasts that were cultured on bone slices (Fuller *et al.*, 1995). However, it is possible that it regulates the migration of a common monocyte-osteoclast progenitor cell from the blood or bone marrow to sites of osteoclast development at the bone surface.

Of the other members of the chemokine family that have been examined for their effects on bone cells, MCP-1α and IL-8 were found to stimulate the motility but suppress the resorptive rate of isolated osteoclasts whereas MIP-1β, MIP-2, and RANTES were without effect (Fuller *et al.*, 1995). However MIP-1α is a potent stimulator of bone resorption that may be involved in the effects of multiple myeloma on bone (Choi *et al.*, 2000).

Interleukin-10

Interleukin-10 is produced by activated T and B lymphocytes (Moore *et al.*, 2001). It is a direct inhibitor of osteoclastogenesis (Owens *et al.*, 1996; Xu *et al.*, 1995) and osteoblastogenesis (Van Wlasselaer *et al.*, 1993). This effect is associated with increased tyrosine phosphorylation of a variety of proteins in osteoclast precursor cells (Hong *et al.*, 2000). The direct effects of IL-10 on RANKL-stimulated osteoclastogenesis are associated with decreases in expression and translocation into the nucleus NFATc1 (Evans *et al.*, 2007) as well as suppressed c-Fos and c-Jun expression (Mohamed *et al.*, 2007). Administration of IL-10 may have utility as a therapy to control wear-induced osteolysis (Carmody *et al.*, 2002).

Treatment of bone marrow cell cultures with IL-10 suppressed the production of osteoblastic proteins and prevented the onset of mineralization (Van Wlasselaer *et al.*, 1993). IL-10 also inhibited the formation of osteoclast-likecells (OCLs) in bone marrow cultures without affecting macrophage formation or the resorptive activity of mature osteoclasts (Owens *et al.*, 1995). This effect appears to involve the production of novel phosphotyrosine proteins in osteoclast precursor cells (Hong *et al.*, 2000). IL-10 also stimulates a novel inducible nitric oxide synthase (Sunyer *et al.*, 1996).

4-1BB is an inducible T-cell costimulatory molecule that interacts with 4-1BB ligand. Treatment of RANKL-stimulated osteoclast precursor cells *in vitro* with 4-1BB ligand enhanced IL-10 production. In addition, expression of IL-10 was greater in RANKL-stimulated wild-type osteoclast precursor cell cultures than in cultured cells from 41-BB-deficient mice (Shin *et al.*, 2006). These results imply that some effects of IL-10 on osteoclasts may be mediated through interactions of 4-1BB with 4-1BB ligand.

Interleukin 12

Interleukin 12 (IL-12) is a cytokine that is produced by myeloid and other cell types. It induces T lymphocytes to express INF-γ (Hsieh *et al.*, 1993). IL-12 has an inhibitory effect on osteoclastogenesis. However, the mechanisms by which this effect occurs *in vitro* is controversial. Some authors have demonstrated direct inhibitory effects of IL-12 on RANKL-stimulated osteoclastogenesis in purified primary osteoclast precursors and RAW 264.7 cells (Amcheslavsky *et al.*, 2006). This effect was associated with inhibition of the expression of NFATc1 in the osteoclast precursor cells. Interestingly, the inhibitory effects of IL-12 on osteoclastogenesis were absent in cells that were pretreated with RANKL (Amcheslavsky *et al.*, 2006). In contrast, others have found that the inhibitory effects of IL-12 on osteoclastogenesis are indirect. One group demonstrated that the inhibitory effects of IL-12 are mediated by T lymphocytes and do not involve production of INF-γ (Horwood *et al.*, 2001). However, a second group disputes this and found inhibition of osteoclastogenesis by IL-12 in cells from T-lymphocyte-depleted cultures and in cells from T-lymphocyte-deficient nude mice (Nagata *et al.*, 2003). The latter authors also found that antibodies to INF-γ blocked some of the inhibitory effect of IL-12 on RANKL-stimulated osteoclast formation.

Interleukin 15

Interleukin 15 (IL-15), like IL-7, is a member of the interleukin 2 superfamily and shares many activities with IL-2 including the ability to stimulate lymphocytes. It has been shown to enhance osteoclast progenitor cell number in culture (Ogata *et al.*, 1999). Production of IL-15 by T lymphocytes has been linked to the increased osteoclastogenesis and bone destruction seen in the bone lesions of rheumatoid arthritis (Miranda-Carus *et al.*, 2006).

Interleukin 17 and Interleukin 23

Interleukin 17 (IL-17) is a family of related cytokines that are unique and contain at least six members (A to F) (Weaver *et al.*, 2007). IL-17E is also called interleukin 25 (Fort *et al.*, 2001). These cytokines are central for the development of the adaptive immune response and the production of a subset of CD4 T lymphocytes with a unique cytokine expression profile.

IL-17A was initially identified as a stimulator of osteoclastogenesis in mixed cultures of mouse hematopoietic cells and osteoblasts (Kotake *et al.*, 1999). It stimulated osteoclastogenesis by inducing prostaglandin synthesis and RANKL. Production of IL-17A in rheumatoid arthritis appears involved in the production of activated osteoclasts and bone destruction in involved joints (Kotake *et al.*, 1999; Lubberts *et al.*, 2000, 2003). Effects of IL-17 on osteoclastogenesis and bone resorption are enhanced by TNF-α, which is also produced in inflamed joints in rheumatoid arthritis (Van bezooijen *et al.*, 1999). Inhibition of IL-17A in an antigen induced arthritis model inhibited the joint and bone destruction that is characteristic and decreased production of RANKL, IL-1β, and TNF-α in the involved lesions (Koenders *et al.*, 2005).

Interleukin-23 (IL-23) is an IL-12-related cytokine composed of one subunit of p40, which it shares with IL-12, and one subunit of p19, which is unique (Kastelein *et al.*, 2007). It is critical for the differentiation of the IL-17-producing subset of T lymphocytes (TH17) along with TGF-β and IL-6 (Bettelli *et al.*, 2006). IL-23 appears to be most important for expanding the population of TH17 T lymphocytes. These are a subset of T lymphocytes that have a high osteoclastogenic potential (Sato *et al.*, 2006). Using a LPS-induced model of inflammatory bone destruction, Sato *et al.* (2006) found markedly decreased loss of bone in mice that were deficient either in IL-17 or IL-23. Hence, production of both IL-23 and IL-17 is involved in the bone loss in this model.

Interleukin 18

Interleukin-18 (IL-18) is similar to IL-1 in its structure and a member of the IL-1 superfamily (Orozco *et al.*, 2007). IL-18 synergizes with IL-12 to induce INF-γ production

(Okamura *et al.*, 1995) and its levels are increased at sites of inflammation such as in joints that are affected by rheumatoid arthritis (Yamamura *et al.*, 2001). It is expressed by osteoblastic cells and inhibits osteoclast formation through a variety of mechanisms. These include its ability to stimulate GM-CSF, which is produced by T cells in response to IL-18 treatment (Horwood *et al.*, 1998). It also stimulates INF-γ production *in vivo* in bone (Kawase *et al.*, 2003), and its inhibitory effects on osteoclastogenesis are enhanced by cotreatment with IL-12 (Yamada *et al.*, 2002). Finally, it has been shown to increase production of osteoprotegerin (Makiishi-Shimobayashi *et al.*, 2001). In IL-18 overexpressing transgenic mice osteoclasts were decreased; although, curiously, so was bone mass. These results indicate that there also may be effects of IL-18 on bone growth (Kawase *et al.*, 2003). IL-18 has been shown to indirectly stimulate osteoclastogenesis through its effects on T lymphocytes (Dai *et al.*, 2004). IL-18 is also a mitogen for osteoblastic cells *in vitro* (Cornish *et al.*, 2003).

Macrophage Migration Inhibitory Factor

Macrophage Migration Inhibitory Factor (MIF) was initially identified as an activity in conditioned medium from activated T lymphocytes that inhibited macrophage migration in capillary tube assays (Baugh *et al.*, 2002). Once purified and cloned (Weiser *et al.*, 1989), it became available for functional studies and was shown to have a variety of activities. In addition to T lymphocytes, it is produced by pituitary cells and activated macrophages. Mice that overexpress MIF globally have high-turnover osteoporosis (Onodera *et al.*, 2006). In contrast, MIF-deficient mice failed to lose bone mass or increase osteoblast or osteoclast number in bone with ovariectomy (Oshima *et al.*, 2006). Hence, MIF appears to be another mediator of the effects of estrogen on bone. Estrogen downregulates MIF expression in activated macrophages (Ashcroft *et al.*, 2003) and a similar response may occur in bone or bone marrow to mediate some of the effects of ovariectomy on bone mass. MIF is made by osteoblasts (Onodera *et al.*, 1996) and its production by these cells is upregulated by a variety of growth factors including TGF-β, FGF-2, IGF-II, and fetal calf serum (Onodera *et al.*, 1999). *In vitro* MIF increases MMP9 and MMP13 expression in osteoblasts (Onodera *et al.*, 2002) and inhibits RANKL-stimulated osteoclastogenesis (Lee *et al.*, 2006).

EFFECTS OF IL-4 AND IL-13 ON BONE

IL-4 is a 19-kDa pleotropic cytokine secreted by activated TH2 cells and mast cells (Howard *et al.*, 1982). Its major functions include the growth and survival of T-helper cells, the activation and growth of B cells, increased expression of class II MHC molecules, and the inhibition of macrophage function (Hart *et al.*, 1989; Stuart *et al.*, 1988). Although IL-4 is not a member of the LIF/IL-6 subfamily of cytokines, it uses a two-chain plasma membrane-expressed receptor similar in many ways to the LIF and IL-11 receptors. Ligand binds to the 140-kDa IL-4 receptor, which is a single-polypeptide chain of 800 amino acids that confers both specificity and determines signaling (Idzerda *et al.*, 1990). The IL-4 receptor heterodimerizes with the common γ-chain of the IL-2 receptor to form a complete and functional receptor (Russell *et al.*, 1993). Binding of IL-4 results in the phosphorylation on tyrosine of cellular substrates. The IL-4 receptor has no intrinsic kinase activity. As is the case for the LIF receptor, Jak1 and Jak3 are tyrosine phosphorylated in response to IL-4, as is Stat 6 (Witthuhn *et al.*, 1994). Jak3 associates with the common γ-chain, suggesting that the IL-4 receptor-specific chain associates with Jak1. It is assumed that the phosphorylation of cellular substrates is mediated by the Jak kinases. IL-4 binding does cause the tyrosine phosphorylation of ligand-specific substrates such as insulin receptor substrates 1 and 2. Activation of these unique substrates is likely to be part of how different ligands confer specific activities to their targets.

IL-4 appears to inhibit bone remodeling. In organ culture, IL-4 antagonized the resorption inducing activity of IL-1, tumor necrosis factor, 1,25-dihydroxyvitamin D_3, and PGE_2 without affecting basal resorption (Watanabe *et al.*, 1990). Addition of IL-4 or IL-13 to calvarial organ cultures suppressed ^{45}Ca release following stimulation with a variety of cytokines (i.e., OSM, IL-1, TNF, and IL-6), PTH, and 1,25-dihydroxyvitamin D_3 (Palmqvist *et al.*, 2006). As mentioned previously, IL-4 inhibits macrophage function. Osteoclasts are in the macrophage lineage, suggesting that at least one of the targets for IL-4 is mature osteoclasts or more likely osteoclast precursors. Addition of IL-4 to cultures of bone marrow cells (source of osteoclast precursors) and stromal cells causes the inhibition of osteoclastogenesis (Shioni *et al.*, 1991). In this system, osteoclastogenesis induced by PTH, PTH-related peptide (PTHrP), 1,25-dihydroxyvitamin D_3, IL-1, and PGE_2 was inhibited by the addition of IL-4 (Lacey *et al.*, 1995). By using this same system, bone marrow macrophages were substituted for unfractionated bone marrow cells with similar results, suggesting that the target for the IL-4 inhibitory activity was the osteoclast precursor. IL-4 and IL-13 decreased RANKL and RANK mRNA and increased OPG mRNA in mouse calvariae (Palmqvist *et al.*, 2006). Induction of RANKL mRNA and a decrease in OPG mRNA induced by vitamin D_3 in osteoblasts were reversed by treatment with IL-4 and IL-13. Osteoclast differentiation from spleen or bone marrow precursors induced with RANKL and M-CSF were decreased by IL-4 and IL-13 treatment (Palmqvist *et al.*, 2006). No inhibition of osteoclast differentiation was seen by using precursors from Stat6 -/- mice. These data suggest that a Stat6-dependent pathway mediates at least part of the action of IL-4 and IL-13. In addition, effects of IL-4 on osteoclastogenesis were demonstrated to occur through direct actions on the peroxisome proliferator-activated receptor gamma 1 (Bendixen *et al.*, 2001).

In vivo bone resorption in mice that were transplanted with a PTHrP- and IL-1-secreting tumor was inhibited by continuous infusions of IL-4 (Nakano *et al.*, 1994). In addition, IL-4 delivery by adenovirus vector was shown to suppress RANKL and IL-17 production and prevent bone resorption in an *in vivo* collagen-induced arthritis mouse model (Lubberts *et al.*, 2000). At least part of this inhibitory effect could be related to the ability of IL-4 to block local prostaglandin synthesis in bone cells (Kawaguchi *et al.*, 1996; Onoe *et al.*, 1996). Further evidence for the role that IL-4 may play in the control of bone remodeling is provided by IL-4-overexpressing transgenic mice (Lewis *et al.*, 1993). These mice have pronounced cortical thinning in the long bones and vertebrae. Although IL-4-overexpressing mice have normal numbers of osteoclasts, their function appeared altered as measured by a decrease in tartrate resistant acid phosphotase (TRAP) activity. Interestingly, these animals have a marked reduction in bone formation. Both the number of cells lining the bone surface and the function of these cells was decreased. IL-4 treatment also decreased bone turnover in ovariectomized mice (Okada *et al.*, 1998).

Hence, osteoblasts, as well as osteoclasts, appear to be a target of IL-4. IL-4 induces the secretion of M-CSF from the murine osteoblast-like cell line MC3T3-E1 as well as primary osteoblasts in a dose-dependent manner (Lacey *et al.*, 1994; Ohara *et al.*, 1994). MC3T3 cells express IL-4 receptors that can be upregulated by treatment with 1,25-dihydroxyvitamin D_3 (Lacey *et al.*, 1993). Primary human osteoblast-like cells and the human osteosarcoma MG-63 also express IL-4 receptors (Riancho *et al.*, 1993; Ueno *et al.*, 1992). Furthermore, IL-4 stimulated the proliferation of human osteoblast-like cells *in vitro* as well as their expression of alkaline phosphatase.

EFFECTS OF THE LIF/IL-6 SUBFAMILY OF CYTOKINES ON BONE

Abundant data have accumulated demonstrating that the LIF/IL-6 subfamily of cytokines has important effects on the regulation of the normal and pathological remodeling cycles. These factors are related based on sequence homology, chromosome location, and receptors (Rose and Bruce, 1991). This subfamily is composed of leukemia inhibitory factor (LIF), oncostatin-M (OSM), IL-6, IL-11, ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), and neuropoietin (Derouet *et al.*, 2004). The data known about these cytokines as they relate to bone are variable. Information is known about which bone cells secrete LIF and IL-6. Receptor expression for all of the cytokines is better described and some of the biological consequences of that binding are known. Substantial information is available on LIF, OSM, IL-6, and IL-11; less is available on CNTF and CT-1; and little is known about CLC and neuropoietin and their receptor expression (Bellido *et al.*, 1997; Elias *et al.*, 1995; Girasole *et al.*, 1994; Horowitz *et al.*, 1994; Jay *et al.*, 1996). Although information is available about the receptors for these cytokines and some of the downstream events that occur following ligand binding in skeletal cells, the signal transduction pathways used by some of these cytokines have been elucidated (Bellido *et al.*, 1994; Horowitz *et al.*, 1996; Levy *et al.*, 1996). These cytokines may interact with their receptors in two settings, both of which can result in signal transduction. The first and more conventional setting is when ligand binds to its appropriate plasma membrane-expressed receptor component. This interaction initiates receptor component assembly, culminating in a functional receptor capable of transmitting a signal to the interior of the cell. This form of ligand–receptor interaction can occur for all of the cytokine subfamily members. The second setting, which involves the interaction between ligand and soluble receptor, is limited to specific members of the subfamily (IL-6, IL-11, and CNTF).

Originally known as differentiation-inducing factor, LIF is a pleotropic cytokine exerting multiple effects in bone. Its effects on bone resorption *in vitro* are variable and, like other members of the group, depend on the model system that is used. LIF is secreted as a diffusible molecule and in an immobilized form associated with the extracellular matrix as a result of alternate promoter usage, again suggesting an important developmental control mechanism (Rathjen *et al.*, 1990). LIF mRNA is found in various rodent and human immortalized cell lines and primary fetal rat calvarial cells (Marusic *et al.*, 1993). It induces bone resorption in cultured mouse calvaria via a prostaglandin-mediated mechanism (Reid *et al.*, 1990). However, in fetal rat and mouse long-bone cultures it inhibits resorption (Lorenzo *et al.*, 1990a; Van Beek *et al.*, 1993). Mice injected with a hematopoietic cell line overexpressing LIF developed cachexia and increased bone turnover with marked bone formation (Metcalf and Gearing, 1989). LIF can either potentate or inhibit the expression of alkaline phosphatase and type I collagen synthesis depending on the cells or assay used, suggesting a role for LIF in osteoblast development (Lorenzo, 1991). Continuous treatment of fetal rat calvarial cell cultures with LIF resulted in inhibition of bone nodule formation (Malaval *et al.*, 1995, 1998). This effect was dependent on the stage of differentiation of the cells. Addition of LIF to the cultures early, during the proliferative stage (days 1 to 4) did not affect nodule formation. However, addition of LIF later, during the differentiation stage (days 1 to 7) resulted in marked inhibition of nodule formation (Malaval *et al.*, 2005). Osteoblasts and adipocytes, arising from a common progenitor, are closely related. Therefore, it was likely that LIF may affect adipocyte differentiation. This is supported by the data showing overexpression of LIF in *in vivo* results in mice with cachexia. Treatment of rat calvarial cells with rosiglitazone (thiazolidinediones, PPARγ agonist) induces increased adipogenesis (Falconi *et al.*, 2007). These data suggest a LIF-regulated switch from osteoblastogenesis to adipogenesis. In contrast, ovariectomized C57BL/6 mice injected with LIF experienced a significant decrease in white fat, serum leptin, and brown fat mass, but had no effect on trabecular bone mineral density (BMD) (Jansson *et al.*, 2006). LIF had variable effects on osteoblast-like cell proliferation. LIF had no effect on the proliferation of the human osteosarcoma cell line MG-63, induced a 40% increase in proliferation in murine MC3T3-E1 cells, and had no effect on authentic murine calvarial osteoblasts (Bellido *et al.*, 1997). The addition of LIF to MC3T3-E1 cells inhibited apoptosis induced by either serum starvation or addition of TNF (Jilka *et al.*, 1998). Treatment of MC3T3-E1 cells with PTH results in the increased transcription of LIF and IL-6 but not 14 other cytokines including IL-1, GM-CSF, and TGFβ-1 (Greenfield *et al.*, 1993) Similarly, treatment of a rat osteosarcoma cell line with PTH resulted in IL-6 secretion (Horowitz *et al.*, 1990). This suggests that some of the effects of PTH may be mediated by LIF and PTH (Grey *et al.*, 1999). Because murine osteoblasts respond to LIF, they must express cell surface receptors. LIF induced signal transduction in non-skeletal cells results in protein tyrosine phosphorylation.

Therefore, appropriate substrates should also be phosphorylated in bone cells. Treatment of the murine osteoblast cell line MC3T3-E1 with LIF resulted in tyrosine phosphorylation of Jak1, Jak2, gp130, and what appears to be the LIFR (Lowe *et al.*, 1995). The fact that osteoblasts respond to LIF and secrete LIF indicates that it is a true autocrine growth factor. However, few data are available on the effects of LIF on primary human osteoblast-like cells, effects that may be particularly important because osteoblast-like cells derived from patients 60 years and older fail to respond to LIF as measured by the induction of phosphoproteins (M. Horowitz, unpublished observation). Whether this reflects an intrinsic unresponsiveness in these cells or is related to other processes is unknown. It is tantalizing to speculate that if this failure were age related, it could account for the onset of osteopenia and possibly osteoporosis. This idea is supported by the observation that mice rendered LIFR negative by homologous recombination develop severe osteopenia with increased osteoclastic activity (Ware *et al.*, 1995). Loss of the LIFR in humans results in Stuve–Wiedemann/Schwartz–Jampel type 2 syndrome (SWS). SWS, which is a severe autosomal recessive condition characterized by bowing of the long bones, cortical thickening, irregularities of the chondro-osseous junction, with rare, thick, and irregular trabeculae, increased numbers of osteoclast and camptodactyly (Dagoneau *et al.*, 2004; Cormier-Daire *et al.*, 1998).

OSM, which is structurally similar to LIF, is a 28-kDa glycoprotein originally isolated from media of phorbal 12-myristate 13-acetate (PMA)-stimulated U937 human histiocytic leukemia cells (Zarling *et al.*, 1986). OSM is secreted by phytohemagglutinin-stimulated T cells, lipopolysaccharide-activated monocytes, and cell cultures from AIDS-related Kaposi's sarcoma for which OSM is a growth factor. OSM mRNA is also found in bone marrow cells. Its expression in bone, however, is currently unknown. OSM, like LIF, induces murine osteoblasts to proliferate and secrete matrix proteins and, of the family members, it is the most potent stimulator of IL-6 (Jay *et al.*, 1996; Bellido *et al.*, 1997). However, it inhibits alkaline phosphatase activity and bone resorption (Jay *et al.*, 1996). OSM has also been reported to inhibit the proliferation of MG-63 cells (Bellido *et al.*, 1997). Anti-Fas antibody-induced apoptosis in MG-63 cells was inhibited by OSM (Jilka *et al.*, 1998). OSM activation of MG-63 cells leads to transcriptional activation of the p21WAF1,CIP1,SDI1 gene, which has been shown to be important in protecting against apoptosis and could explain the antiapoptotic effects of OSM (Lacey *et al.*, 1997; Wang *et al.*, 1996). Transgenic mice that overexpress bovine OSM develop osteopetrosis (Malik *et al.*, 1995). Although the overall syndrome is similar to the picture seen in the LIF-overexpressing mice, it does not have the associated increase in bone resorption. In fact, the osteopetrosis seen in both the LIF- and the OSM-overexpressing mice is just the opposite picture to that seen in the LIFR knockout mice. Continuous treatment of fetal rat calvarial cells with mOSM caused a significant reduction in bone nodule formation similar to LIF (Malaval *et al.*, 2005). In contrast to LIF, treatment of rat calvarial cells with OSM during the early proliferative phase of *in vitro* culture resulted in a marked increase in nodule formation. This effect appears to be mediated through the OSM-specific receptor (OSMR). OSM treatment of the rat osteosarcoma cell line ROS17/2.8 or osteoblast from long-bone explants inhibited growth and differentiation (Chipoy *et al.*, 2004). This inhibitory effect was restricted to mature osteoblasts and differentiated osteosarcoma cells because OSM stimulated osteoblast differentiation in early but not late bone marrow mesenchymal stem cells. It should be noted that some of the differences in response to these cytokines may be caused by the anatomical source of the cells. The ability and type of response of calvarial cells (mouse or rat) to cells from long bones may be very different. Treatment of human adipose mesenchymal stem cells isolated from subcutaneous fat with OSM resulted in a decrease in adipocyte differentiation and an increase in osteoblast differentiation (Song *et al.*, 2007). OSM is the only member of the LIF/IL-6 family of cytokines that increases osteoprogenitor development, which may help to explain its anabolic effects *in vivo*. Mice deficient in the OSMR had a 75% increase in trabecular bone volume in the long bones and vertebrae with little change in cortical bone (Sims *et al.*, 2007). This was associated with a decrease in osteoclast number and bone resorption. Bone formation was also reduced as evidenced by a decreases in osteoid thickness, osteoblast surfaces, and bone formation rate. Interestingly, marrow adipocytes were increased 4 fold (Sims *et al.*, 2007). Treatment of a mouse stromal cell line with OSM caused increased mineralization and a decrease in fat production. These data are, for the most part, consistent with the data from the OSMR null mice and that reported by Malaval *et al.* (2005).

Human OSM induces tyrosine phosphorylation in both primary human and mouse osteoblast-like cells. Three JAK family members (Jak1, Jak2, and Tyk2) are tyrosine phosphorylated in response to OSM in calvarial osteoblastic cells, a response similar to that observed in nonskeletal cells (Levy *et al.*, 1996). However, human osteoblast-like cells express a number of additional phosphoproteins in the 150- to 200-kDa range not found in the mouse. These differences may be caused by the mouse cell's use of the LIFR + gp130 and the alternate receptor, or of the OSMR + gp130 complex versus the human cell's use of only the OSMR, because human cells do not express the LIFR (Gearing and Bruce, 1992; Rose *et al.*, 1994; Hermanns *et al.*, 2000; Heinrich *et al.*, 2003). These data suggest that differences exist between the ability of these ligands to bind and activate osteoblasts, and the complexity may be increased by differences in species and age.

CT-1, a 21.5-kDa protein, was originally isolated from differentiated mouse embryonic stem (ES) cells (Pennica *et al.*, 1995a). The amino acid sequence of CT-1 is similar to LIF (24% homology) and CNTF (19% homology). mRNA for CT-1 is expressed in heart, liver, and kidney with less in brain and none in spleen. Its expression in bone is currently unknown, CT-1 is a potent inducer of myocyte hypertrophy, as are LIF, IL-11, and OSM, whereas IL-6 and CNTF are much less effective. CT-1 is inactive in the B9 assay (detects IL-6) and inhibits the differentiation of mouse ES cells (Pennica *et al.*, 1995b). CT-1 binds the LIFR directly and requires gp130 to effect signal transduction (Pennica *et al.*, 1995b). No α component of the receptor has been identified. Based on the receptor component composition, CT-1 should activate osteoblasts. Addition of CT-1 to calvarial osteoblastic cultures induces numerous tyrosine-phosphorylated proteins in a time-dependent manner (M. Horowitz, unpublished observation). Because CT-1 uses the LIFR + gp130, it seems reasonable that the defects observed in heart development in the gp130 knockout mouse owe, at least in part, to the failure of CT-1 to function. CT-1 must also be considered a candidate, as are LIF and OSM, for the cytokines contributing to the maintenance of normal bone remodeling, which is profoundly altered in the LIFR knockout mice.

The second setting for the interaction of ligand and receptor is restricted to IL-6, CNTF, and IL-11 by the ability of their receptors to be cleaved from the membrane and function in soluble form (sIL-6R, sCNTFR, and sIL-11R). It is these α components that confer a level of specificity to the interaction of ligand and cell. Soluble receptor can bind ligand, forming a complex that can interact with the appropriate β-subunits to trigger signaling. Addition of premixed sIL-6R plus IL-6 to murine calvarial osteoblasts results in their activation. This indicates that for IL-6 this interaction can occur in solution. This interaction can also occur on the cell membrane, as is the case for IL-11 and CNTF interacting with their receptor on calvarial osteoblasts. This is due to the presence of the α components for IL-11 and CNTF being expressed on the cell membrane whereas the IL-6 α component is absent. This type of interaction may induce signal transduction in cells that would not normally be responsive to these cytokines because of lack of the appropriate α components on their membrane surface. It is thought that the major effect of IL-6 is its colony-stimulating activity on osteoclast precursors (Roodman, 1992). This is an important issue because estrogen inhibits cytokine-induced IL-6 secretion by bone cells and osteoclastogenesis (Girasole *et al.*, 1992). Antibodies to IL-6 or estrogen blocked the increased formation of osteoclasts in ovariectomized mice (Jilka *et al.*, 1992). However, Tamura has reported that IL-6 does not directly induce osteoclast formation in coculture experiments (Tamura *et al.*, 1993). This failure can be overcome by the addition of sIL-6R. IL-11, OSM, and LIF (OSM > IL-11 > LIF); all directly induce osteoclast formation in this system. Because of the design of the studies, the target of the cytokines could not be determined. However, treatment of primary mouse osteoblast cultures with IL-11 resulted in the upregulation of RANKL gene expression (Yasuda *et al.*, 1998). Increased RANKL expression would explain the increase in osteoclast formation in coculture induced by all four cytokines. We have shown that IL-6 does not directly induce tyrosine phosphorylation in primary mouse or human osteoblasts, but this failure can be overcome by the addition of sIL-6R (Horowitz *et al.*, 1994). However, it has been demonstrated that treatment of murine osteoblastic cells with dexamethasone induced a marked increase in IL-6 receptor expression (Udagawa *et al.*, 1995). This suggests that osteoblastic cells may be the target for IL-6 under the appropriate conditions. Therefore, the target cell in bone for IL-6 remains ambiguous. The ambiguity over the role of IL-6 in bone is heightened by the differences reported in the ability of IL-6 to induce bone resorption (Ishimi *et al.*, 1990; al-Humidan *et al.*, 1991). Because osteoblasts secrete IL-6, it is possible that IL-6 may function as an autocrine or paracrine factor, but this could only occur in the presence of sIL-6R or an increase in cortisol levels. The fact that other members of the subfamily also induce osteoclast formation supports our hypothesis that these factors play an important role in the regulation of bone remodeling. Although the LIFR has no α component, LIFR can be detected in the circulation (Layton *et al.*, 1992). Unlike the agonist effect of sIL-6R + IL-6, sLIFR + LIF may have an antagonistic effect (Gearing, 1993). Whether this is because of α versus β receptor component usage or downstream events is unknown. Equally unknown is the effect of sLIFR + LIF on bone cells.

IL-11 is a 23-kDa stromal cell-derived cytokine that causes the maturation of hematopoietic precursor cells (Quesniaux *et al.*, 1993). The IL-11-specific receptor is 24% homologous with the IL-6R, suggesting that it also occurs in soluble form (Hilton *et al.*, 1994). IL-11, unlike IL-6, appears to function in the estrogen-replete state to induce osteoclast formation and bone resorption and therefore may regulate normal remodeling (Girasole *et al.*, 1994). We have demonstrated that IL-11 is secreted by primary human osteoblasts and induces tyrosine phosphorylation in primary mouse osteoblasts (Elias *et al.*, 1995; Horowitz *et al.*, 1994). This suggests that, unlike IL-6, but like LIF, IL-11 is a true autocrine factor for osteoblasts. This is supported by our demonstration that IL-11 induces IL-6 secretion by primary osteoblastic cells (Horowitz *et al.*, 1994). The IL-11 receptor, which is 32% and 30% identical by amino acid composition to the CNTF and IL-6 receptors, respectively, is expressed by day 9 in the developing embryo (Neuhaus *et al.*, 1994). The highest expression is in mesenchymal cells, particularly in regions containing chondro- and osteoprogenitors. However, no IL-11R mRNA was detected in mature osteoblasts, chondroblasts, odontoblasts, or osteocytes. This suggests that IL-11R is a marker of early bone development. MC3T3-E1 cells treated with IL-11 resulted in the phosphorylation of Jak1, Stat1, and Stat3, the inhibition of cellular proliferation, and stimulation of alkaline phosphatase activity (Shih *et al.*, 2000). Treatment of fetal rat calvarial cells with IL-11 inhibited bone nodule formation (Malaval *et al.*, 2005). IL-11 stimulated BMP through Stat3, leading to osteoblast differentiation, and inhibited adipogenesis in bone marrow stromal cells (Takeuchi Y *et al.*, 2002). Mice made null for both isoforms of the IL-11 receptor (IL-11Rα1 and IL-11Rα2) had significantly increased trabecular bone volume (Sims *et al.*, 2005). This was associated with decreased bone resorption and formation *in vivo*. Culture of IL-11R -/- bone marrow with RANKL and M-CSF to induce osteoclast differentiation resulted in fewer osteoclasts in the mutant bone marrow than in controls. In contrast, overexpression of IL-11 in transgenic mice resulted in stimulation of bone formation with increased cortical thickness and bone strength (Takeuchi *et al.*, 2002). Increased IL-11 expression did not affect osteoclast differentiation and bone resportion. Injection of IL-11 into 7-month-old ovariectomized mice that were actively losing bone restored BMD to sham levels and was as good as injected PTH (Ma *et al.*, 2006). Trabecular architecture, including trabecular number and trabecular separation, improved in the IL-11-treated mice. Cortical area increased by increasing periosteal and endocortical mineralizing surfaces and reducing endocortical bone resorption.

One way in which these ligands are related is by the use of a common receptor. This receptor is composed of multiple components and expression of different components allows the cell to discriminate between ligands. Therefore, a clear understanding of how this receptor functions cannot be separated from the significance of how multiple ligands sharing a common receptor regulate cell function. The receptor is composed of a set of proteins that are assembled in a defined order and that have specific functions. However, the members of the subfamily utilize different combinations of the receptor components. Each subfamily member appears to possess at least three receptor-binding sites. The first site binds the α component of the receptor. The receptors for IL-6, IL-11, and CNTF have ligand-binding α components whereas the receptors for OSM, LIF, and CT-1 lack this component (Davis *et al.*, 1991).

The other two binding sites on the ligand are required for interaction with the β1 and β2 components of the receptor. The β1 component is a transmembrane protein, which forms a complex with the ligand and the α component, or with the ligand alone, if the α component is absent. The β1 component has been found in the receptor of all subfamily members that have been examined. However, it is not necessarily the same molecule for every member. OSM, CNTF, and CT-1 use the same β1 molecule that was originally identified for LIF, known as LIFRβ or LIFR (Gearing *et al.*, 1992). LIFR is now known to be the low-affinity receptor for LIF (Fig. 1).

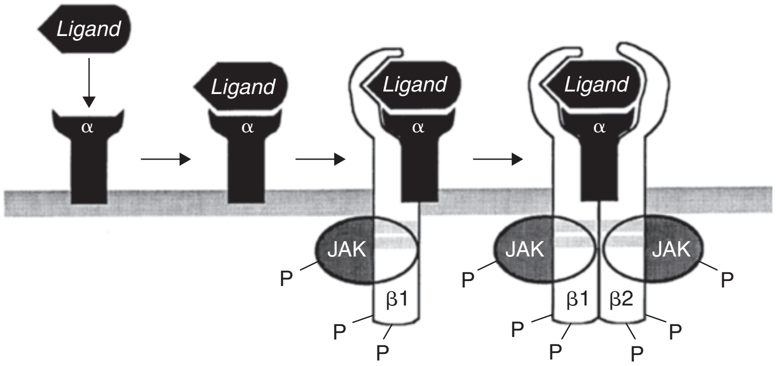

FIGURE 1 LIF/IL-6 cytokine receptor with component assembly including ligand, α component, β1 component, β2 component (gp130), and JAK kinase. P indicates tyrosine phosphorylation sites; cross-hatched portion in β component represents box 1 and 2 regions where JAK kinase is associated.

The β2 component has been identified as gp130. GP-130 was originally identified as a signal transducer for IL-6 and is now known to be a common and obligatory component of the receptor for all of the cytokines in the subfamily and is required for signal transduction (Ip *et al.*, 1992). GP-130 has been referred to as a high-affinity converting subunit. An example of this is cell activation with LIF, an event that requires LIF, LIFR, and gp130. LIF can bind LIFR and transmit a suboptimal signal. However, the presence of gp130 allows for high-affinity binding, optimal signal transduction, and cell activation. With the exception of OSM, it appears that gp130 is the last member of the receptor components to bind the ligand and form the completed and functional receptor. As can be seen in Table I, CNTF, LIF, OSM, and CT-1 use the LIFR subunit as the β1 component and gp130 as the β2 component. IL-6 and IL-11 use two gp130 molecules. Like LIF, OSM uses gp130 as a high-affinity converting subunit. However, unlike LIF, OSM binds gp130 preferentially. OSM may also use the OSM alternate receptor (OSMR), which is similar in structure to LIFR (32% homology) and requires gp130 for signaling (Mosley *et al.*, 1996). The OSMR is expressed on primary murine calvarial osteoblasts (Horowitz *et al.*, 1996). LIF does not bind the OSMR (Fig. 2).

It has been demonstrated in nonskeletal cells that signal transduction stimulated by this subfamily of cytokines is mediated by tyrosine kinases. With respect to bone cells, two major examples can be cited to demonstrate the importance of tyrosine phosphorylation in bone remodeling. Both examples involve altered bone remodeling because of genetic defects in some aspect of the process involving tyrosine phosphorylation. The first, as demonstrated by the oplop mouse, has a defect in the secretion of biologically active M-CSF, which can no longer signal through its receptor, c-fins, a receptor tyrosine kinase (Yoshida *et al.*, 1990). The second example involves c-src, a nonreceptor tyrosine kinase whose gene deletion by homologous recombination, like the oplop defect, results in osteopetrosis (Soriano *et al.*, 1991).

TABLE I Cytokine Receptor Composition

Cytokine (ligand)	Subunit		
	α	β1	β2
IL-6	IL-6R	None	gp130
IL-11	Il-11R	None	gp130
LIF	None	LIFR	gp130
OSM	None	LIFR	gp130
OSM	None	OSMR	gp130
CNTF	CNTFR	LIFR	gp130
CT-1	None	LIFR	gp130
CLP	CNTF	LIFR	gp130
NP	CNTF	LIFR	gp130

The JAK family (Janus or just another kinase) is associated with the receptors for the LIF/IL-6 subfamily and other cytokines including interferon-γ, erythropoietin, growth hormone, IL-3, and GM-CSF (Silvennoninen *et al.*, 1993). The JAK family presently includes five members: Jak1–4 and Tyk2, which range in size from 130 to 137 kDa. In mammals, Jak1, Jak2, Jak3, and Tyk2 are used. These kinases can be distinguished from other nonreceptor tyrosine kinases in that they have no SH2 or SH3 (src homology) domains and contain two catalytic domains, one being the classical kinase domain and the second, a kinase-related domain that is nonfunctional (Firmbach-Kraft *et al.*, 1990; Wilks *et al.*, 1991). Different JAK kinases are activated depending on cell type and ligand bound and may phosphorylate different substrates, representing a second level of discrimination by the cell (Stahl, 1994).

The LIF/IL-6 subfamily of cytokines uses Jak1, Jak2, and Tyk2. IL-12 uses a gp130-related receptor but not gp130 itself. Cytokines whose receptors are homodimers, such as growth hormone, prolactin, erythropoietin, and thrombopoietin, use Jak2. Signal transduction is initiated by ligand binding to its receptor, which causes receptor multimerization and an apparent conformational change in the receptor complex, allowing for activation of the associated JAK kinases and phosphorylation of three groups of proteins. The first group includes the β-chains of the receptor (LIFR, gp130, and OSMR). Second, the kinase itself becomes phosphorylated. The third group includes a family of cytoplasmic proteins. In both skeletal and nonskeletal cells some of these cytoplasmic proteins are transcription factors belonging to the STAT family (signal

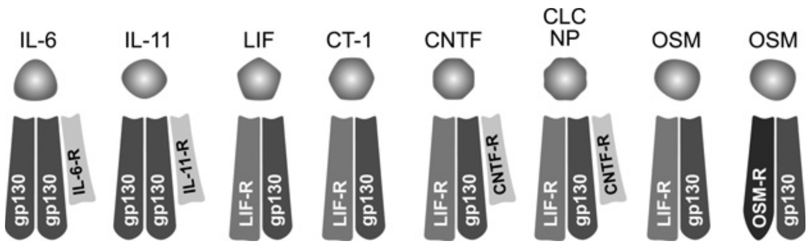

FIGURE 2 Receptor composition of the LIF/IL-6 family of cytokines.

transducers and activators of transcription). In addition to the STAT proteins, a group of adaptor proteins (i.e., Shc) may also become phosphorylated on tyrosine. Tyrosine phosphorylation of She is important because it provides the bridge between activated receptor chains and the mitogen-activated protein kinase (MAPK) pathway. Phosphorylated tyrosine residues and their flanking amino acids on the cytoplasmic domains of the receptor chains form docking sites where proteins like the STATs, She, and protein tyrosine phosphatases bind and become phosphorylated (Stahl *et al.*, 1995). These proteins recognize the phosphorylated tyrosine through their SH2 domains. Because the JAKs autophosphorylate, their phosphorylated tyrosines may also serve to dock additional proteins. JAK-deficient mice have been generated with multiple phenotypes (Neubauer *et al.*, 1998; Parganas *et al.*, 1998; Rodig *et al.*, 1998). Jak1-deficient mice show perinatal lethality, are small at birth, have defects in lymphopoiesis but not myelopoiesis, and fail to respond to the LIF/IL-6 subfamily of cytokines. Jak2 deficiency results in embryonic lethality because of the absence of erythropoiesis. Loss of Jak3 results in a SCID-like syndrome (Park *et al.*, 1995; Thomis *et al.*, 1995). Apparently, the skeletons of these mice have not been examined.

At the present time, six members of the STAT family have been described (Darnell *et al.*, 1994). Once phosphorylated, these proteins homo- or heterodimerize and translocate to the nucleus, by an unknown mechanism, and bind appropriate DNA sequences, resulting in gene transcription (Shuai *et al.*, 1993). A DNA promoter site in genes activated by IL-6 is similar to the IFN-γ-activated site (GAS), which mediates transcriptional induction of IFN-γ-responsive genes (Pearse *et al.*, 1993). Nonskeletal cells treated with IL-6 tyrosine phosphorylate Stat1 or related proteins with subsequent binding to GAS and IL-6 sites (Bonni *et al.*, 1993; Sadowski *et al.*, 1993). CNTF induces phosphorylation of STAT proteins with subsequent translocation to the nucleus (Bonni *et al.*, 1993). Because CNTF, as well as other family members, activates early-response genes, a comparison of promoter sequences was developed as a potential target sequence for STAT proteins. When nuclear extracts from CNTF-treated cells (nonskeletal) were incubated with oligonucleotides to the STAT protein-recognizing sequence, binding occurred as detected by DNA-protein mobility-shift assay. This binding did not occur in the absence of CNTF and could be blocked (super shift) by the addition of anti-p91 (anti-Stat1α) (Bonni *et al.*, 1993). At present, only limited data are available relating to phosphorylation of cytosolic transcription factors and subsequent gene activation in bone cells. We have shown by immunoprecipitation that OSM induces tyrosine phosphorylation of both Stat1 α and Stat3 in primary mouse osteoblastic cultures (Levy *et al.*, 1996). Stat1 α and Stat3 are also phosphorylated in MC3T3 cells in response to LIF (Lowe *et al.*, 1995). Expression of a dominant negative Stat3 in a stromal/osteoblastic cell line that normally supports osteoclastogenesis results in the failure of those cells to further support osteoclast formation (O'Brien *et al.*, 1999). The inhibition of osteoclastogenesis by the dominant negative Stat3 occurs following stimulation by OSM, IL-6 + sIL-6R, and IL-1 but not by parathyroid hormone or 1,25-dihydroxyvitamin D_3. In the same system the dominant negative Stat3 blocked the ability of OSM or IL-6 + sIL-6R to induce expression of RANKL, indicating an important role for Stat3 in gp130-mediated osteoclast formation. Stat3-deficient mice have a severe defect in development and die early in fetal life (Takeda *et al.*, 1997). A conditional knockout of Stat3 has been developed (Takeda *et al.*, 1998). These mice demonstrate the importance of Stat3 in IL-2, IL-6, and IL-10 signaling. Their bone phenolype(s), if any, have not been reported. Although the data are extremely limited at present, CNTF appears not to play a major role in bone cell activation. Of the family members, CNTF is the poorest at

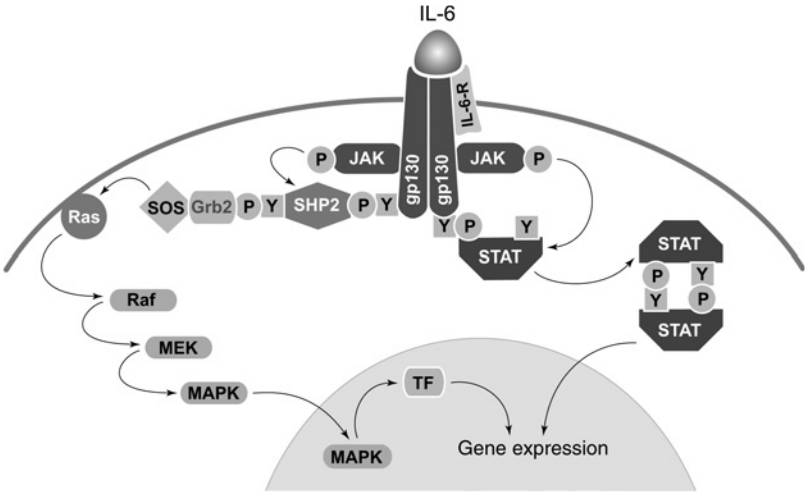

FIGURE 3 LIF/IL-6 cytokine signaling.

inducing IL-6 secretion from primary cultured osteoblastic cells (Horowitz *et al.*, 1994). However, CNTF did induce cell proliferation, increase alkaline phosphatase activity, and induce IL-6 secretion in MC3T3 cells (Bellido *et al.*, 1995). One pathway that has been shown to be activated by tyrosine phosphorylation in nonskeletal cells is Ras, a small GTP-binding protein that functions to relay signals to a group of kinases referred to as mitogen-activated protein kinases (MAPKs) or extracellular signal-regulated kinases (ERKs) (Bollag and McCormick, 1991). Activation of the MAPK cascade leads to the stimulation of transcription factors resulting in specific biological consequences. In nonskeletal cells it is known that OSM and IL-6 phosphorylate and activate MAPK (Amaral *et al.*, 1993; Kumar *et al.*, 1994). Our data show induction of a 43- to 45-kDa phosphorylated protein in human and mouse osteoblasts following OSM stimulation, which we have recently shown is MAPK (Horowitz and Levy, 1995). Tyrosine kinase pathways are linked to the Ras signaling pathway via Grb2, a small adaptor protein that contains domains referred to as src homology (SH2 and SH3), which are involved in protein–protein interactions. Grb2 associates with the guanine nucleotide exchange factor for Ras, SOS (the mammalian homologue of the *Drosophila* son of sevenless gene product), via interactions between its SH3 domain and proline-rich sequences in SOS. These Grb2–SOS complexes then associate with tyrosine kinase receptors through binding of the SH2 domain of Grb2 to tyrosine residues in growth factor receptors that become phosphorylated following ligand binding and cellular activation. The tripartite complex of receptor/Grb2/SOS aids in translocating SOS from the cytoplasm to the plasma membrane where it is placed in close proximity to its target, Ras, converting Ras from the GDP to the biologically active, GTP-bound state. Another adaptor protein, Shc, contains an SH2 domain that has been shown to bind to tyrosine-phosphorylated receptors in nonskeletal cells (Pelicci *et al.*, 1992). Shc also contains an additional phosphotyrosine-binding domain (PTB) that is capable of interacting with other phosphorylated proteins (Kavanaugh and Williams, 1995). Subsequent to cytokine binding, Shc becomes tyrosine phosphorylated and can interact with phosphorylated cytokine receptors, thereby enabling its interaction with Grb2 and activation of the Ras pathway. OSM stimulation of human osteoblasts results in tyrosine phosphorylation of Shc (Horowitz and Levy, 1995). The presence of tyrosine-phosphorylated MAPK supports the idea that family members may be stimulating the Ras pathway as well as the JAK/STAT signaling pathway. The tyrosine kinases that are responsible for phosphorylating Shc upon cytokine stimulation may be members of the JAK family or other cytoplasmic tyrosine kinases such as the src-family of tyrosine kinases.

As with other types of cytokine signaling, it is as important, if not more so, to turn off the signaling cascade as it is to turn it on. This can be accomplished by the activation, through phosphorylation on tyrosine residues, of protein tyrosine phosphatases. Once activated these proteins dephosphorylate other phosphorylated proteins, in particular, tyrosine kinases, resulting in deactivation. As mentioned previously, protein tyrosine phosphatases dock to phosphorylated tyrosine residues on the cytoplasmic domains of the cytokine receptor through their SH2 domains, resulting in phosphorylation of the phosphatase. Although there is clear evidence that protein tyrosine phosphatases are activated in response to the LIF/IL-6 family of cytokines (PTPase, Shp1) few if any direct data are available as to whether this negative regulation occurs in bone cells (Stahl *et al.*, 1995). In addition to the negative regulatory activity of phosphatases, a new family of proteins has been recently identified that can inhibit JAK-STAT signaling. The first member of this family (CIS/CIS1) inhibited the signaling of IL-3 and erythropoietin (Yoshimura *et al.*, 1995). A second member of the family was identified and is referred to as JAB (JAK-binding protein), SOCS1 (suppressor of cytokine signaling), or SSI-1 (STAT-induced STAT inhibitor) (Endo *et al.*, 1997; Naka *et al.*, 1997; Starr *et al.*, 1997) and is a negative regulator of IL-6 signaling. At present seven members of this family have been identified. In some cases (JAB and CIS3) inhibit the kinase activity by directly binding to the JAKs and in others cases (CIS/CIS1) the STATs are inhibited. Thus, ligand binding results not only in activation of the signaling pathway, but also in its inactivation by a negative feedback loop. Whether this family of proteins downregulates cytokine activation in bone cells remains to be determined.

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Part II

Molecular Mechanisms of Metabolic Bone Disease.

Chapter 59

Skeletal Effects of Nitric Oxide: Novel Agent for Osteoporosis

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INTRODUCTION

Nitric oxide (NO) is a water-soluble, gaseous radical molecule that moves freely across cell membranes. It is enzymatically produced by the oxidation and cleavage of one of the terminal nitrogen atoms of the amino acid L-arginine. The reaction is dependent on electrons donated by the cofactor NADPH and requires oxygen, yielding L-citrulline as a co-product (Fig. 1). Nitric oxide is involved in a variety of physiological and pathophysiological processes including vasodilatation, neurotransmission, the cytotoxic effects of various pathogens, and the genesis of inflammatory diseases. These broad-ranging actions are determined largely by the site, the rate, and the quantity of NO generated, and by the nature of the environment into which it is released. The reactivity of NO is influenced in particular by the presence of reactive oxygen intermediates and the activity of anti-oxidant defense systems (Moncada and Higgs, 1993; Knowles and Moncada, 1994; Gross and Wolin, 1995). Generation of NO from conversion of L-arginine to L-citrulline is illustrated in Fig. 1.

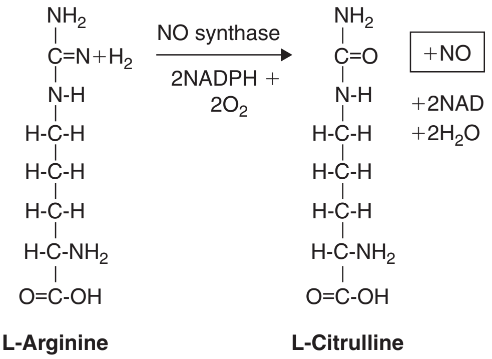

FIGURE 1 Generation of nitric oxide (NO) from L-arginine via nitric-oxide synthase enzyme.

A family of three related enzymes, the nitric oxide synthases (NOS), regulates the synthesis of NO. These are characterized as a neuronal form (type 1; nNOS) originally isolated from brain, an endothelial form (type 3; eNOS) originally isolated from bovine aortic endothelial cells (Pollock *et al.*, 1991), and an inducible form (type 2; iNOS) originally isolated from murine macrophages (Xie *et al.*, 1992). Both eNOS and nNOS are expressed constitutively and are characterized by highly regulated, rapid but low-output NO production that has a tonic physiological function (Moncada and Higgs, 1993; Knowles and Moncada, 1994). For example, when a compound that NOS is administered intravenously (e.g., L-NAME), one could observe an instant rise in blood pressure in animals (Yallampalli and Wimalawansa, 1998). Table I illustrates the differences and commonalities of three NOS isoenzymes.

Conversely, iNOS is generally not active under normal physiological conditions, but can be induced in a number of different cell types in response to infection, inflammation, or traumatic injury (Moncada and Higgs, 1993; Knowles and Moncada, 1994; Gross and Wolin, 1995). In contrast to rapid activation and deactivation of the constitutive enzymes eNOS and nNOS, iNOS expression is induced over several hours, requiring transcription of mRNA and *de novo* protein synthesis. Once activated, iNOS is capable of generating sustained high levels of NO locally over many hours (Gross and Wolin, 1995). In these circumstances NO can exacerbate the inflammatory response as seen in septic shock and rheumatoid arthritis, and it may also contribute to localized cell and tissue damage (Gross and Wolin, 1995).

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TABLE I Chromosomal and Cellular Localization of NOS Isoenzymes, and Their Activities[a]

NOS Type	Name	Chromosomal Location	Predominant Location	NO Output	Calcium-Dependent Activation
NOS-1	nNOS (bNOS) (*Constitute*)	12	Brain NANC neurons	Low	Dependent Cytosolic/membrane
NOS-2	iNOS inducible	17	Macrophages Neutrophils	High	Independent Cytosolic
NOS-3	eNOS (*Constitute*)	7	Endothelium Smooth muscles	Low	Dependent Membrane bound

[a] *Adapted from* Wimalawansa (2008).

Although nNOS, eNOS, and iNOS catalyze the same reaction to generate NO from L-arginine, they are distinct enzymes. Each is the product of a separate gene, and they share only 50% to 60% homology. In addition, each is subject to widely differing mechanisms of regulation (Moncada *et al.*, 1991; Hukkanen *et al.*, 1999). General characteristics of NOS isoenzymes are presented in Table I and in more detail in Table II. All three isoforms exhibit a bidomain structure containing consensus sequences along the C-terminal domain for binding for several cofactors including NADPH, FAD, FMN, and calmodulin. The arginine-binding site is retained within the N-terminal domain, which also contains a heme group and a consensus site for binding tetrahydrobiopterin. All cofactors are required for full activity and in their native, catalytically competent state, all three isoforms are homodimers (Griffith and Stuehr, 1995).

The activities of the constitutive NOS enzymes (nNOS and eNOS) are also dependent on mobilization of intracellular Ca^{2+}. Comparisons of sequence homologies reveal that NOS enzymes bear resemblance to cytochrome P450 reductase, sharing around 60% sequence similarity along their C-terminal domain (Bredt *et al.*, 1991; Griffith and Stuehr, 1995). This has been instrumental in elucidating the catalytic mechanism of NOS showing that the C-terminal domain, like cytochrome P450, is essentially an electron transporter. Bound calmodulin acts as a gate to regulate flow of electrons from the C-terminal domain to the N-terminal domain, where they are donated to the heme group, and their energy is used to drive the generation of NO (Griffith and Stuehr, 1995). Whereas the C-terminal domains of NO synthase isoenzymes are very similar, the N-terminal domains exhibit some variations. Most notably, eNOS possesses consensus sequences for myristoylation/palmitoylation at its N terminus and is important in determining its subcellular trafficking, locating the enzyme to membranes and in particular the caveolae (Garcia-Cardena *et al.*, 1996).

The molecular regulation of iNOS is particularly complex and occurs at several sites in the gene expression pathway with both transcriptional and post-transcriptional mechanisms (Ganster and Geller, 2000). Expression of iNOS can be induced by various "proinflammatory" cytokines including tumor necrosis factor alpha (TNF-α), interleukin 1-beta (IL-1β), and interferon-gamma (IFN-γ), and the effects of these inductive stimuli are mediated by the NF-κB and Jak-STAT signaling pathways (Damoulis and Hauschka, 1997; Ganster and Geller, 2000). Downregulators of iNOS expression include glucocorticoids such as dexamethasone, which inhibits cytokine-induced iNOS mRNA at the transcriptional level (Ganster and Geller, 2000). On the other hand, the cytokine TGF-β downregulates iNOS expression at the post-transcriptional level by inducing instability in iNOS mRNA. There is also evidence for a negative feedback loop whereby NO downregulates iNOS gene expression as well as inhibits the activity of iNOS protein (Ganster and Geller, 2000).

Although the eNOS gene is considered to be constitutively expressed, there are a number of additional factors that exert positive or negative effects on its expression. The activity of eNOS is stimulated by a number of agonists including estrogen, vascular endothelial growth factor (VEGF), and shear or mechanical stress (Klein-Nulend *et al.*, 1998; Fox *et al.*, 1996). In fact VEGF expression is stimulated by NO (Frank *et al.*, 1999). Also involved are activation of various signaling pathways, particularly those resulting in mobilization of intracellular Ca^{2+} and phosphorylation of eNOS (Gratton *et al.*, 2000). It is clear that in addition to stimulating the activity of eNOS protein, shear stress, estrogen, VEGF (Frank *et al.*, 1999), as well as TGF-β and fibroblast growth factor (FGF), upregulate eNOS expression at the level of transcription. Conversely, TNF-α and hypoxia downregulate eNOS gene expression by inducing instability in eNOS mRNA (Kleinert *et al.*, 2000).

Nitric oxide donors such as nitroglycerine and nitrates have been used as therapeutic agents for the past century, and the only significant adverse effect is headache. When the body cannot generate adequate amounts of NO for its biological homeostasis, administration of

TABLE II General Molecular and Biochemical Characteristics of NOS Enzymes and Their Association with Bone

	nNOS [NOS I]	iNOS [NOS II]	eNOS [NOS III]
Molecular weight (kDa)	160 Active form ~260 homodimer	130 Active form ~260 homodimer	135 Active form ~260 homodimer
Gene size and structure	160 kb, 29 exons, 28 introns	21 kb, 26 exons, 25 introns	37 kb, 26 exons, 25 introns
Chromosome	12	17	7
Cell pro-type	Neurons	Macrophages	Endothelial
Subcellular localization	Cytosolic/membrane	Cytosolic	Membrane
Calcium/calmodulin dependent	+	−/+	+
Typical characteristics	Low output, regulated by protein activation	High output, transcription regulation	Low output, regulated by protein activation
Bone expression (mRNA/protein)	Equivocal	1.Osteoblasts (Ralston *et al.*, 1994; Hukkanen *et al.*, 1995; Helfrich *et al.*, 1997) 2.Osteoclasts (Brandi *et al.*, 1995; Fox and Chow, 1998; Helfrich *et al.*, 1997)	1.Osteoblasts (Raincho *et al.*, 1995; Fox and Chow, 1998; Armour and Ralston, 1998; O'Shaughnessy *et al.*, 2000) 2.Osteoclasts (Brandi *et al.*, 1995; Helfrich *et al.*, 1997) Osteocytes (Fox and Chow, 1998; Zaman *et al.*, 1999)

exogenous NO or prolongation of the actions of endogenous NO are practical methods of supplementation. Examples of the former are patients with angina pectoris, coronary artery disease, hypertension, osteoporosis, or gastrointestinal motility disorders; an example for the latter is erectile dysfunction in men (use of phosphodiesterase 5 inhibitors). Postmenopausal NO deficiency is rectified by hormone replacement therapy, enhancing local production of NO (Wimalawansa, 2007). Declining local NO production secondary to estrogen deficiency in postmenopausal women and perhaps in older men could be one of the key reasons for age-related increased incidences of cardiovascular events and sexual dysfunction. Thus, in addition to supplementation of NO compounds in acute situations such as alleviating angina and erectile dysfunction, chronic NO therapy may potentially lead to a decrease in cardiovascular event rates and improvement in urogenital as well as skeletal health.

During the 1980s, NO was identified as a significant biological signaling molecule that regulates a wide range of important cellular physiological functions. Discovery of NO as the endothelium-derived relaxing factor has opened the door to a wide research arena (Angus and Cocks, 1989; Ignarro *et al.*, 1981; Moncada *et al.*, 1991). Nitroglycerine and nitrates are common heart medications that dilate blood vessels, decrease blood pressure, and improve circulation, and control angina and heart failure. Nitric oxide-donors are cost-effective and have beneficial effects in multiple body systems. Administration of exogenous NO donor compounds are a practical way to supplement NO.

Circulating NO products are significantly lower in postmenopausal women compared to premenopausal women (Stacey *et al.*, 1998; Rosselli *et al.*, 1995), and this is corrected after initiation of hormone replacement therapy (HRT; Rosselli *et al.*, 1995). Because menopause can lead to NO deficiency, there is a plausible biological basis for use of NO replacement or supplementation therapy in menopause. Studies have shown that the beneficial effects of estrogen on bone are predominantly mediated via NO (Wimalawansa *et al.*, 1996). Therefore, it makes sense to supplement NO directly for postmenopausal women, because HRT has potential (albeit small) adverse effects in postmenopausal women as reported from the Women's Health Initiative study.

NITRIC OXIDE BIOCHEMISTRY

Nitric oxide is synthesized in biological systems by a complex set of enzymes, the nitric oxide synthases (NOS; Bredt and Snyder, 1990; see Table 1). Smaller quantities of NO are also produced by non-enzymatic chemical reductions of inorganic nitrate and during acidic-reducing conditions (Weitzberg and Lundberg, 1998). Cyclic guanosine

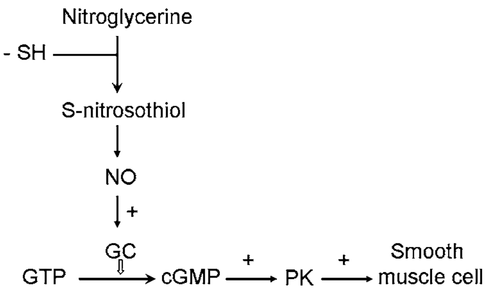


FIGURE 2 The necessity of the thiol group (–SH) in activation of the nitroglycerine molecule to release NO at target tissues.

monophosphate (cGMP) is synthesized from the nucleotide guanosine triphosphate (GTP) by the enzyme guanylate cyclase (Muscara *et al.*, 1998, Murad *et al.*, 1978). Cyclic GMP serves as the second messenger for several key compounds and peptides such as atrial natriuretic peptide (ANP) and NO, and it even elicits the light response by the rods in the retina. Some of the effects of cGMP are mediated through protein kinase G (PKG), a cGMP-dependent protein kinase that phosphorylates target proteins in cells.

Glyceryl trinitrate (GTN), or nitroglycerine, is one of the oldest synthetic drugs that are still widely used in clinical practice. It releases its active moiety NO or an NO-congener such as *S*-nitrosothiol (SNO), within or very close to the cell membranes of target cells such as vascular or penile smooth muscle cells. This results in activation of guanylate cyclase (GC) leading to smooth muscle cell relaxation (Munzel, 2001). Sulfhydryl groups (–SH) are essential for release of the NO moiety from nitroglycerine (Fig. 2). The sulfhydryl requirement for NO release in cell relaxation can explain in part nitrate tolerance: that is, the loss of clinical efficacy over a short period of time, especially with frequent administration of nitroglycerine (Carini *et al.*, 2001). This is a major problem for long-term use of nitrates (Munzel, 2001; Nakamura *et al.*, 1999) but can be overcome by intermittent dosing of nitrates. Figure 2 illustrates the requirement of thiol (–SH) groups containing compounds for activation of nitroglycerine to releasing NO at local target sites.

Figure 3 illustrates the several potential mechanisms involved in the development of nitrate tolerance in humans (i.e., losing its efficacy) *in vivo*. Attention to these factors is necessary to overcome this difficulty during longer-term administration of nitroglycerine to maintain its intended actions (efficacy).

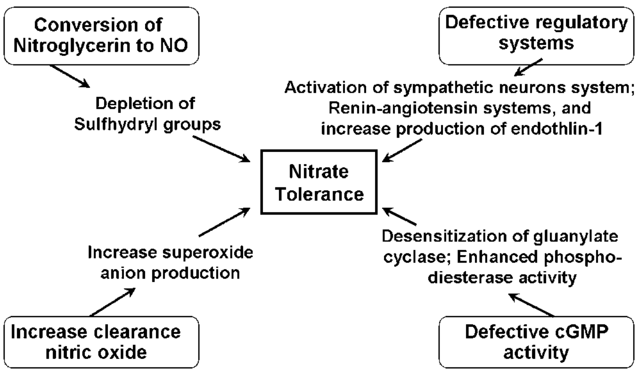

Conversion of Nitroglycerin to NO
Defective regulatory systems
Depletion of Sulfhydryl groups
Activation of sympathetic neurons system; Renin-angiotensin systems, and increase production of endothlin-1
Nitrate Tolerance
Increase superoxide anion production
Desensitization of gluanylate cyclase; Enhanced phospho-diesterase activity
Increase clearance nitric oxide
Defective cGMP activity

FIGURE 3 Potential methods of developing desensitization to nitroglycerine and nitrates (Wimalawansa, 2008).

The signaling functions of NO begin with its interactions with guanylate cyclase on cell membranes, or following diffusion into cells. The binding sites can be either a metal ion in the protein, or one of its sulfur atoms: e.g., on a cysteine (Wiesinger, 2001; Luo and Cizkova, 2000). In either case, binding triggers an allosteric change in the protein that in turn triggers the formation of a "second messenger" within the cell. The most common protein target for NO is guanylate cyclase, the enzyme that generates the second messenger cGMP (Murad *et al.*, 1978). In the presence of hemoproteins, such as hemoglobin or myoglobin, NO may promote or inhibit oxidative reactions, depending on the relative fluxes of O_2^-, H_2O_2, and NO (Jourd'heuil *et al.*, 1998). This balance can be a critical event in protection of tissue damages associated with a variety of pathological conditions, such as postischemic myocardial damage, chronic smoking, post-traumatic brain injury, hemorrhagic disorders, and chronic inflammation.

NO SIGNALING

Biology and Chemistry of NO

Although NO might be regarded chemically as a simple molecule, its biochemistry and its actions in biological systems are often complex, and this accounts in part for the wide range of biological processes that are influenced by NO. Nitric oxide products represent several related species, including its higher oxides and derived oxidants. For the purpose of this review it is necessary to consider only the NO-derived oxidant peroxynitrite, with all other actions generalized as NO.

Nitric oxide is eventually oxidized to nitrite and nitrate in biological systems. In aqueous buffers and culture conditions, nitrite is the principal oxidation product of NO (Ignarro *et al.*, 1993), whereas *in vivo* NO is in general almost completely oxidized to nitrate and excreted. These end products have proved to be useful markers for evidence of NO biosynthesis within biological samples (Wimalawansa, 2007). Nitrate and nitrite can be detected by the Greiss reaction, which relies on the reduction of nitrate to nitrite after the addition of the enzyme nitrite reductase or a metallic catalyst to the sample, the resultant color changes being then detectable spectroscopically (Green *et al.*, 1982; Stuehr and Marletta, 1987). This technique, however, is quite insensitive, requiring the accumulation of micromolar

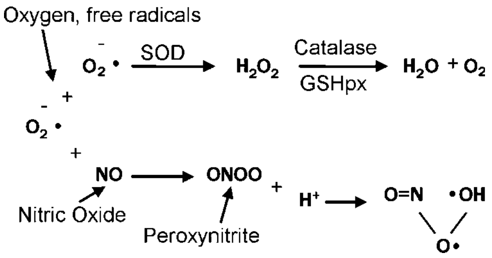


FIGURE 4 Generation of peroxynitrite from nitric oxide.

amounts of nitrite. For greater sensitivity, a chemiluminescence analyzer can be used to measure NO levels. In this assay, a strong reducing agent is used to reduce the nitrite or nitrate present in the sample of interest back to NO. The NO is then reacted with ozone, which results in the generation of light or chemiluminescence, with the amount of light being directly proportional to NO levels (Archer, 1993).

Perhaps one of the better-understood reactions of NO in biological systems is its combination with superoxide anion ($O^{-}{}_{2}$) to form peroxynitrite anion ($ONOO^{-}$; Beckman *et al.*, 1990). Both molecules show a high affinity for each other, the reaction proceeding several-fold more quickly than catalysis of superoxide anion dismutation by superoxide dismutase (SOD). Free radical NO itself is a potent oxidant (Freeman, 1994). Peroxynitrite is a very powerful oxidant and can either augment or abrogate some of the biological actions associated with NO (Beckman *et al.*, 1994a). Induction of iNOS together with the formation of peroxynitrite is thought to play an important part in osseous prosthetic failure (Hukkanen *et al.*, 1997). Figure 4 demonstrates the alternate path of NO metabolism leading to generation of peroxynitrite.

Cellular Targets of NO

NO does not require specific receptors to communicate its physiological actions; its small size and lipophilic nature allow it to traverse plasma membranes without the need for a specific transporter. In biological systems, the principal cellular targets for NO are proteins containing transition metal ions. Soluble guanylate cyclase is perhaps the most significant of these metal-ion proteins, with NO demonstrating high affinity for the heme moiety of the enzyme, forming a transient and reversible nitrosyl-heme group. Binding of NO induces a conformational change in the enzyme stimulating the catalysis of cGMP formation.

Nitric oxide is also rapidly bound to free hemoglobin, resulting in the formation of methemoglobin and nitrate and termination of the activity of NO. This quenching action explains in part the rise in blood pressure in patients following intravascular hemolysis. It has been proposed that hemoglobin acts as a biological "sink" for NO (Gross and Wolin, 1995), but Jia *et al.* (1996) reported that NO can *S*-nitrosylate hemoglobin to form *S*-nitrosohemoglobin, and this, on the other hand, is important for preserving or potentiating the vascular actions of NO. *S*-Nitrosylation of proteins by NO is also an important intracellular signaling mechanism, activating for example the small G-protein p21^{ras} and the ensuing MAP kinase cascade (Lander, 1997).

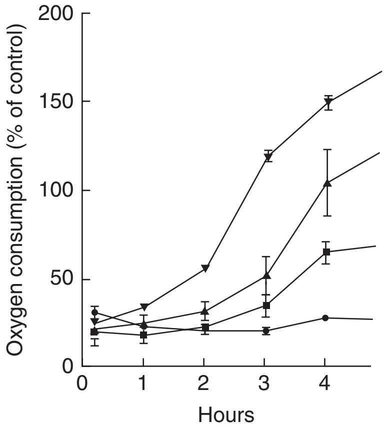


FIGURE 5 Effects of exposure to the NO donor, DETA-NO on oxygen consumption of J774 cells (Orsi *et al.*, 2000).

Nitric oxide will also bind and inhibit various iron-sulfur (Fe-S) cluster proteins (Hibbs *et al.*, 1990). Many Fe-S proteins play a significant role in energy metabolism as well as in growth, and NO therefore is able to affect cellular cytotoxicity and cytostasis (Freeman, 1994). In particular, NO inhibits key enzymes involved in mitochondrial respiration, including mitochondrial aconitase, NADH-ubiquinone oxidoreductase, and NADH-succinate oxidoreductase (Hibbs *et al.*, 1990; Stuehr and Nathan, 1989), as well as ribonucleotide reductase (Kwon *et al.*, 1991), which is the rate-limiting enzyme in DNA synthesis.

Following prolonged exposure to NO, cells exhibit a decrease in oxygen utilization as a consequence of two synergistic actions: persistent inhibition of mitochondrial oxygen consumption and production of a hypoxic microenvironment following extraction of oxygen from extracellular medium. It is uncertain whether or not these striking effects occur *in vivo,* but should they do so, they are likely to be associated with pathological effects (Beltran *et al.*, 2000; Orsi *et al.*, 2000; Fig. 5). However, this could in part explain how myocardial oxygen consumption is decreased (leading to improvements in angina and heart failure) following administration of therapeutic amounts of NO supplements to cardiac patients.

Nitric oxide and superoxide anion demonstrate a strong affinity, reacting to form peroxynitrite (Beckman *et al.*, 1990). Peroxynitrite is a powerful oxidant with the potential to cause tissue and cellular damage; its products are potent nitrating agents with a particular affinity for protein tyrosine residues (Beckman *et al.*, 1994a; Freeman, 1994) that is capable of interfering with tyrosine kinase signaling

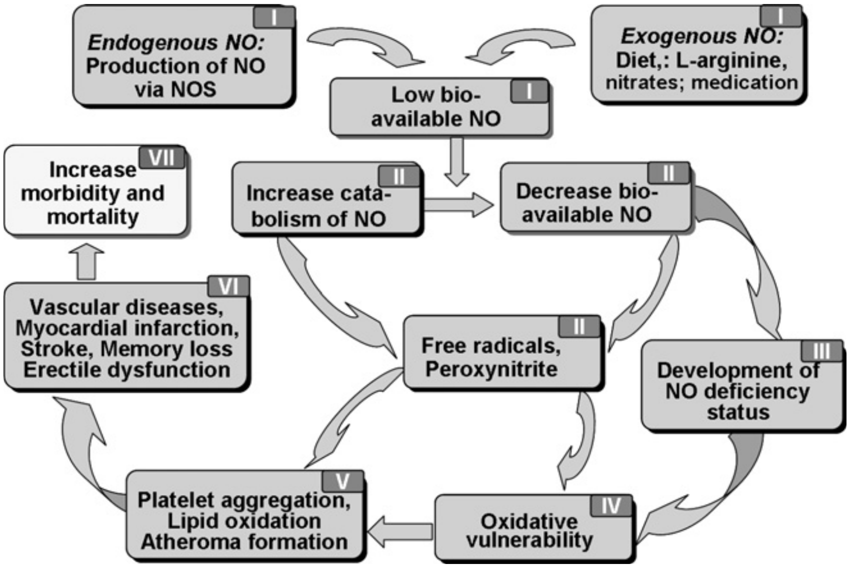

Endogenous NO:
Production of NO via NOS
I
Exogenous NO:
Diet,: L-arginine, nitrates; medication
I
Low bio-available NO
I
Increase morbidity and mortality
VII
Increase catabolism of NO
II
Decrease bio-available NO
II
Vascular diseases, Myocardial infarction, Stroke, Memory loss Erectile dysfunction
VI
Free radicals, Peroxynitrite
II
Development of NO deficiency status
III
Platelet aggregation, Lipid oxidation Atheroma formation
V
Oxidative vulnerability
IV

FIGURE 6 Cycle of disease vulnerability due to relative nitric oxide deficiency (production or bio-availability): Interactions and the balances between NO generation, free radicals, scavenging molecules, and disease statuses.

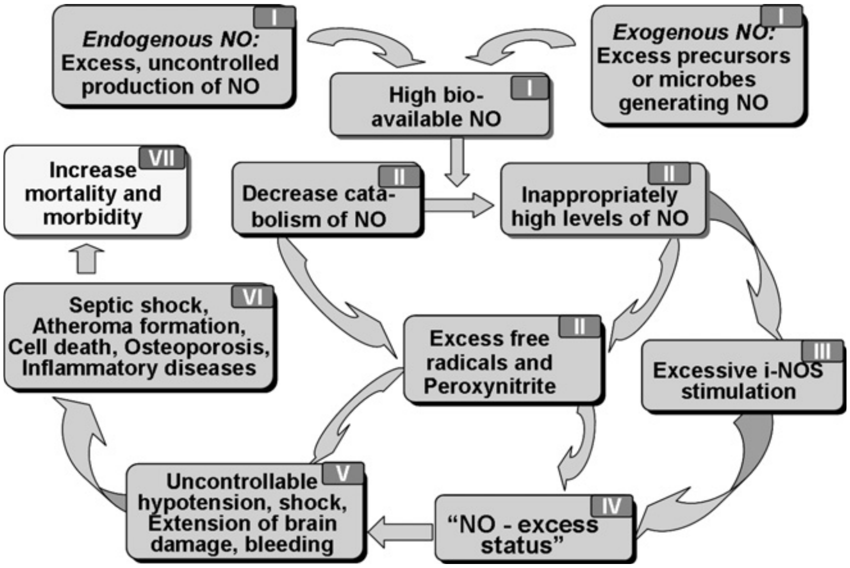

Endogenous NO:
Excess, uncontrolled production of NO
I
Exogenous NO:
Excess precursors or microbes generating NO
I
High bio-available NO
I
Increase mortality and morbidity
VII
Decrease catabolism of NO
II
Inappropriately high levels of NO
II
Septic shock, Atheroma formation, Cell death, Osteoporosis, Inflammatory diseases
VI
Excess free radicals and Peroxynitrite
II
Excessive i-NOS stimulation
III
Uncontrollable hypotension, shock, Extension of brain damage, bleeding
V
"NO - excess status"
IV

FIGURE 7 Vicious cycle of vulnerability to high levels of NO: diseases secondary to excessive generation, or synthesis of inappropriately high levels of NO. Figure also demonstrates the interactions and imbalances between NO productions and free radicals enhancement leading to development of serious disease statuses.

pathways. Evidence for endogenous peroxynitrite activity has been provided through the immunochemical (Beckman *et al.*, 1994b) and biochemical detection of nitrotyrosine species (Kaur and Halliwell, 1994) in tissues and fluids.

Nitric oxide or NO species, in particular peroxynitrite, can damage DNA bases, either by oxidation or by base deamination. This damage to DNA can directly stimulate the activation of the enzyme poly-ADP ribose polymerase (PARP), which facilitates DNA repair, by a process that utilizes both NADH and ATP (Zhang *et al.*, 1994). However, prolonged activation of PARP and in particular the continued consumption of NADH and ATP can lead to rapid energy depletion and result in cell death. This may be one mechanism by which generation of high levels of NO locally is able to induce cell death by enhanced apoptosis. Increased production of NO also correlates with increased expression of the proapoptotic proteins P53 and caspases and downregulation of bc12.

Over the years, the role of NO in disease status has been under-estimated. Figure 6 illustrates the interactions and the balance between NO generation, its bioavailability, free radicals, scavenging molecules such as free hemoglobin, and disease statuses. Impairment of generation of NO, or its excessive degradation leading to sub-optimal levels of active No moiety can contribute to the development of several disease status.

Figure 7 illustrate the development of vicious cycle of disease processes secondary to excessive generation leading to inappropriately higher levels of NO (usually synthesized via NOS enzyme. Similar process can also be sustained via exogenous administration of high doses of nitrates. Figure also demonstrates the interactions and balances between NO productions, free radicals enhancement leading to development of serious disease statuses.

NITRIC OXIDE IN BONE

NOS Isoforms

A large number of antibodies and probes are now available to investigate the expression and localization of NOS enzymes. On the whole, many of the studies performed on bone tissues and cultured cells have resulted in similar descriptions of NOS expression and localization (Helfrich *et al.*, 1997). However, there has been some controversy, with some groups failing to find expression or localization where others had. In part, such discrepancies can be reconciled by the lack of standardization between studies. Many of the antibodies raised against different NOS enzymes may be species- or even tissue-specific, and some may be cross-reactive (Coers *et al.*, 1998). This is also confounded by variations in the cell lines studied, differences in culture conditions, or differences in the differentiation status of the cultures.

A number of RT-PCR and immunocytochemical investigations performed on whole bone preparations and cultured bone-derived cells from both human and rodents confirmed that eNOS and iNOS are the principal NOS isoforms expressed in bone (Helfrich *et al.*, 1997; Fox and Chow, 1998; MacPherson *et al.*, 1999). Although, some investigators have shown expression of nNOS mRNA in bone tissues by RT-PCR (Pitsillides *et al.*, 1995; Helfrich *et al.*, 1997), there is little evidence for the presence of nNOS protein in bone. However, nNOS gene knockout mice have specific bone phenotypes (Moradi-Bidhendi *et al.*). Whether this is due to the nNOS-containing nerves present within the bone regulating bone cell activity or bone blood flow has not been addressed yet. As discussed later, nNOS knockout mice also have altered responses to osteogenic hormones such as estrogen.

From the studies performed to date, there is consensus that under normal, physiological conditions eNOS is the predominant NOS enzyme expressed in the bone and is present in endothelial cells within the bone tissues, bone marrow stromal cells, osteoblasts, osteocytes, and osteoclasts (Brandi *et al.*, 1995; Helfrich *et al.*, 1997; Fox and Chow, 1998; MacPherson *et al.*, 1999). Expression of eNOS varies according to developmental stage, with eNOS mRNA and protein being most abundant in the bones of neonates and subsequently declining with age (Hukkanen *et al.*, 1999). Robust eNOS immunostaining has been demonstrated in cuboidal columnar osteoblasts lining trabecular and endosteal bone from neonates and to a lesser extent in osteocytes and multinuclear osteoclasts located in resorption lacunae.

Expression of NOS Isoforms

Expression of eNOS in osteoblasts, osteocytes, and osteoclasts becomes more diffuse with age. eNOS enzyme is also present in hypertrophic and degenerating chondrocytes of the epiphyseal growth plate of all ages, but particularly in the first 4 weeks of the neonatal period. Chondrocytes within the proliferation zone, however, are generally negative for eNOS expression and staining. NOS expression during fetal development in murine and human fetal long bones (mouse embryonic day 14 and human 8- to 12-week-old abortuses) has shown robust immunostaining, principally in hypertrophic and degenerating chondrocytes, but not in the proliferation zones. There is multiple evidence to suggest that NO is directly involved in osteoblast function and cell differentiation (Danziger *et al.*, 1997; Inoue *et al.*, 2000; Hikiji *et al.*, 1997; Wimalawansa, 2008). On the other hand, iNOS induced excess production of NO can lead to osteoblast apoptosis (Damoulis and Hauschka, 1997; Wimalawansa, 2007).

iNOS is generally not thought to be expressed in unstimulated bone cells and bone tissues, and expression is generally associated with pathological conditions. Nevertheless, some researchers have reported the presence of low levels of iNOS transcripts in unstimulated whole bone and cultured osteoblast-like cells by RT-PCR (Hukkanen *et al.*, 1995, 1999; Riancho *et al.*, 1995; Helfrich *et al.*, 1997), and others have described low-level iNOS expression in untreated cultures of a human preosteoclastic cell line (Brandi *et al.*, 1995). There is also evidence to indicate that in some tissues including bone, iNOS is expressed transiently and likely to have a physiological function. Immunostaining for iNOS protein was seen in sections of neonatal bone, but not in older animals, and localized to both osteoblasts and osteoclasts, where it was compartmentalized toward the ruffled border. A role for iNOS in regulating osteoclast activity was also suggested on the basis of *in vivo* administration of NOS inhibitors (Kasten *et al.*, 1994) that was subsequently supported by additional studies (Tsukahara *et al.*, 1996; Turner *et al.*, 1997).

iNOS is expressed in bone during the neonatal period. Expression of iNOS in bone later in life is usually associated with inflammatory stimulus. The expression of iNOS mRNA and protein have been demonstrated in cultured rodent and human osteoblast-like cells after cytokine stimulation (Ralston *et al.*, 1994, 1995; Hukkanen *et al.*, 1995). The level of expression of iNOS by osteoblasts is also quantitative and correlates with the type and combination of cytokine stimuli (Damoulis and Hauschka, 1994).

In general, combinations of two or more cytokines are more potent than single cytokines in stimulating iNOS activity. The synergism between IFN-γ, TNF-α, and IL-1β is particularly effective for iNOS induction in osteoblasts (Hukkanen *et al.*, 1997). Moreover, rodent osteoblasts seem to be more responsive to cytokine-stimulated iNOS induction than human osteoblasts.

Cytokines and iNOS

The evidence for cytokine-stimulated iNOS expression by osteoclasts is less definitive. Some researchers have shown marked iNOS expression in rodent and avian osteoclastic cell lines and comparatively weaker expression in a human preosteoclastic cell line after cytokine stimulation *in vitro* (Brandi *et al.*, 1995; Hukkanen *et al.*, 1995, 1997). Others have described marked cytokine-induced iNOS expression in an avian osteoclastic cell line (Sunyer *et al.*, 1996). Conversely, some failed to detect iNOS by *in situ* hybridization or immunocytochemistry in osteoclast cultures even after cytokine stimulation (Helfrich *et al.*, 1997). The reasons for such differences are unclear, but there are corollaries with other cells of leukocyte lineage. Induction of iNOS expression can be readily achieved in rodent monocyte macrophages after cytokine challenge, and the response is often exaggerated, whereas induction of iNOS in human monocyte macrophages is much more tempered (Sakurai *et al.*, 1995). Data from author's laboratory indicate that stimulated osteoblast cells can mediate osteoclastic iNOS induction. Others have reported that NO stimulated cGMP production in osteoclasts (Dong *et al.*, 1999). However, iNOS-induced higher local expression of NO together with cytokines is thought to be responsible for osteoblast apoptosis (Jilka *et al.*, 1998).

Human cells are also responsive to combinations of cytokines that are distinct from those described for rodent cells. One possible explanation for these idiosyncrasies, other than differences between cell lines or differentiation status, might lie with the "distinctness" of the human iNOS gene, which shares only 80% homology with the rodent gene. This has added relevance considering that the constitutive NOS isoforms (eNOS and nNOS) are very highly conserved, with greater than 93% homology between rodent and human.

Other important sites of iNOS expression include the articular joint, where iNOS is expressed and localized to chondrocytes, and the synovium of arthritic but not in normal subjects (Sakurai *et al.*, 1995), again suggestive of cytokine-driven expression of iNOS in chondrocytes. NO also activates cyclooxygenase enzymes (Salvemini *et al.*, 1993). Collectively, these observations demonstrate that NOS isoforms, in particular eNOS and iNOS, are widely expressed in bone tissue. These observations are consistent with that involvement of NO in many aspects of embryonic bone growth, modeling, and remodeling under both physiological and pathophysiological conditions.

NOS Gene-Deficient Mice

Gene knockout mice represent powerful tools for studying possible functions and targets of regulatory factors involved in physiological and pathological processes. Murine gene knockouts have been created for all three isoforms of NOS (Huang *et al.*, 1993, 1995; MacMicking *et al.*, 1995; Wei *et al.*, 1995). In all cases the mutant mice were found to be viable, fertile, and identical in general appearance but not behavior, to their wild-type counterparts.

Mice deficient in nNOS were the first knockouts to be created and revealed that the nNOS gene-deficient mice are largely resistant to brain damage induced by vascular stokes (Huang *et al.*, 1993), confirming the importance of nNOS-derived NO in mediating traumatic brain injury and stroke damage. It is also apparent that nNOS knockout male mice are aggressive (Nelson *et al.*, 1995). The observations of iNOS gene-deficient mice were also in keeping with what might have been expected, corroborating the participation of iNOS in mediating inflammatory responses with iNOS gene-deficient mice demonstrating attenuated microbicidal and tumoricidal responses (MacMicking *et al.*, 1995; Wei *et al.*, 1995).

The findings from mice deficient in eNOS are also in accord with what might have been expected. These mice had an elevated blood pressure, and aortic rings isolated from these animals did not display a relaxation response when challenged with acetylcholine. This demonstrated the important role of eNOS-derived NO in mediating vascular homeostasis (Huang *et al.*, 1995). Nevertheless, induction of a hypotensive effect following administration of a NOS inhibitor has been shown to be reversed by infusion of excess L-arginine (Huang *et al.*, 1995). This suggests that blockers of NO synthesis might have independent effects on the vasculature or that other isoforms of NO synthase contribute to the maintenance of blood pressure. The prime candidate for this is nNOS, which is known to be present in the vasomotor centers of the central nervous system and hence may be involved in neuronal regulation of blood pressure and in nerves supplying blood vessels.

NOS-Deficient Mice as a Model

NOS gene-deficient mice represent somewhat useful models for studying the role of NO in bone metabolism, circumventing the lack of isoform specificity of the current generation of NOS inhibitors and providing a rigorous test of previous proposed actions of NO on bone. It is inevitable that after inactivation of a NOS gene, there will be compensation by over-expression of other two NOS genes, clouding the interpretation from these knockout mouse models. Moradi-Bidhendi, *et al.* demonstrated that male nNOS knockout mice have a marked reduction in urinary nitrate (Fig. 8). In addition, female nNOS knockout mice fail to respond to the stimulatory effect of estrogen based on urinary nitrate

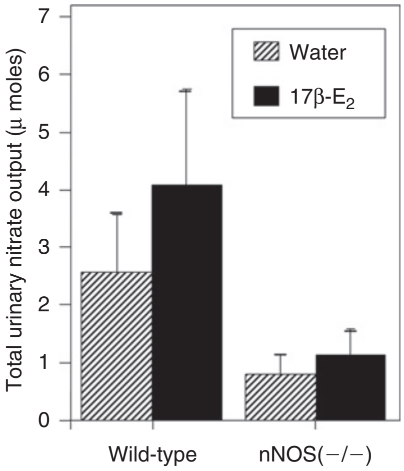


FIGURE 8 Marked difference of the total urinary nitrate output (mean) in c57/bI/6 wild-type mice versus nNOS null (–/–) knockout mice. nNOS(–/–) knockout mice showed a significantly lower nitrate output than wild-type controls. Animals were between 8 and 12 weeks of age and maintained on a low nitrate diet throughout the experiment. Values are mean±SEM, $n = 6$ (**$p < 0.01$) using Student's *t*-test (Moradi-Bidhendi, Mancini, Forte, Cafferkey, Benjamin, and MacIntyre).

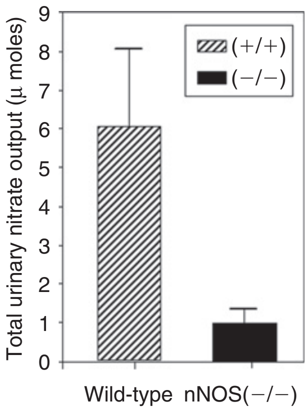


FIGURE 9 The mean urinary total nitrate output of c57/bI/6 wild-type and nNOS (–/–) knockout mice following administration of estrogen (administration of 10μg of 17/β-E$_2$), or placebo. Control c57/b1/6 mice showed a significant increase in urinary nitrate output in comparison to nNOS knockout mice, while there were change in the nNOS (–/–) mice. Animals were between 8 and 12 weeks of age and maintained on a low-nitrate diet throughout the experiment. Values are mean±SEM, $n = 6$ (**$p < 0.01$) using Student's *t*-test.

measurements (Fig. 9). Interestingly, male eNOS knockouts have urinary nitrate outputs similar to wild-type controls, and females respond normally to estrogen with a cyclic increase in urinary nitrate excretion. These findings imply that nNOS is mainly responsible for urinary nitrate and that nNOS is stimulated by estrogen, thus explaining the normal increase in nitrate after administration of estrogen to eNOS knockouts. Further, it is conceivable that the aggressive behavior of nNOS knockouts resembles the effect of estrogen deficiency on cerebral nNOS.

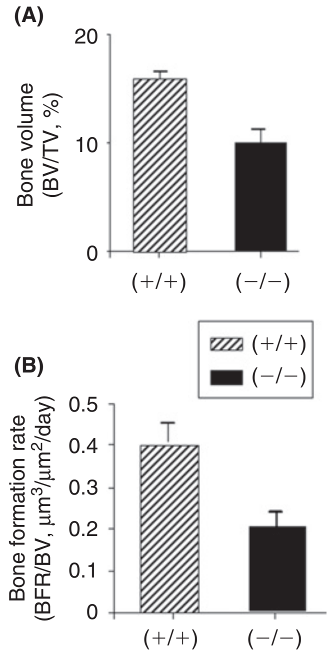


FIGURE 10 Histomorphometry analysis of femurs from 6-week-old eNOS gene knockout mice and age-matched wild types. Both bone formation rate and bone volume are significant reduced in eNOS knockouts. Values are mean±SEM, $n = 7$ (*$p < 0.05$; **$p < 0.01$).

eNOS knockout mice are viable and fertile, and in terms of gross physical appearance (stature, gait, and anatomy) are unremarkable, but aggressive behavior has been reported (Demas *et al.*, 1999). However, closer examination of the long bones demonstrated that both the femur and the tibia of eNOS knockouts were significantly shorter (1 to 2 mm) than the corresponding wild-type. Histomorphometry analysis of the same bones showed a marked reduction in bone formation rate as well as bone volume (Fig. 10). eNOS activity is likely to be required for the maintenance of physiological bone mass (Aguirre *et al.*, 2001; Armour *et al.*, 2001).

Interestingly, the consequences of eNOS gene deficiency were most pronounced in young (6- to 9-week-old) adults, and by 12 to 18 weeks bone phenotype was restored toward wild type. Regardless of eNOS expression, bone turnover was significantly higher in young mice than old mice, and similar observations have been made on rats and humans, demonstrating that bone modeling/remodeling activity is related to sexual maturity. Moreover, we have shown that eNOS expression correlates with skeletal development and is most abundant in the bones of neonates and young adults, decreasing markedly in older animals (Hukkanen *et al.*, 1999).

These data suggest that eNOS expression and activity is important during phases of rapid bone growth or turnover, such as in the period from neonate through to sexually mature adult and the attainment of peak bone mass. eNOS gene deficiency does not represent a severe or irreversible impediment to skeletal growth. Upregulation of eNOS activity is important in mechanical load-induced bone growth (Pitsillides *et al.*, 1995; Turner *et al.*, 1996; Klein-Nulend *et al.*, 1995; Zaman *et al.*, 1999) and fracture healing (Corbett *et al.*, 1999a, , 1999b; Diwan *et al.*, 2000). Thus, eNOS knockout mice are likely to exhibit defective or protracted bone repair mechanisms following traumatic injury. NO also seems to involved in control of expression of tartrate-resistant acid phosphatase (TRAP). Inhibition of osteoclast acid phosphatase markedly decrease bone resorption (Zaidi *et al.*, 1989). Overexpression of TRAP is associated with increased bone turnover (Angel *et al.*, 2000), and TRAP-deficient mice have an osteopetrotic phenotype (Hayman *et al.*, 1996).

Bone Cell Biology of NOS Gene Knockout Mice

Osteoblasts from eNOS knockouts, unlike wild-type cultures, were unresponsive to the mitogenic effects of estradiol over a range of concentrations, providing further evidence that the eNOS-NO signaling pathway is important in mediating the osteogenic actions of estrogen (O'Shaughnessy *et al.*, 2000). Moreover, these cells demonstrated an attenuated chemotaxis response and failed to migrate along a TGF-β gradient (Aguirre *et al.*, 2001)—the latter is a potent cytokine in recruiting osteoblasts to remodeling sites (Pfeilschifter *et al.*, 1990). Taken together, these data illustrate that distinct components of the osteoblast phase of the bone remodeling cycle are altered in eNOS knockout mice, strongly implicating NO-dependent signaling via eNOS in the regulation of osteoblast growth, differentiation, recruitment, and extracellular matrix synthesis. Studies by Armour and coworkers (Armour *et al.*, 2001) also showed that ovariectomized eNOS knockout mice have a significantly diminished anabolic response to high doses of exogenous estrogen.

There is evidence that bone vasculature can modify bone formation through intraosseous pressure (Kelly and Bronk, 1990) and factors released from endothelial cells including NO can modulate bone cell function (Collin-Osdoby, 1994). The potential impact of the persistent hypertension associated with eNOS gene deficiency (Huang *et al.*, 1995) on bone turnover needs to be thoroughly evaluated.

Studies *in vitro* and *in vivo* using iNOS gene knockout mice (van't Hof *et al.*, 2000) demonstrated that IL-1-induced NO production by iNOS was important for promoting osteoclast formation and activity. Moreover, osteoblast-bone marrow cocultures revealed that osteoblasts were the principal source of NO regulating osteoclast activity, particularly in osteoclast precursors, and was dependent on NFκB translocation and DNA binding. Importantly, the defects in IL-1-induced bone resorption could be reversed by exogenous supply of the NO donor SNAP (van't Hof *et al.*, 2000). Initial studies on iNOS knockouts have revealed marked reductions in bone formation and volume similar to the abnormalities described in eNOS knockouts (Aguirre *et al.*, 2001). However, the underlying mechanism is distinct and appears to relate to increased osteoclast number and activity and support the NOS inhibitor (iNOS; Kasten *et al.*, 1994; Tsukahara *et al.*, 1996; Turner *et al.*, 1997). iNOS knockout mice have a three- to fourfold compensatory increase in eNOS mRNA expression in whole bone preparations. Such a phenomenon is expected and is not unusual in NOS gene knockouts (Huang and Fishman, 1996).

NO Donor Compounds and Osteoporosis

Unlike some other disease entities, osteoporosis can be easily diagnosed and treated. Because osteoporosis leads to fracture, the keys to preventing fractures are early detection of the disease by measuring bone mineral density (BMD), a bone-healthy lifestyle, avoiding medications that contribute to bone loss, elimination of secondary causes of bone loss, prevention of falls and injuries, and effective use of antiosteoporotic agents (Wimalawansa *et al.*, 1997).

In the past, many postmenopausal women relied on HRT after menopause to reduce the risk of heart disease and osteoporosis fractures. However, the Women's Health Initiative (WHI) study confirmed that although it is highly effective in preventing fractures, HRT could also slightly increase the risk of stroke, heart disease, and breast cancer in some patients. Another barrier to HRT treatment is the high cost, breakthrough bleeding, and breast tenderness, especially in older women. Thus, finding a cost-effective alternative therapy for osteoporosis with few or no adverse effects would be very useful. One such exciting opportunity is to use NO donor therapy such as nitroglycerine; the original postulation of the role of NO in bone was demonstrated in 1987 (Fig. 11; Wimalawansa, 1988).

Role of Nitric Oxide in Bone Metabolism

During the past decade, significant advances have been made in understanding cellular mechanisms involved in bone metabolism leading to identification of novel therapeutic agents. These include those with antiresorptive (also known as anticatabolic) and anabolic agents. However, it is essential to consider multiple interactions of these chemical entities *in vivo* for proper understanding as well as to develop new therapeutic agents. Key interactions between cytokines, hormones, and NO in bone are illustrated in Fig. 12.

Nitric oxide has been shown to regulate osteoclasts (Dong *et al.*, 1999; Inoue *et al.*, 1995), cells responsible for bone resorption that belong to the monocyte lineage. Bone formation and resorption are key processes in the constant remodeling that occurs in bone tissue in order to keep it healthy and to repair bone microdamage. Uniquely, the NO donor nitroglycerine has a beneficial effect on controlling

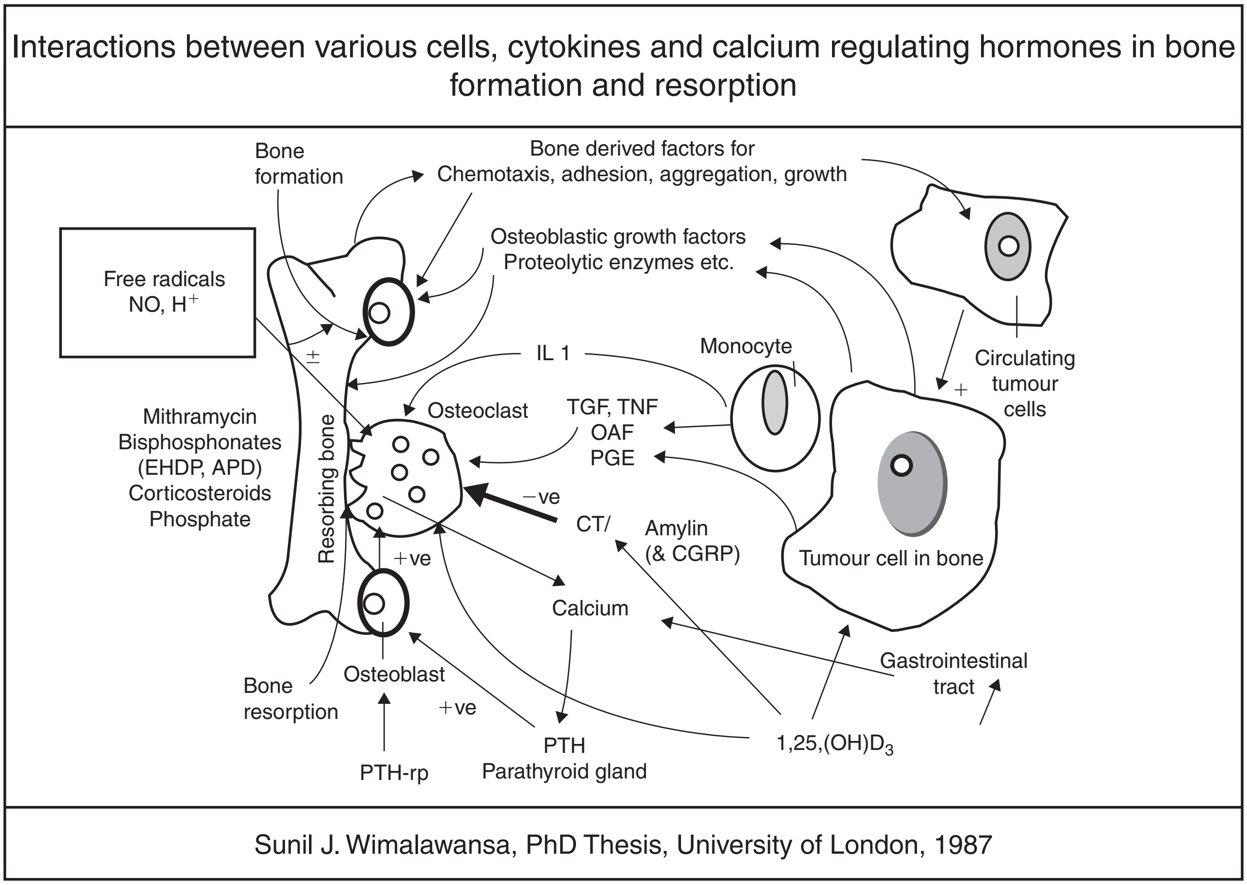

Interactions between various cells, cytokines and calcium regulating hormones in bone formation and resorption
Bone formation
Bone derived factors for Chemotaxis, adhesion, aggregation, growth
Osteoblastic growth factors
Proteolytic enzymes etc.
Free radicals
NO, H$^+$
±
IL 1
Monocyte
Circulating tumour cells
+
Osteoclast
TGF, TNF
OAF
PGE
Mithramycin
Bisphosphonates
(EHDP, APD)
Corticosteroids
Phosphate
Resorbing bone
−ve
CT/
Amylin
(& CGRP)
Tumour cell in bone
+ve
Calcium
Bone resorption
Osteoblast
+ve
Gastrointestinal tract
PTH-rp
PTH
Parathyroid gland
1,25,(OH)D$_3$
Sunil J. Wimalawansa, PhD Thesis, University of London, 1987

FIGURE 11 First postulation of involvement of NO in bone metabolism (Wimalawansa, 1988). NO = nitric oxide; PTH = Parathormone, PTH-rp = PTH related peptide; CT = calcitonin; PGE = Prostaglandin; CGRP = calcitonin gene-related peptide; D_3 = vitamin D; IL = interleukin; OAF = osteoclast activating factors.

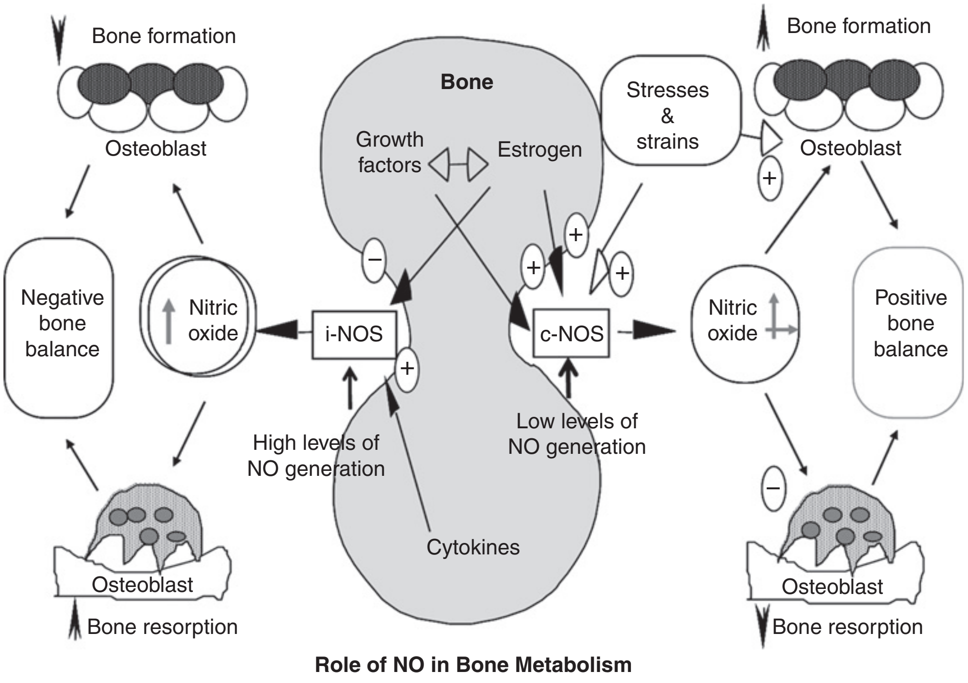

Bone formation
Osteoblast
Bone
Growth factors
Estrogen
Stresses & strains
Bone formation
Osteoblast
+
Negative bone balance
Nitric oxide
−
+
+
+
+
i-NOS
c-NOS
Nitric oxide
Positive bone balance
+
High levels of NO generation
Low levels of NO generation
Cytokines
−
Osteoblast
Bone resorption
Osteoblast
Bone resorption
Role of NO in Bone Metabolism

FIGURE 12 Key interactions between sex-steroid hormones, cytokines, and growth factors generating nitric oxide (NO) via iNO and c-NOS, and their effects on osteoblasts and osteoclasts (Wimalawansa, 2007).

bone resorption (decreased osteoclastic activity), while also having positive effects on bone formation (enhanced osteoblastic activity; Wimalawansa, 2007, 2008). For postmenopausal women who cannot tolerate or afford HRT treatment, nitroglycerine therapy may become an affordable and cost-effective option in the future (Wimalawansa, 2007).

Furthermore, supplementary studies demonstrated that nitroglycerine has beneficial additive effects on BMD when coadministered with some agents including vitamin D, calcitonin and bisphosphonates (Wimalawansa, 2008; D. Wimalawansa *et al.*, 2008). However, because the mechanism of action of these agents in bone cells is different from that of NO, it is not surprising that there is an added beneficial effect when any one of these agents are coadministered with a NO donor on the BMD. For example, the effects of statins on BMD reported to be additive to those of the bisphosphonate agent risedronate when these two agents are coadministered (Tanriverdi *et al.*, 2005). It appears that NO donors have the potential to treat bone loss and fragility in women (Wimalawansa *et al.*, 1996). Taken together, these data suggest that long-term therapy with NO will not only increase BMD (prevention of osteoporosis), but also likely decrease fracture rates (treatment of osteoporosis). Because the final common pathway of actions of sex-steroid hormones, in particular estrogen, and statins (Garrett *et al.*, 2001), as well as essential fatty acids, seems to be mediated via NO at least in bone cells, no such additive effect is expected. *In vitro* and *in vivo* animal data suggest positive effects of bone cell metabolism of estrogens and statins as well as essential fatty acids. Fig. 13 illustrates the postulated final common pathway of these quite different compounds on bone metabolism.

Osteoclast Effects

Osteoclasts express both eNOS and iNOS (Brandi *et al.*, 1995; Sunyer *et al.*, 1996; Helfrich *et al.*, 1997; Fox and Chow, 1998; MacPherson *et al.*, 1999), and accordingly, these cells demonstrate sensitivity to low physiological levels of basal NO synthesis generated via eNOS and nNOS, as well as with higher levels of NO synthesis via iNOS following stimulation by cytokines (Evans and Ralston, 1996). The effects of NO on osteoclasts are also biphasic; NO can either suppress or stimulate osteoclast-mediated bone resorption (see Fig. 12).

Some researchers have demonstrated that exposure of cultured osteoclasts to NO-donor compounds inhibit cell spreading and caused profound inhibition of bone resorption measured by the bone slice assay (Brandi *et al.*, 1995; MacIntyre *et al.*, 1991). Although the concentrations of NO used were supraphysiological, these findings have subsequently been confirmed by several additional *in vitro* and *in vivo* studies. Chronic *in vivo* administration of the NOS inhibitor compounds in rats, in particular aminoguanidine, was accompanied by marked reductions in bone mass (Kasten *et al.*, 1994; Tsukahara *et al.*, 1996; Turner *et al.*, 1997). These data suggest that NO is an endogenous inhibitor of osteoclast activity, and hence they are consistent with the profound inhibitory effect of NO donors on osteoclastic bone resorption (Wimalawansa, 1988; MacIntyre *et al.*, 1991; Kasten *et al.*, 1994). These findings will have profound impact not only on our understanding bone cell biology, but also on future drug developments. Figure 14 demonstrate the effects of low and high doses of NO donors on osteoclast-mediated pit formation on bone slices in compression compared to osteoclasts cultured in control medium.

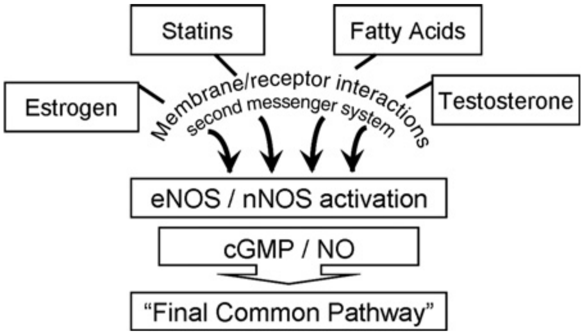


FIGURE 13 Proposed interactions and final common pathway of sex-steroid hormones, free fatty acids, and statins in bone (Wimalawansa, 2008).

However, the reported selectivity of aminoguanidine for iNOS and the apparent absence of any significant effects following administration of other nonselective NOS inhibitors has led to the somewhat misleading suggestion that iNOS is the principal source of NO in the regulation of bone cells. Another potentially incorrect conclusion was made based on NOS inhibitor studies that iNOS might be expressed constitutively in bone. Some researchers have failed to elicit expression of iNOS in bone cells (Riancho *et al.*, 1995; Hukkanen *et al.*, 1995), which is against the inference just mentioned, but is consistent with studies demonstrating low-level expression of iNOS transcripts in unstimulated whole bone preparations and in bone cell cultures (Brandi *et al.*, 1995; Helfrich *et al.*, 1997).

Interactions of NO and Cytokines with Osteoclasts

Cytokines including IL-1β, TNF-α, and IFN-γ are powerful regulators of bone resorption (Mundy, 1993) and also are potent stimulators of iNOS (Ganster and Geller, 2000). It has been shown that cytokine-induced iNOS activity exerts marked effects on osteoclastic bone resorption (Lowik *et al.*, 1994; Brandi *et al.*, 1995; Riancho *et al.*, 1995; Ralston and Grabowski, 1996). Apparently, cytokine-induced NO synthesis can either stimulate or inhibit osteoclastic bone resorption and correlates with the apparent level of iNOS induction or activation (Ralston and

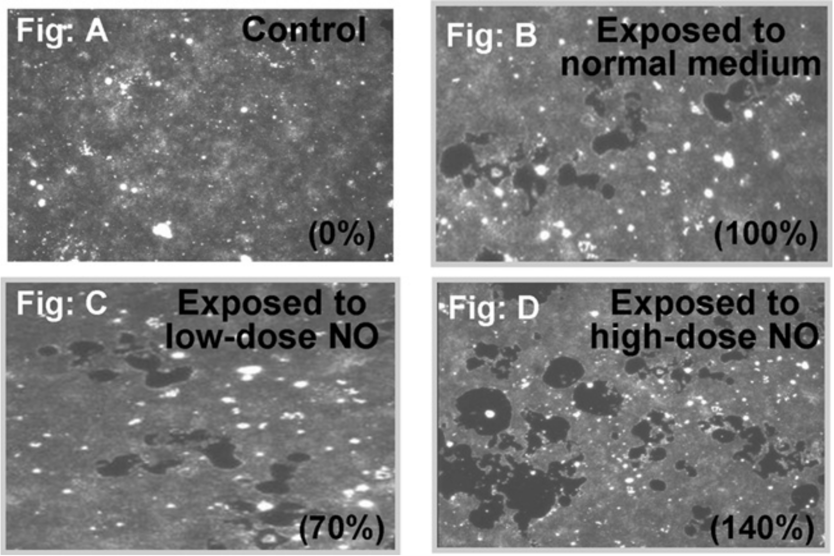


FIGURE 14 Bone slice-pit-resorption assay demonstrating the effects of adding low and high doses of NO donors to culture medium in comparison with a control (Wimalawansa, unpublished).

Grabowski, 1996). However, when added singly, these cytokines produce very different results. IL-1β and TNF-α exert a more modest inductive effect on iNOS expression and activity, and this is manifest by inhibition of the pro-resorptive effects of these cytokines by NOS inhibitors (Lowik *et al.*, 1994; Ralston *et al.*, 1995). These studies also demonstrated synergism between NO and prostaglandin E_2, another important regulator of bone resorption (Ralston *et al.*, 1995; Hukkanen *et al.*, 1999). This is consistent with recent observations on bone marrow cocultures from iNOS-gene-deficient mice, revealing that iNOS is essential for mediating the bone-resorptive effects of IL-1β (van't Hof *et al.*, 2000). IL-1β induced bone resorption as well as osteoclastic apoptosis is thought to be NO-dependent (perhaps in a dose-dependent fashion; van't Hof and Ralston, 1997; van't Hof *et al.*, 2000).

Expression of eNOS and the activity of this constitutive enzyme have been suggested to be associated with stimulating bone resorption (Brandi *et al.*, 1995; Ralston *et al.*, 1995). Supporting evidence was reported with the administration of a general NOS inhibitor to untreated neonatal rodent osteoclasts blocking resorption, suggesting that tonic release of NO or moderate induction of NO synthesis is an important facet of osteoclast resorptive function. Nevertheless, it is unlikely that this is the case; in fact, constitutive production of NO via eNOS (and nNOS) together with osteoprotegerin seems to be the key protective mechanism keeping excess osteoclastic activity under control (Wimalawansa, 2007). Furthermore, *in vivo* NOS inhibitors have shown to suppress adjuvant arthritis (Stefanovic-Racic *et al.*, 1993, 1994).

Although it is clear that NO has a key role in the regulation of osteoclastic bone resorption, the cellular source(s) that contribute to this process are still controversial. There is evidence to show that preosteoclastic and osteoclast cell lines have a low basal NO synthesis (Brandi *et al.*, 1995), which can be further stimulated by fluid shear stress (McAllister *et al.*, 2000). In response to cytokine stimulation, iNOS expression is induced in avian, rodent, and human osteoclastic cell lines (Brandi *et al.*, 1995; Sunyer *et al.*, 1996) and is accompanied by marked elevation in NO synthesis, as suggested by nitrite accumulation (Sunyer *et al.*, 1996). Such data would be consistent with an autocrine role for NO in osteoclast function. However, other investigators have argued against this after failing to detect appreciable levels of iNOS expression in osteoclasts even after cytokine stimulation (Helfrich *et al.*, 1997). Moreover, this same group of investigators have implicated the osteoblast as being the predominant source of inducible NO affecting osteoclast activity based on bone organ culture or osteoblast bone marrow coculture experiments (van't Hof and Ralston, 1997; van't Hof *et al.*, 2000).

Mechanisms of Action of NO in Osteoclasts

The mechanisms of action of NO on osteoclasts are quite varied and also dependent on the stage of cell development. Exposure to high levels of NO associated with iNOS activation is known to induce damage and death in a number of cell types. Consistent with this, cytokine-stimulated NO synthesis (i.e., high NO levels) initially will enhance activity of osteoclast, but later diminishes proliferation of preosteoclasts (Brandi *et al.*, 1995) and may involve induction of apoptotic cell death (van't Hof and Ralston, 1997). Although suggestive, the data are not convincing that osteoblasts are more resistant to NO-induced apoptosis in

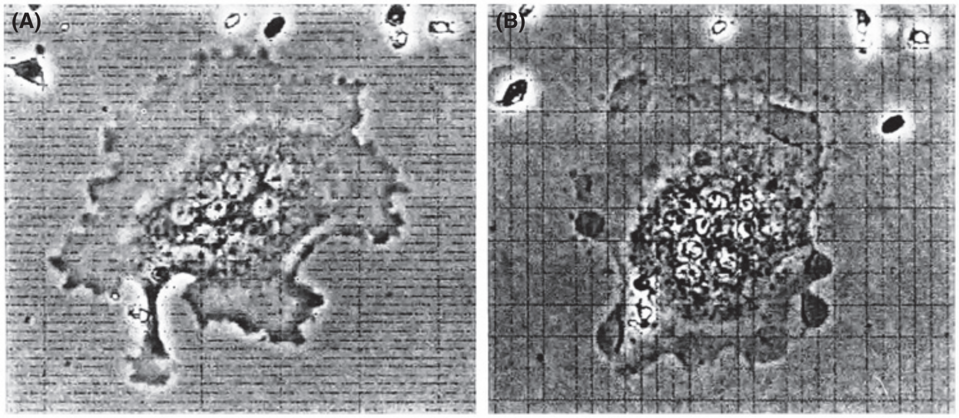


FIGURE 15 Video-microscope image of a primary rat osteoclast. **(A)** A typical rat osteoclast showing large cytoplasmic area and numerous nuclei. **(B)** The same cell after addition of a nitric oxide donor showing reduced cell area (MacIntyre, 2000).

comparison to osteoclasts (van't Hof and Ralston, 1997). In fact, data from author's laboratory suggest exactly the opposite. The upshot of this is that very high levels of NO *in vitro* may cause a reduction in the formation of mature osteoclasts and suppression of bone resorption. However, at higher levels of NO, virtually none of the osteoclasts or osteoblasts will survive. Finally, it is doubtful that such high levels of NO will ever be achieved *in vivo*, and this seems to be an *in vitro* pharmacologic artifact.

In mature osteoclasts NO has been shown to both stimulate and inhibit activity: a bidirectional effect (Brandi *et al.*, 1995) related to changes in the local concentration of NO. Exposure of mature osteoclasts to NO stimulates contraction and causes the cells to become detached from the underlying bone surface (MacIntyre *et al.*, 1991; Brandi *et al.*, 1995). This effect is illustrated in isolated rat osteoclasts (Fig. 15). The contraction of isolated osteoclasts is induced by fluxes in calcium ion concentration and appears to be mediated by a calcium-sensitive isoform, eNOS, because NOS inhibitors largely prevent the calcium-induced osteoclastic contraction (Brandi *et al.*, 1995). It is now known that this action of nitric oxide is largely mediated by cGMP (Wimalawansa, 2008), although this was not apparent in initial studies using slowly permeable cGMP analogues such as 8-bromo- or dibutyryl-cGMP (MacIntyre *et al.*, 1991). However, in more recent studies using rapidly permeable cGMP analogues, 8-pCPT-cGMP has been shown to produce a dramatic and rapid osteoclast contraction. This is also seen after exposure to NO donors and is largely, although not entirely, prevented by guanylate cyclase inhibitors (Mancini *et al.*, 1998). The calcium-stimulated effect of NO is highly likely to be physiological, and during osteoclastic bone resorption, in which periodic calcium-induced detachment occurs, this contracting and detaching effect likely forms an essential part of osteoclastic bone resorption and also is involved in osteoclast movement on bone surface.

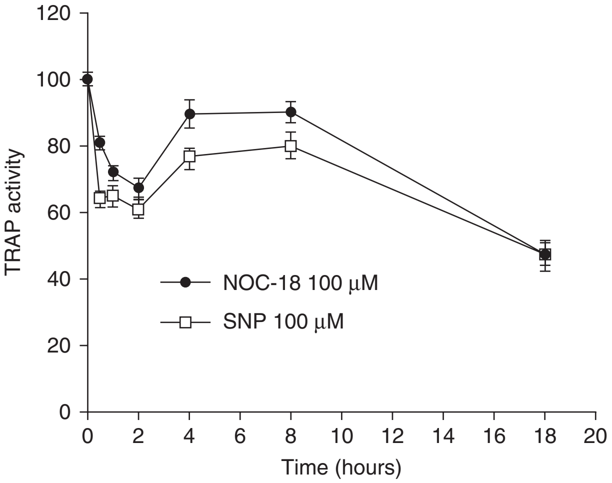


FIGURE 16 Effect of NO donors on human type 5 acid phosphatase activity. The two NO donors, SIN-1 and NOC-18, used in this experiment are both able to decrease TRAP activity over time, with a maximum reduction observed after 18 hours. Values are the mean of three different experiments and are expressed as percentage of control (Mancini L, Cox T, MacIntyre I).

NO inhibits the activity of recombinant tartrate-resistant acid phosphatase (Fig. 16), and this effect might contribute to the inhibitory action of NO on osteoclastic bone resorption. TRAP is mainly localized in resorption vacuoles, suggesting that it is secreted by the osteoclasts at the site of active resorption, although the mechanism by which it promotes bone resorption is still unclear. However, it is established that inhibition of acid phosphatase by chemical and immunological means can abolish osteoclastic bone resorption (Zaidi *et al.*, 1989). Further, acid phosphatase knockout mice have mild osteopetrotic phenotype, whereas genetically engineered mice overexpressing acid phosphatase have an increased bone turnover (Hayman *et al.*, 1996; Angel *et al.*, 2000). Thus, acid phosphatase seems to be important for bone resorption and osteoclast activity and the finding that NO can inhibit TRAP activity, possibly by binding to the iron moiety of the protein; this could account for some of the inhibitory actions of NO on osteoclastic bone resorption.

Osteoblast and Osteocyte Effects

Synthesis of NO by cells of the osteoblast lineage came from studies in responses of these cells to cytokine stimulation. Several groups independently demonstrated induction of iNOS mRNA and protein expression in cultures of both rodent and human primary osteoblasts and osteoblast cell lines after cytokine challenge (Damoulis and Hauschka, 1994; Lowik *et al.*, 1994; Ralston *et al.*, 1994; Hukkanen *et al.*, 1995; Riancho *et al.*, 1995). Activity of iNOS and synthesis of NO by these cells was confirmed by marked increases in the accumulation of nitrite, one of the oxidation products of NO biosynthesis, in the culture medium. Furthermore, these studies revealed a rank order of potency for induction of iNOS by cytokines.

By including inhibitors of NOS activity in the culture medium during or after cytokine-induced NO production, it has been possible to examine the effects of NO on osteoblast function. However, the blanket conclusions that NO causes profound inhibition of cell proliferation and DNA synthesis (Ralston *et al.*, 1995; Hukkanen *et al.*, 1995; Evans and Ralston, 1996) and osteoblast cell apoptosis (Damoulis and Hauschka, 1997; Jilka *et al.*, 1998) may not be correct. One cannot conclude from any of these experiments that NO causes osteoblastic suppression, whereas NOS inhibitors as well as very high levels of NO produced following cytokine stimulation can accomplish both.

Because of the higher levels of NO generated, cytokine-induced NO synthesis by iNOS leads to reductions in the expression and activity of osteoblast differentiation as suggested by the decrease in osteoblastic markers alkaline phosphatase and osteocalcin (Ralston *et al.*, 1995; Hukkanen *et al.*, 1995), as well as formation of mineralized bone nodules (Buttery, Hughes and Hukkanen, unpublished observations; Fig. 17). NO donors also have been demonstrated to stimulate expression of cyclooxygenase 2 and prostaglandin (PGE_2) synthesis; this might be relevant to osteoblastic control of osteoblast-mediated bone resorption (Hughes *et al.*, 1999).

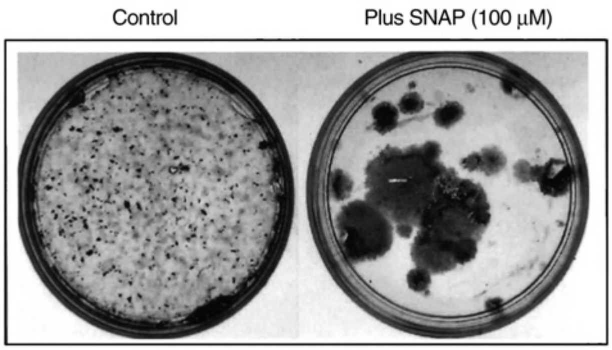


FIGURE 17 Effects of exogenous NO on rat osteoblast differentiation and formation of mineralized bone nodules. Exposure to SNAP (12 hours) at the time of cell seeding severely inhibited subsequent cell growth and differentiation (MacIntyre, 2000).

There are also some physiological processes that are stimulated by osteoblast/osteocyte constitutive NO synthesis by eNOS. Some have demonstrated basal, constitutive NO synthesis by osteoblasts, which promoted cell proliferation and could be augmented by 1,25-dihydroxy vitamin D_3 (Riancho *et al.*, 1995). NOS inhibitors also blocked the new bone formation induced by mechanical strain in rodent models (Fox *et al.*, 1996; Turner *et al.*, 1996). Furthermore, osteocytes and osteoblasts rapidly produce NO when exposed to mechanical strain or pulsatile fluid flow *in vitro* (Pitsillides *et al.*, 1995; Klein-Nulend *et al.*, 1995; Turner *et al.*, 1996), and this is now known to be dependent on the expression and activation of eNOS (Klein-Nulend *et al.*, 1998; Zaman *et al.*, 1999). This again confirmed that eNOS activation is physiological and indeed beneficial for the skeletal system.

Such a role for eNOS in sensing and responding to mechanical loading or shear flow is consistent with the regulation of this enzyme (Kleinert *et al.*, 2000). More recently, McAllister and Frangos (1999) have examined the kinetics of NO release from osteoblasts exposed to both steady and transient fluid shear stress. The results demonstrated two distinct biochemical pathways with transient flow stimulating a burst of NO that was dependent on G-protein activation and calcium mobilization, whereas steady flow produced sustained NO release that was G-protein- and calcium-independent. Such studies are important because they not only confirm the involvement NO in transduction of osteogenic signals, but also provide an insight into how perturbations in this signaling pathway might contribute to bone physiology and pathology. There are ample *in vitro* (Danziger *et al.*, 1997) and *in vivo* animal data (Wimalawansa, 2000c, 2008; Wimalawansa *et al.*, 1996) as well as human data (Wimalawansa *et al.*, 2000a; Wimalawansa, 2007) to support a key role of NO in bone metabolism.

Estrogen and NO

NO generated by cells of the osteoblast lineage is important in mediating the anabolic effects of estrogen on bone (Wimalawansa *et al.*, 1996). Although the major effect of estrogen has been thought to be its ability to inhibit osteoclastic bone resorption, estrogen has also been shown to have a direct effect on osteoblast-like cells (Ernst *et al.*, 1989). The presence of functional estrogen receptors in cells of the osteoblast lineage (Eriksen *et al.*, 1988; Komm *et al.*, 1988) suggests that estrogen compounds such as

17β-estradiol (E_2) stimulate bone formation *in vivo* by a direct action on osteoblasts (Chow *et al.*, 1992a, 1992b; Tanko-Yamamoto and Rodan, 1990; Samuels *et al.*, 1999). Indeed, this independent anabolic effect of estrogen on osteoblast cells is one of the possible explanations of the additive effects on BMD observed when two classical anti-resorptive agents (e.g., estrogen and bisphosphonate) are administered to postmenopausal women (Wimalawansa, 1995, 1998). Estrogen enhances bone cell eNOS and nNOS activity, acting via estrogen alpha receptor in bone cells (Bonnelye and Aubin, 2005), while NO directly regulates bone metabolism (Chae *et al.*, 1997; Hikiji *et al.*, 1997).

Studies *in vitro* have shown that 17β-estradiol enhances both proliferation and differentiation of cultured osteoblasts (Qu *et al.*, 1998; Ernst *et al.*, 1988; Scheven *et al.*, 1992). Although a link between estrogen and stimulation of eNOS had been established in the vascular system (Hayashi *et al.*, 1995; Weiner *et al.*, 1994), the first studies to demonstrate a link between the osteogenic effects of estrogen and NO in bone were those of Wimalawansa and coworkers (Wimalawansa *et al.*, 1996). They demonstrated that the NO donor compound nitroglycerin, long used in the treatment of ischemic heart disease, could prevent and also reverse the bone loss induced by ovariectomy in a rat model (Wimalawansa *et al.*, 1996; Wimalawansa, 2000c).

The results of subsequent studies demonstrated upregulation of eNOS expression and activity in a human osteoblast cell line after stimulation by 17β-estradiol (Armour and Ralston, 1998). 17β-Estradiol dose-dependently stimulated osteoblast (primary human and rat osteoblast cultures) proliferation and differentiation as assessed by alkaline phosphatase activity and bone nodule formation (Hayashi *et al.*, 1995; O'Shaughnessy *et al.*, 2000; Fig. 18), and in particular the trabecular bone (Tanko-Yamamoto and Rodan, 1990). This is abolished in the presence of inhibitors of NOS.

Additionally, 17β-estradiol increased total eNOS enzyme expression in rat osteoblasts and stimulated increases in NO metabolite levels (Fig. 19); once again this could be abolished by NOS inhibitors. A final and important finding from these studies is the lack of response to 17β-estradiol in animals treated with NOS blockers (Wimalawansa *et al.*, 1996; Wimalawansa, 2000c) and in primary osteoblasts cultures from eNOS gene knockout mice. Collectively these observations suggest that the stimulatory effect of estrogen on osteoblast proliferation and differentiation relies on local production of NO by bone cells via the eNOS (and nNOS) isoforms (Wimalawansa, 2007).

Estrogen has immunomodulating as well as anti-inflammatory actions. Following natural menopause and after oophorectomy, serum IL-1, IL-6, IL-8, TNF-α, and GM-CSF levels increase (Pacifici *et al.*, 1991), while TGF-β level increase in bone (Finkelman *et al.*, 1992). Estrogen replacement therapy reverses these cytokine levels (Fig. 20).

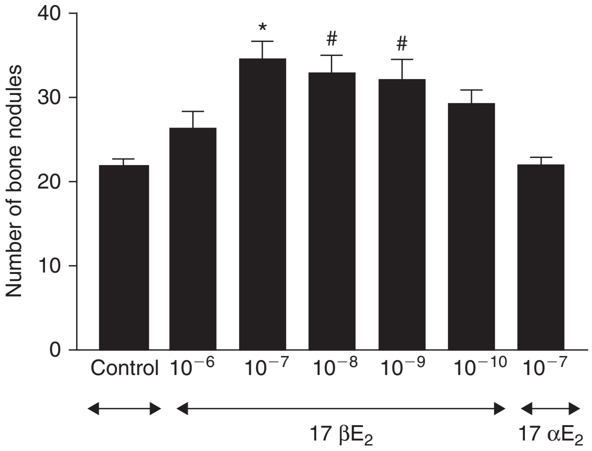


FIGURE 18 Quantitative assessment of rat osteoblast differentiation by *in vitro* formation of mineralized bone nodules. 17β-Estradiol has a dose-dependent effect on rat osteoblast differentiation. α-Estradiol did not have any effects on osteoblast differentiation (negative control). Values are means±SEM of six different experiments (**$p < 0.001$; *$p < 0.01$).

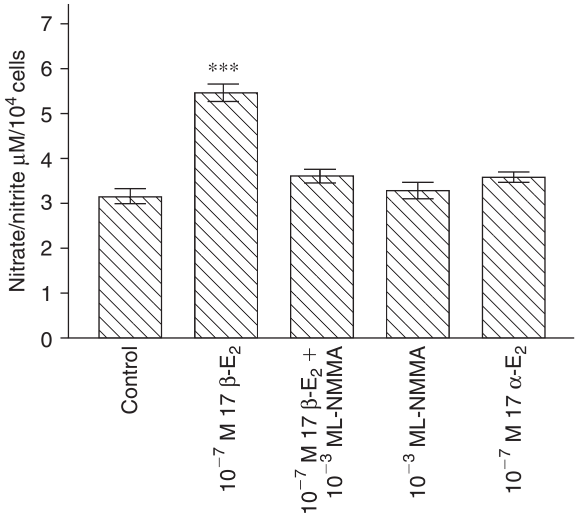


FIGURE 19 Analysis of NOS activity in osteoblasts by nitrite accumulation. Estradiol stimulates eNOS activity, which was blocked by the addition of the NOS inhibitor l-NMMA. Values are means±SEM of six different experiments (***$p < 0.01$ versus control).

Estrogen is known to increase TGF-β and stimulate eNOS and nNOS isoenzymes (Finkelman *et al.*, 1992; Ascroft *et al.*, 1997). This is in contrast to glucocorticoids that suppress the production of TNF-α as well as many other proinflammatory cytokines but do not prevent bone loss and, in fact, worsen bone loss. These data suggest that in addition to the production and suppressions of these cytokines, other second messengers must be involved *in vivo*. We proposed that NO is one such candidate for this (see Figs. 12 and 13).

Similar results to those just described are also seen after exogenous administration of NO donor compounds to osteoblast cultures. NO donors increase cGMP production,

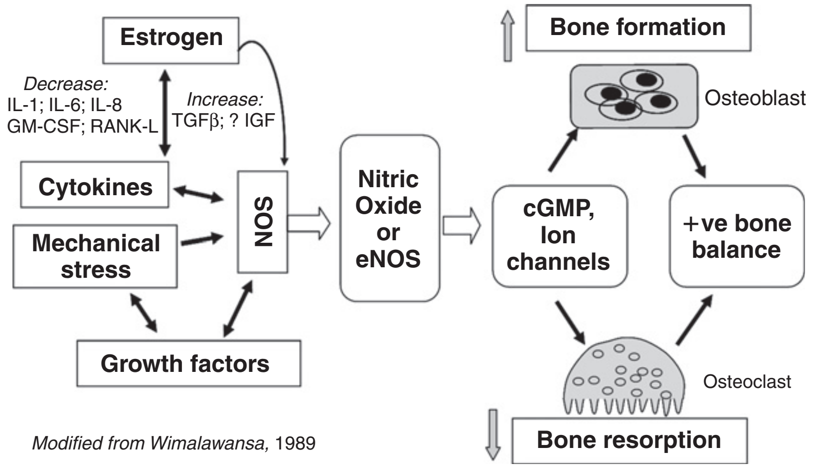


FIGURE 20 Schematic representation of interactions between estrogen, cytokines mechanical stress and growth factors on NO generation from eNOS/nNOS (physiological levels) and iNOS (pathological levels of NO); and their effects on skeletal homeostasis (Wimalawansa, 2007).

alkaline phosphatase activity, osteocalcin expression, and *in vitro* bone nodule formation (Chae *et al.*, 1997; Hikiji *et al.*, 1997; Otsuka *et al.*, 1998). Many researchers have found that whereas slow release and generation of low concentrations of NO stimulated osteoblast replication and alkaline phosphatase activity, rapid release and high concentrations of NO inhibited proliferation and induced apoptosis (Mancini *et al.*, 2000; Wimalawansa, 2007). These observations help to emphasize a fundamental aspect of the biological chemistry of NO, showing that alterations in the kinetics of NO synthesis, perhaps differential regulation of eNOS versus iNOS activity, can evoke profoundly different biological responses. These studies also demonstrated that the biphasic effects of NO on osteoblasts are mediated by the second messenger cGMP (Mancini *et al.*, 2000; Wimalawansa, 2007).

Interestingly, an estrogen-induced antioxidant effect is also due to suppression of superoxide anion production via release of NO (Arnal *et al.*, 1996). Estrogen indeed increases synthesis of eNOS (Thompson *et al.*, 2000; Hernandez *et al.*, 2000; Guetta *et al.*, 1997). Statins also activate protein kinase Akt, leading to an increased NO production by eNOS (Kureishi *et al.*, 2000). Furthermore, essential fatty acids and their metabolism enhance eNOS activity (see Fig. 13). Therefore the mechanism of all three of the compounds, estrogen, statins, and essential fatty acids, in improving skeletal health is likely to be mediated by augmenting eNOS activity. This is further supported by the fact that raloxifene, a selective estrogen receptor modulator (SERM), is also shown to dose-dependently increase the release of NO from endothelial cells (Simoncini and Genazzani, 2000). The data further support that eNOS activation and NO release from endothelial cells, osteoblasts, and osteocytes tonically inhibits osteoclastic activity. Unlike estrogen and NO donors, nitroglycerine and statins may have different paths of action on osteoblasts. So, the combination of these two agents at least in theory could have an added benefit on the skeleton.

Results confirm that NO is generated by osteoblasts via eNOS and iNOS and has an important role in osteoblast as well as osteoclast function. As a general rule of thumb, transient low-level production of NO regulated by eNOS is associated with stimulation of osteoblast activity and bone formation (see Fig. 20). whereas more sustained or high-output NO synthesis associated with iNOS expression and activity inhibits osteoblast function. The latter can lead to bone loss.

Bone tissue including bone marrow contains an abundance of endothelial cells that are capable of inducing eNOS activity. Similarly, some of the nerve terminals also contain nNOS. Many of these lie in proximity to osteoblasts and osteoclasts and so can influence their activity. Because of the sheer numbers of endothelial cells present in bone, it is conceivable that eNOS activity generated from these cells will have a constant homeostatic influence on bone cells.

The effects of NO on osteoblasts and osteoclasts are dependent on the local concentration of NO, and hence the level of stimulation of eNOS/nNOS and the iNOS. Therefore, we hypothesized that if these physiological amounts of NO can be delivered to bone cells via the bloodstream using NO donor therapies, the beneficial effects of NO on bone should be possible to obtain. Our data suggests that at low (physiological) concentrations, NO promotes osteoblast cell proliferation and activity and decreases bone-resorbing activity by osteoclasts. However, at higher concentrations (e.g., proinflammatory cytokine-induced iNOS stimulation and generation of higher levels of NO), NO enhances bone resorption and suppresses osteoblast activities (Wimalawansa, 2007).

NO Donors

NO donors can be used to characterize NO-mediated effects and provide a means of supplying “controlled” levels of NO from an exogenous source to mimic endogenous NO synthesis in *in vitro* or *in vivo* biological systems. The kinetics of NO release varies among NO donor compounds. Some release NO spontaneously in aqueous buffers, whereas others require active metabolic uptake, presence of thiol compounds, or degradation (see Figs. 2 and 3). Compound-specific susceptibility to changes in pH, oxygen, light, and temperature and formation of different by-products and metabolites during decomposition or metabolism also vary.

One of the most commonly used NO donor compounds is nitroglycerine (glyceryl trinitrate, GTN). It is an organic nitrate with more than 100 years of therapeutic use, particularly in cardiovascular disease (Wimalawansa, 2008). It appears to require active metabolic uptake and degradation by cells to release NO and to be biologically effective. Therein lies one of the drawbacks of its use, with cells potentially becoming tolerant after repeated exposure, requiring consistently higher dosages. Nitroglycerin does have several advantages; NO is a safe agent and has been used in clinical practice for several decades, it can readily be used *in vivo*, it is economical to use, and it has been shown to be effective in the prevention or reversal of estrogen-depleted bone loss in rodent models and osteopenia in human subjects (Wimalawansa *et al.*, 1996; Wimalawansa, 2000b, 2000c). Rapid-acting nitrates such as GTN and orally administered nitrates such as isosorbide dinitrate (ISDN, ISMN), although they may be effective, are associated with unacceptable incidences of headaches.

S-Nitroso-*N*-acetylpenicillamine (SNAP), *S*-nitrosoglutathione (SNOG), 3-morpholinosydnonomine (SIN-1), and sodium nitroprusside (SNP) are some of the other NO donor compounds that have been used in a number of *in vitro* experiments, including in cultured bone cells, and some *in vivo* experiments. All seem to liberate NO or NO adducts more or less spontaneously on contact with aqueous buffers. However, this can potentially “flood” the culture medium with NO, making difficult to quantify cellular responses. Careful adjustment of donor concentration may overcome this problem somewhat by creating a NO gradient that may mimic both physiological and pathophysiological responses *in vitro* (Damoulis and Hauschka, 1997; Otsuka *et al.*, 1998; Hughes *et al.*, 1999; O’Shaughnessy *et al.*, 2000; Aguirre *et al.*, 2001). These compounds are, however, too toxic to be administered in humans.

The final class of compounds, the diazeniumdiolates or NONOates, are perhaps the most useful for mimicking endogenous NO synthesis in *in vitro* studies. Although the NONOates release NO spontaneously, their rate of decomposition can be controlled. This enables the kinetics and concentrations of NO liberated into the culture medium to be more accurately predicated and adjusted to correlate more closely with the activity of eNOS or iNOS (Otsuka *et al.*,1998; Mancini *et al.*, 2000).

Nitroglycerine

Nitroglycerine is the preferred NO donor to be used for prevention and treatment of osteoporosis in humans. NO donors can be administered in many ways: tablets, sprays, gels, and creams, and also intravenously. Nitroglycerine is 1,2,3-propanetriol, an organic vasodilator nitrate. Nitroglycerine ointment has been on the market for more than 50 years, and the mild nature of its adverse effects has been well documented. It is supplied as a USP 2% in 60-g tubes (as the current market leader Nitrobid, Fougera Co., a division of Altana Pharmaceuticals). It is recommended to be stored between 15°C and 30°C (59–86°F). The chemical structure of nitroglycerine is illustrated in Fig. 21.

It is also important to note that ED_{50} of NO required for activation of each biological organ system is quite different (Wimalawansa, 2007). For example, the average nitroglycerine dose used in clinical practice to treat angina ranges

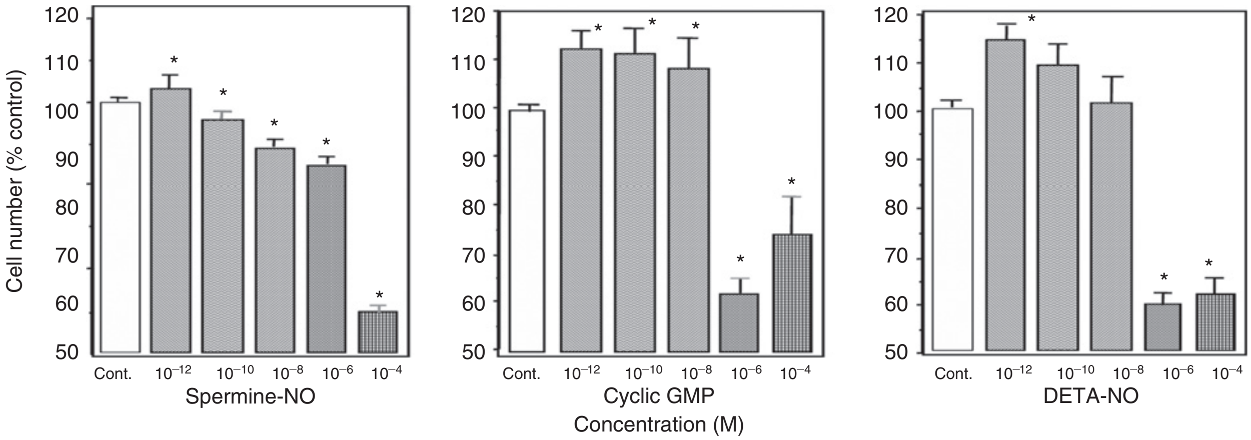


FIGURE 21 Dose-dependent cell-proliferation effect on various NO donor compounds (Wimalawansa, 2008).

from 15 to 30 mg, two to three times a day (i.e., over 60 mg of nitroglycerine daily, or its bioequivalent), whereas the dose of nitroglycerine required for skeletal health is only 15 to 25 mg, once a day, about one fourth of the standard dose use in clinical practice. Figure 22 illustrates data from a cross-sectional study demonstrating dose-effect of NO donor nitroglycerine on BMD in comparison to those who are not on nitrates.

Whereas doses below 30 mg equivalent of nitrates are beneficial for bones, doses over 40 mg of nitroglycerine/day, as demonstrated in Fig. 22, seem to be detrimental to skeletal tissues (Wimalawansa, 2007). The nitroglycerine dose used in the first human clinical study in 1997 demonstrated an equivalent efficiency of nitroglycerin to estrogen (Premarin; Wimalawansa, 2000b, 2007), and in the NOVEL clinical study (2002) about 20 mg nitroglycerine/day was utilized (Wimalawansa, 2007). That dosage represents about one fourth of the average dose that routinely used in cardiac patients with angina. The osteoporosis prevention studies just mentioned used once a day application of Nitro-Bid ointment containing 2% nitroglycerine that delivered about 20 mg nitroglycerine a day. If the efficacy is established, then use of nitroglycerine for prevention of bone loss will cost less than 10% of most of the currently approved anti-osteoporosis therapies (Wimalawansa, 2008).

Local NO levels can be raised either by supplements via an exogenous NO donor (e.g., nitroglycerine), or by preventing its rapid breakdown locally via inhibition of specific phosphodiesterase enzymes (Wimalawansa *et al.*, 2008). In fact, some inhibitors of phosphodiesterase have been shown to increase BMD by promoting bone formation (Kinoshita *et al.*, 2000). It may be possible in the future to target treatment to up-regulate osteoblast and osteocyte specific eNOS activity, enhancing its effectiveness completely eliminating its adverse effects. Expression of NOS isoenzymes are detailed elsewhere (MacPherson *et al.*, 1999; Kleinert *et al.*, 2000).

If nitroglycerine were eventually approved for prevention and treatment of osteoporosis, it would become the most cost-effective therapy for this disorder. The only significant adverse effect of nitroglycerine is the occurrence of mild headaches in about 20% of the subjects who use this ointment. This is comparable to the background incidence of headaches reported among U.S. adults over a period of time (*MMWR Morb. Mortal. Wkly Rep.* 55, 77 [2006]). However, with the orally administered nitrates, the incidence of headaches may be as high as 50% or more, and hence these are unlikely to have a practical utility. Therefore, for the use in chronic conditions such as osteoporosis, a percutaneous route seems to be the best choice.

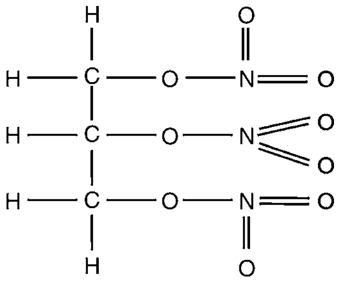


FIGURE 22 The structure of glyceryl trinitrate (GTN; nitroglycerine).

Inhibition of NO Synthesis

Research directed toward the controlling of NO synthesis (and hence NOS inhibitors) has flourished in the past decade, particularly in view of the roles that NO production may play in various pathophysiological states. Following the initial discovery that N^G-monomethyl-L-arginine (L-NMMA) could inhibit the synthesis of NO, a variety of N^G-substituted L-arginine derivatives and related compounds have been investigated as inhibitors of NO synthesis. Structures of couple of these NOS antagonistic compounds are illustrated in Fig. 23.

L-NMMA and N^G-N^G-dimethyl-L-arginine (ADMA) are naturally occurring inhibitors of NO synthesis with an equal affinity for the constitutive and inducible isoforms of NO synthase (Vallance *et al.*, 1992). Other commonly used inhibitors of NO synthesis include N^G-nitro-L-arginine (L-NNA), N^G-L-argininemethylester (L-NAME), N^G-iminoethyl-L-ornithine (L-NIO), and L-canavine. Some of these are reported to demonstrate rank-order specificity for particular isoforms (Knowles and Moncada, 1994). For example, L-NNA, L-NIO, and L-canavine are more selective for iNOS than for either nNOS or eNOS. Aminoguanidine (AG), has been much used as a somewhat selective inhibitor of iNOS including studies on bone (Kasten *et al.*, 1994; Tsukahara *et al.*, 1996) and is reported to be about 20-fold more selective for iNOS than the constitutive isoforms. However, none of these inhibitors

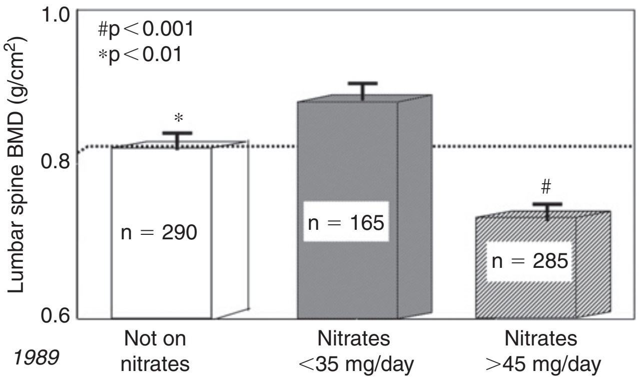


FIGURE 23 Effects of taking low or high doses of NO donors on BMD in comparison with a control group not taking nitrates or any other agents affecting bone metabolism (modified from Wimalawansa, 2007).

is sufficiently specific in its selectivity while also having effects independent of its NOS inhibition (Peterson *et al.*, 1992) to allow unequivocal assignment of a specific cellular function to a particular NOS isoform.

In Vitro Cell Biological Studies

Nitric oxide appears to play an autocrine and paracrine regulatory role in bone cell metabolism. In addition, NO modulates the activity of both osteoblasts and osteoclasts *in vitro*. Number of studies suggest that NO may have an anabolic effect on bone tissues and aid osteoblast-induced mineralization in all cultures (Wimalawansa, 2007). Thus, NO donors increase osteoclast cell proliferation, osteocalcin synthesis, and the formation of a mineralized matrix by osteoblasts *in vitro*. As expected, NOS inhibitors have an antiproliferative effect on osteoblastic cells *in vitro*. Also not surprisingly, the release of large amounts of NO from iNOS in cytokine-stimulated cells also has an antiproliferative effect on osteoblasts (Armour *et al.*, 2001), increases osteoblast apoptosis, and enhances osteoclast-mediated bone resorption (Lowik *et al.*, 1994; Armour *et al.*, 1999). Therefore, NO appears again to have a biphasic effect on bone forming cells (Brandi *et al.*, 1995): In low concentrations it promotes bone formation, whereas it may have an inhibitory effect at higher concentrations (Fig. 24).

Studies have demonstrated production of NO by osteoclasts in response to a rise in intracellular Ca^{2+}, leading to a retraction of cells and inhibition of bone resorption (Silverton *et al.*, 1999). Some authors have suggested that NO effects on bone cells are not mediated via cGMP (MacIntyre *et al.*, 1991), but overwhelming evidence suggests otherwise (Wimalawansa, 2007). Estrogen is known to regulate eNOS in osteoblasts (Armour and Ralston, 1998) and osteoblasts produce NO (Ralston *et al.*, 1994), whereas cytokine-induced inflammation (Hukkanen *et al.*, 1995) enhances iNOS activity (Armour *et al.*, 1999). NO compounds have been shown to decrease bone resorption *in vitro*, and similar effects have been demonstrated with L-arginine (Fini *et al.*, 2001). Furthermore, the inhibition of NOS activity leads to enhanced bone resorption (Broulik *et al.*, 2003; Kasten *et al.*, 1994; see Fig. 18).

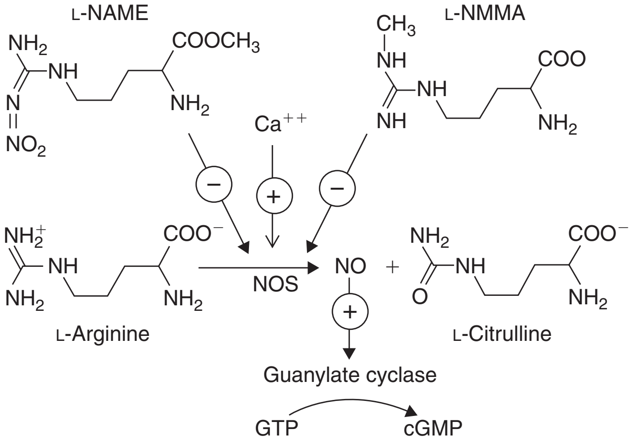


FIGURE 24 Structures of two commonly used NOS-antagonistic compounds *in vitro* cell culture and some *in vivo* animal research (Wimalawansa, 2008).

Interestingly, TNF-α-dependent osteoclastic survival is thought to be iNOS-dependent (Lee *et al.*, 2004), although some researchers have suggested otherwise (Rogers *et al.*, 1996). Bone cells, in particular osteoclasts (Silverton *et al.*, 1999) but also osteoblasts (Ralston *et al.*, 1994) as well as bone marrow cells, produce NO (Punjabi *et al.*, 1992). Mechanical strain on cells also produces NO (Pitsillides *et al.*, 1995; Sterck *et al.*, 1998; Burger and Klein-Nulend, 1999; Turner *et al.*, 1996) . The nitric oxide-cGMP pathway also seems to be involved in the mechanism of bone resorption by cyclosporin as well (Wimalawansa *et al.*, 2000c).

Enhanced expression of NO via iNOS in inflammatory conditions, e.g., lipopolysaccharide-induced bone resorption, can be alleviated with NOS inhibitors. This inflammation-associated bone loss has been predominantly due to augmentation of cytokine-induced matrix metalloproteinase 1 (MMP-1) production in osteoblasts and subsequent activation of osteoclasts (Grabowski *et al.*, 1996; Lin *et al.*, 2003). *In vitro* studies using high doses of NO also led to rapid osteoclast cell death. Indeed, NO donors inhibit osteoclast formation in mouse bone marrow cultures, an experimental system frequently used to study factors regulating osteoclastogenesis. It is possible that this effect is mediated by the NO-induced apoptosis of osteoclast progenitors, perhaps via peroxynitrite (see Fig. 4) and/or diminished osteoclast requirement, at physiological levels of NO. Nevertheless, the inhibition of osteoclast activity seems to be the predominant effect of NO under normal conditions (Wimalawansa, 2007).

NO Is a Mediator in Mechanical Stimulation

Nitric oxide is a key mediator in osteoblastic stimulation following exercise, shear-strain and most mechanical stimulations (Rodan *et al.*, 1975; Fox and Chow, 1998; Fox *et al.*, 1996). Pulsating fluid flow simulating canaliculi and shear stress releases NO that leads to osteoblastic stimulation (Klein-Nulend *et al.*, 1995, 1998). In addition to releasing NO, fluid shear stresses also release prostaglandins (McAllister *et al.*, 2000), perhaps via preosteoclast cells (McAllister and Frangos, 1999).

Mechanical stimuli are important during growth and subsequent bone homeostasis. This is manifested by lower peak bone mass in children who are physically inactive, and by the decrease in bone mass in adults following bed rest, inactivity, or exposure to microgravity. The mechanisms involved in the transduction of the effect of mechanical forces have not been elucidated, but data suggest the influence of prostaglandins and NO. Indeed, the inhibition of NO synthesis *in vivo* impairs the bone formation induced by mechanical loading of rat tibiae (Turner *et al.*, 1996). Furthermore, the exogenous NO donors can potentiate the osteogenic effect of loading.

In Vivo Studies with NO

In vivo studies suggest that the predominant effect of NO is on osteoclast cells, but anabolic effects on osteoblast cells are also important (Wimalawansa, 2007). Whereas NOS inhibitors such as aminoguanidine or L-NAME cause bone loss in rats, NO donors at physiological doses prevent ovariectomy-induced and glucocorticoid-induced bone losses (Wimalawansa, 2000b, 2000c) in rats and estrogen deficiency-induced bone loss in women. This further suggests physiological roles of NO in bone metabolism. Furthermore, eNOS- and/or nNOS-deficient mice have reduced bone formation (Aguirre *et al.*, 2001), whereas iNOS activation enhances bone resorption see Fig. 8). NO also seems to play a role in angiogenesis, particularly in vascular tissues in bone (Ziche, 2000).

NO also seems to mediate local vasoreactive activity during fracture healing (Corbett *et al.*, 1999b). Moreover, studies showed that although the NO-donor nitroglycerine by itself had no significant effect on the bone in adult rats (hormonally normal male or female rats), it prevented the loss of bone induced by methylprednisolone (Wimalawansa *et al.*, 1997; Wimalawansa and Simmons, 1998). These studies suggest that NO-releasing compounds may have a protective effect even on glucocorticoid-induced osteoporosis. The relevance of this to humans is yet to be studied.

We and others have demonstrated that nitroglycerine prevents both ovariectomy and corticosteroid-induced bone loss, as assessed by BMD, bone weight, and bone histomorphometry in rats (Wimalawansa *et al.*, 1997; Wimalawansa and Simmons, 1998; Wimalawansa, 2000b). Further, using the NO synthase inhibitor, L-NAME, using animal models of bone loss, it has been demonstrated that almost all beneficial effects of estrogen on bone can be blocked (Wimalawansa *et al.*, Figure 25).

Changes in the expression of eNOS have also been shown to influence growth plate chondrocytes as well as osteoblast activity in the metaphyses, especially in the lactation-induced bone loss model (Aguirre *et al.*, 2003). Using the adult ovariectomized (OVX) rat model, we and others have demonstrated positive effects of topically applied nitroglycerine (Fougera, NY 2% ointment) on indicators of bone metabolism including BMD, biochemical markers of bone turnover, and bone histomorphometry (Wimalawansa *et al.*, 1997). In these experiments, 17β-estradiol was used as positive control and compared its effects with nitroglycerine (Wimalawansa *et al.*, 1996). Data revealed that the skeletal effects of nitroglycerine are equivalent to those of estrogen in preventing as well as in treating OVX-induced bone loss.

Although applications of nitroglycerine once daily (0.2 mg/kg) were highly effective in the prevention of OVX-induced bone loss, multiple applications of the same dose were ineffective (Wimalawansa, 2007). The beneficial effects of nitroglycerine are biphasic and maximal between 0.2 and 0.5 mg/kg. Below 0.2 mg/kg as well as above 0.5 mg/kg the skeletal effects were not different from a placebo (i.e., relatively narrow therapeutic window for its skeletal benefits). Interestingly, nitroglycerine applied in the morning hours was more efficacious than when applied in

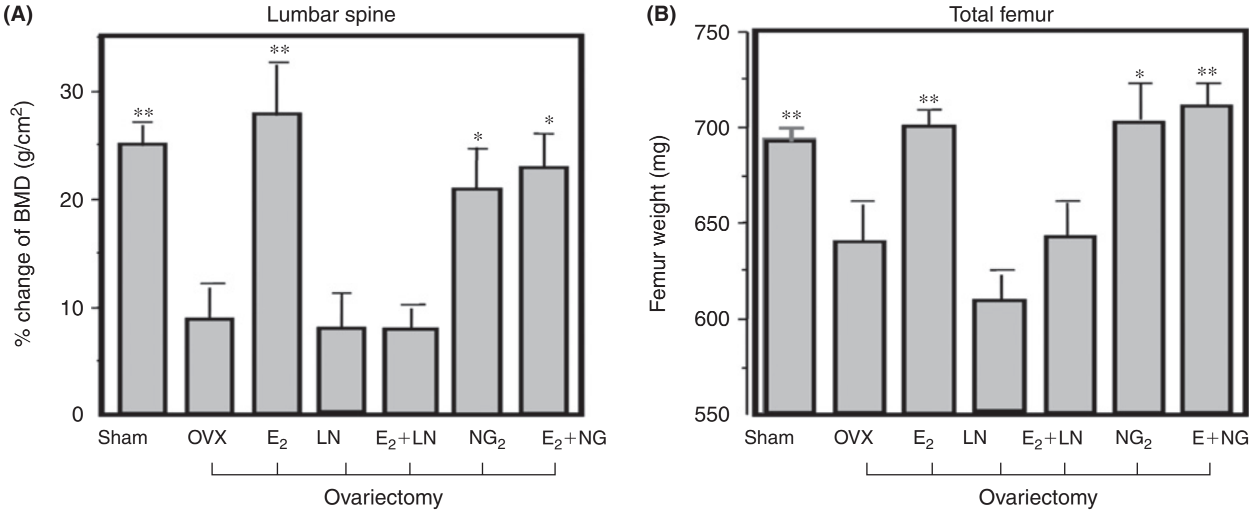


FIGURE 25 Effects of NO 17β-estradiol and NOS-antagonist l-NAMA on BMD in ovariectomized rats. Beneficial effects of estrogen are negated in the presence of l-NAME (NOS-blocks), suggesting that at least in part, estrogenic effects on bone are mediated via the NO/cGMP pathway (Wimalawansa *et al*., 1996).

the evening. Figure 26 illustrates the dose- and Figure 27, frequency-dependent effects of nitroglycerine on BMD in female Wistar rats.

Our animal studies have demonstrated a similar efficacy of NO donor therapy in male rats. Among castrated rats, preservation of BMD by either testosterone or estrogen administration is blocked by concomitant administration of the NOS blocker L-NAME. These data suggests that NO therapy is also likely to help in males, especially those males suffering from hypogonadism and older males suffering from idiopathic bone loss. Accordingly, the use of nitroglycerine is likely to have advantages over estrogen replacement therapy or SERMs in postmenopausal women, and testosterone and selective androgen receptor modulators (SARMs) in men.

Another study demonstrated that nitroglycerine was superior to more than 10 other NO donors. Also shown were the additive effects on BMD when nitroglycerine was coadministered with vitamin D, calcitonin, and bisphosphonates (Wimalawansa *et al.*, 2008). Because the final common pathway is the same, no such additive effects were expected or seen when nitroglycerine was coadministered with sex-steroid hormones such as estrogen, progestogens, or testosterone.

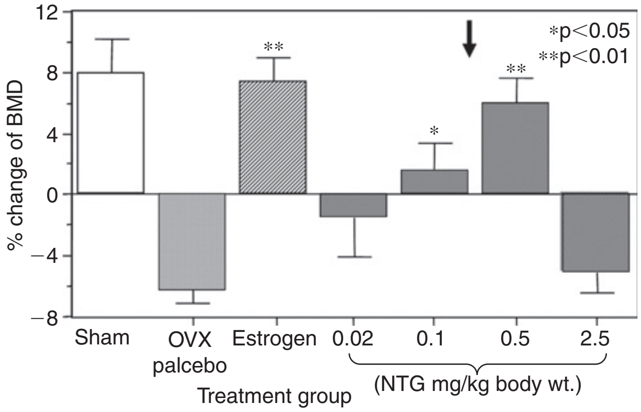


FIGURE 26 Dose-dependent effect of NO donor nitroglycerine on lumbar spine BMD in ovariectomized, female Wistar rats (Wimalawansa *et al.*, 2000a).

Overall, the results indicate that at low doses, nitroglycerine has marked beneficial effects on bone metabolism. Cyclosporine A is known to cause bone loss (Movsowitz *et al.*, 1988). The interactions of cyclosporine A with the bone cellular nitric oxide–cGMP pathways has also been shown to induce bone loss (Wimalawansa *et al.*, 2000c). No donor therapy can prevent the glucocorticoid-induced bone loss (Wimalawansa *et al.*, 1997). The same authors also demonstrated that NO therapy can prevent the glucocorticoid-associated histomorphometric changes in bone (Figure 28).

Using the ovariectomized rat model we also demonstrated that estrogen and the NO donor therapy with nitroglycerine were also equally effective in restoring or maintaining bone strength. Figure 29 illustrates the femoral neck ultimate load [force/load (N); displacement(N/mm)] in four groups of rats (sham operated; ovariectomized placebo therapy; estrogen-treated; nitroglycerine-treated). Both estro gen and nitroglycerine therapy significantly improved the bone strength.

L-NAME, the NO synthase inhibitor, prevents synthesis and release of NO via a NOS enzyme, abrogating the beneficial effects of estrogen in bone (Wimalawansa *et al.*, 1996). These suggest that some part of the beneficial effects of estrogen in bone is mediated through NO. Furthermore, effects of NO on bone cells are highly dose dependent; at low concentrations activation of eNOS or nNOS promotes

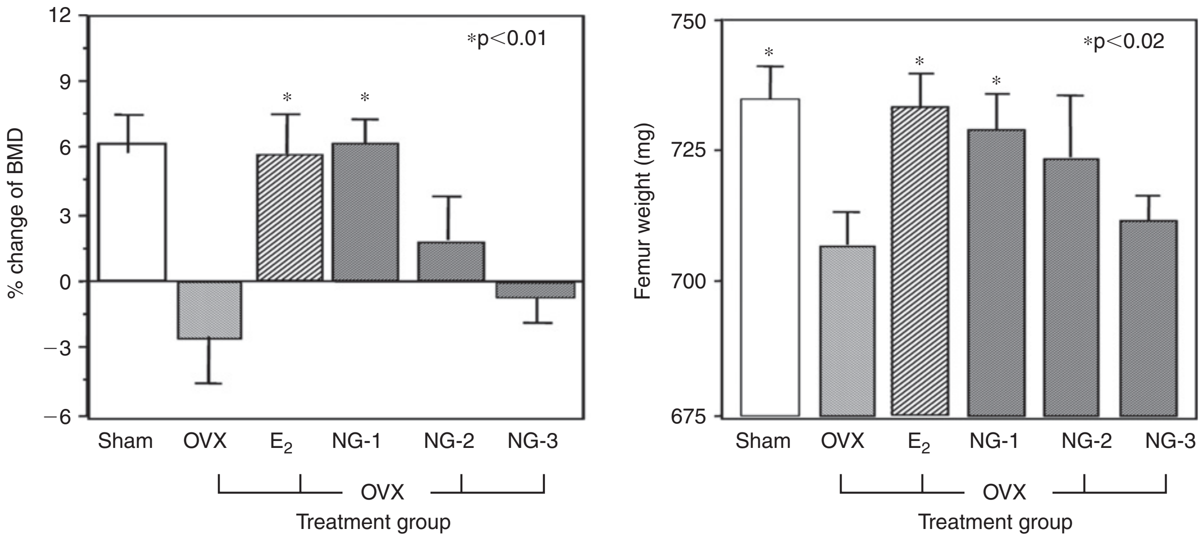


FIGURE 27 Frequency-dependent effect of NO donor nitroglycerine on lumbar spine BMD (trabecular bone) and femur weight (cortical bone) in ovariectomized rats (Wimalawansa *et al.*, 2000a).

osteoblast activity and cell proliferation and control of osteoclastic bone resorption, whereas at higher concentrations induction of iNOS activity with inflammatory cytokines leads to loss of osteoblastic activity and enhances osteoclastic activity leading to bone loss (Wimalawansa, 2007).

Potential Molecular Targets for the Action of NO in Bone Cells

NO signaling is able to activate various signal transduction pathways in different cell types. Despite this, there is a paucity of information of the detailed signaling mechanisms of NO in bone cells (Margolis and Wimalawansa, 2006). The guanylate cyclase-cGMP pathway is a classical target for NO, and there is a substantial body of evidence to show that the cGMP-dependent signaling is crucial to normal bone formation (Wimalawansa, 2007). Early studies demonstrated that cGMP was important in transducing the anabolic effects of mechanical loading (Rodan *et al.*, 1975) and more recently those of natriuretic peptides on bone formation (Suda *et al.*, 1996, Mericq *et al.*, 2000).

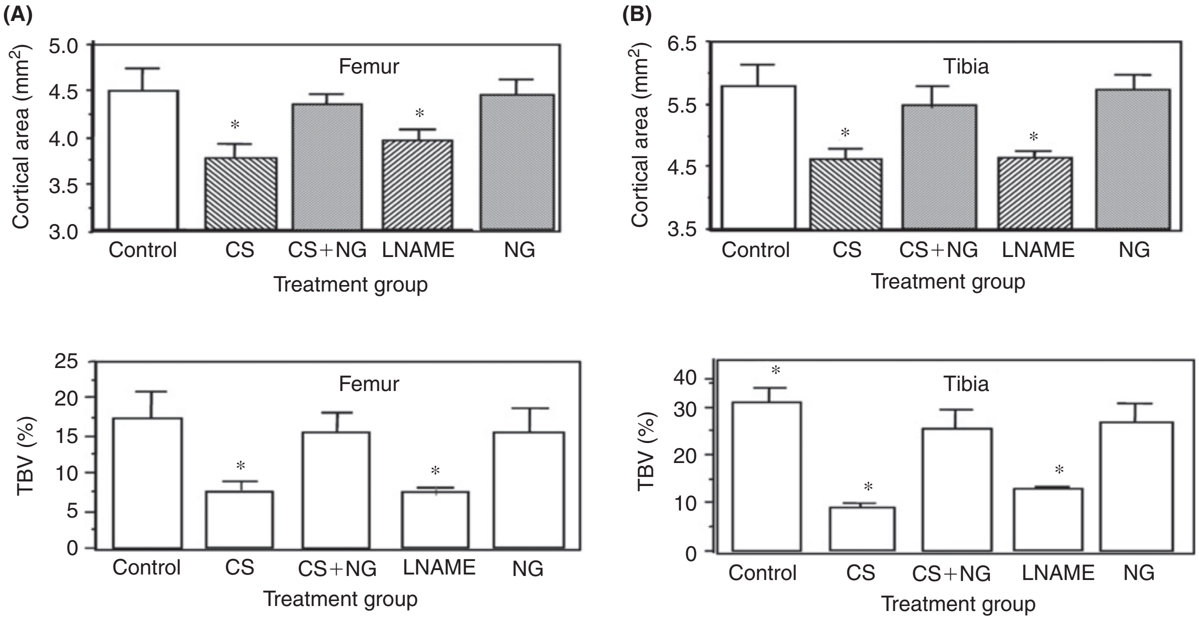


FIGURE 28 Effects of NO donor nitroglycerine on bone histomorphometry—cortical area and TV/BV% in comparison to control in glucocorticoid-treated rats. The effect of blocking the effects of NOS using the NOS inhibitor l-NAME is similar to that of glucocorticoid on the bone structure. Glucocorticoid-induced bone loss and associated histomorphometric changes can be prevented with appropriate doses of NO donor therapy in rats (Wimalawansa *et al.*, 1997).

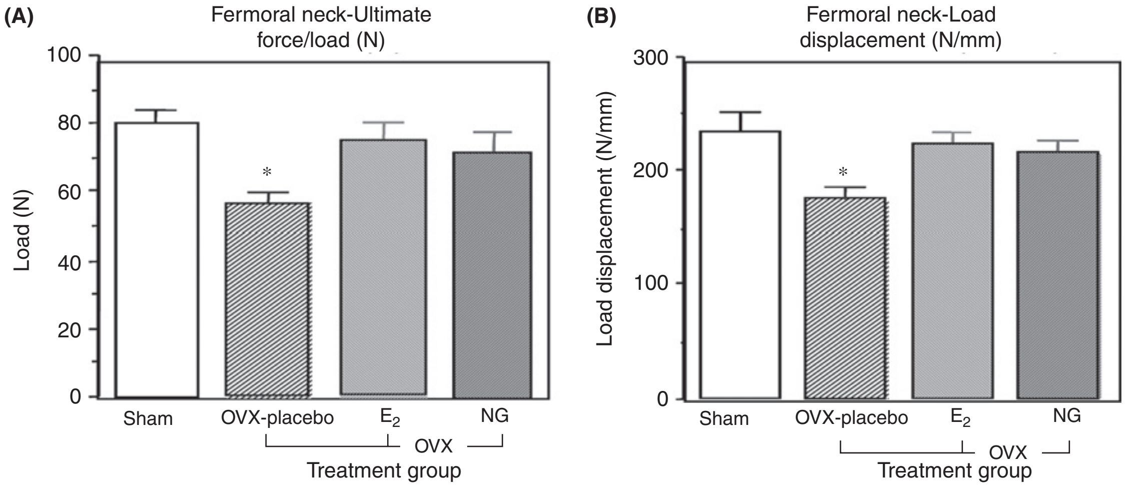


FIGURE 29 Effects of NO donor nitroglycerine on bone strength in comparison to 17β-estradiol (Wimalawansa, unpublished).

cGMP analogues increase the expression of markers of osteoblast differentiation alkaline phosphatase and osteocalcin and augment formation of mineralized bone nodule in rat calvaria cell cultures (Inoue *et al.*, 1995). The marked bone abnormalities and dwarfism seen in mice lacking protein G kinase type II is particularly compelling evidence for the role of cGMP, and a role for NO, in endochondral ossification (Pfeifer *et al.*, 1996). Osteoblast cultures stimulated with NO donors showed increased alkaline phosphatase activity, osteocalcin expression, and *in vitro* bone nodule formation that depended on cGMP synthesis (Chae *et al.*, 1997; Hikiji *et al.*, 1997; Otsuka *et al.*, 1998; Mancini *et al.*, 2000), demonstrating the association between NO and cGMP in bone cells.

The role of cGMP in mediating osteoclast function is less conclusive. Initial studies suggested cGMP production is not required for the functional modulation of osteoclasts by NO (MacIntyre *et al.*, 1991; Ralston and Grabowski, 1996). However, more recent studies using newer generation cGMP analogues and guanylate cyclase inhibitors have demonstrated that stimulation of cGMP by low concentrations of NO does regulate osteoclast function, increasing cell contraction and detachment from bone surfaces (Mancini *et al.*, 1998). In addition, Dong *et al.* (1999) showed that NO-dependent cGMP production reduced transport of HC1 osteoclast membranes and inhibited activity.

In addition to the cGMP pathway, NO is a potent stimulator of p21^{ras} activity and MAP kinase signaling (Lander, 1997). Although there is no direct evidence for NO-dependent activation of this pathway in bone cells, it has been shown that the MAP kinases are involved in stimulating osteoblast proliferation and differentiation (Matsuda *et al.*, 1998) and in regulating expression of the osteoblast transcription factor Cbfa-1 (Xiao *et al.*, 2000). MAP kinase activation is also involved in RANK ligand-induced osteoclast differentiation (Matsumoto *et al.*, 2000).

Expression and activity of VEGF is fundamental to the process of endochondral ossification stimulating chondrocyte cell death, cartilage remodeling, and angiogenesis (Gerber *et al.*, 1999). However, there is no direct evidence to support the involvement of NO in mediating the actions of VEGF in bone, but there is evidence to suggest that NO facilitates the angiogenic effects of VEGF in other cell types (Papapetropoulos *et al.*, 1997; Frank *et al.*, 1999; Ziche, 2000). As such, NO-VEGF signaling might also be important in the angiogenic response (Frank *et al.*, 1999) that is crucial to fracture healing.

Phase II Studies Using NO Donor Compounds

The rationale for using NO compounds for osteoporosis has been developed after many *in vitro* and *in vivo* studies conducted over the past two decades (Wimalawansa, 1988, 2000b, 2000c, 2007; Wimalawansa *et al.*, 1997). Circulating nitrate levels increase in the presence of HRT (Rosselli *et al.*, 1994) and androgen replacement therapy (Wimalawansa, unpublished), as well as calcitonin therapy (Tas *et al.*, 2002). Circulating nitrate (NO) levels fluctuate according to the menstrual cycle (Cicinelli *et al.*, 1996), whereas administration of estradiol increases plasma NO levels (Cicinelli *et al.*, 1998). Results from these animal and human studies indicate that nitroglycerine has marked beneficial effects on bone metabolism. Some of these beneficial effects of established antiosteoporosis agents such as estrogen, testosterone, anabolic steroids, and SERMS as well as statins are likely to mediate via the cGMP/NO pathway (see Fig. 13).

The first human clinical study to evaluate the beneficial effects of the NO donor nitroglycerine in prevention of oophorectomy-induced bone loss was conducted in the mid-1990s (Wimalawansa, 2000b). Based on animal data,

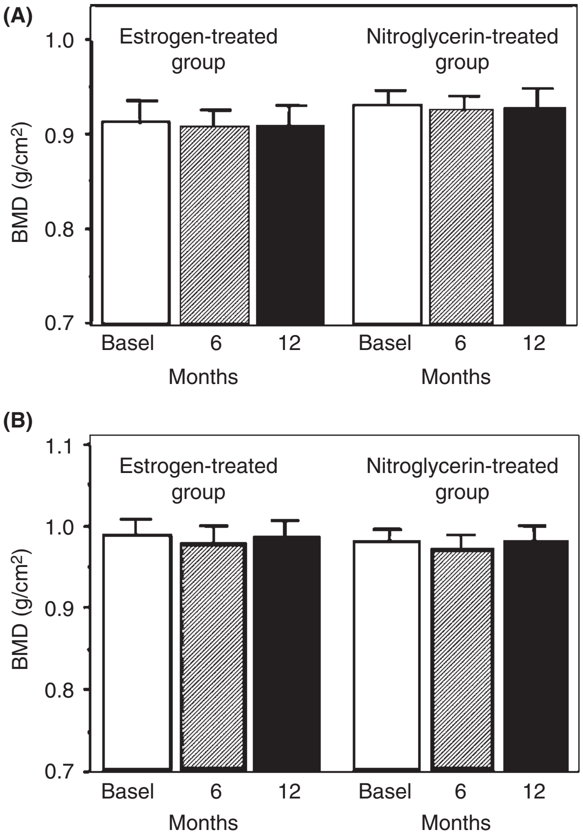


FIGURE 30 Comparable effects of NO donor nitroglycerine with estrogen (Premarin) in prevention of bone loss in oophorectomized women. Women were randomly allocated to receive either Premarin (standard therapy) or percutaneously administered nitroglycerine. BMD and the biochemical markers of bone turnover were examined at the baseline and at 6-month- and 12-month time points. There were no differences of BMD between the two groups, or from their respective baselines to 6- and 12-month time points in each treatment group (Wimalawansa, 2000b).

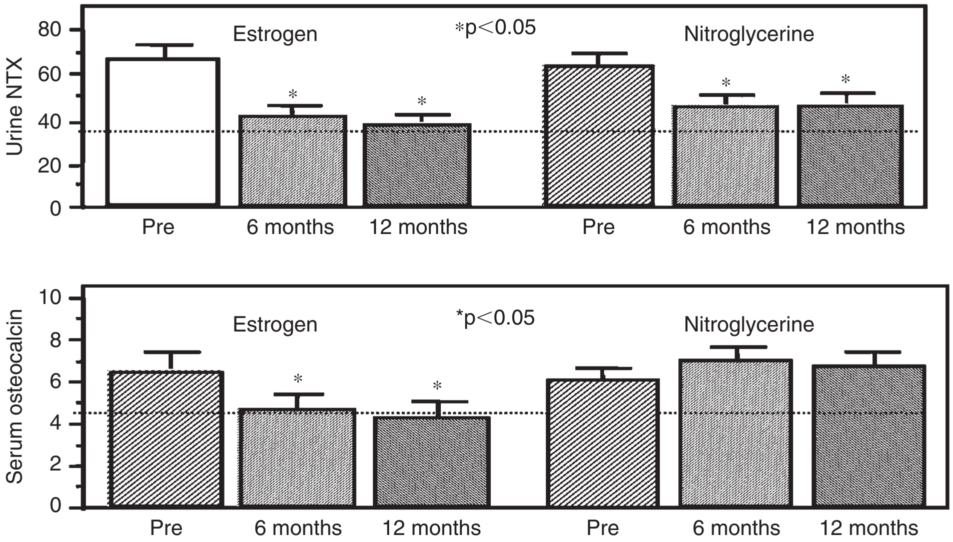


FIGURE 31 Changes of biochemical markers of bone turnover in response to estrogen (Premarin) versus topically administered nitroglycerine ointment (for the details of the experiment see the legend of Fig. 30). While a bone resorption marker (N-telopeptide) decreased as expected with any antiresorptive agent, the bone formation marker osteocalcin was suppressed only with estrogen therapy (as a coupling effect). This suggests that a dissociation of bone formation from resorption, which is favorable for gaining bone, occurred in the presence of the NO donor nitroglycerine (Wimalawansa, 2000b).

this 1-year controlled, randomized human study was conducted to assess the efficacy of topically administered nitroglycerine ointment in comparison with oral estrogen, using BMD and biochemical markers as endpoints. Data from this pilot study established an equipotent effect of nitroglycerine to estrogen HRT, preventing oophorectomy-induced bone loss in women; that is, NO donor therapy prevented accelerated bone loss in early menopause. Figure 30 illustrates the equipotency of NO with estrogen in maintaining the BMD in oophorectomized women.

Figure 31 illustrates the differential (i.e., an example of dissociation of bone resorption from bone formation) effects of NO versus estrogen in oophorectomized women. Note that whereas the bone resorption marker N-telopeptide is suppressed with both estrogen and NO, the bone formation marker osteocalcin is only suppressed with estrogen.

Supplementation with the NO precursor L-arginine may be effective (Visser and Hoekman, 1994), but the amount of L-arginine that must be ingested to achieve these biological effects is so high that it makes this approach impractical. A cross-sectional study also supported the role of NO in enhancing BMD (Jamal *et al.*, 1998). More importantly, a larger nationwide case-controlled study conducted in Denmark, studying 124,655 subjects with fractures in comparison to 373,962 gender-matched controls, revealed a 15% reduction of hip fractures in those using organic nitrates Fig. 32). The authors speculated that use of nitrate may protect against osteoporosis and its fractures.

Phase III Study with NO Donor Nitroglycerine

Based on the pilot study data described earlier, a single-center, large, randomized, double-blind, placebo-controlled clinical study funded by the National Institutes of Health was conducted to assess the effectiveness of topically administered nitroglycerine. This clinical trial, known as the NOVEL [**N**itroglycerine as an **O**ption: **V**alue in **E**arly bone **L**oss] study, was designed to resolve the questions: Can nitroglycerine stop bone loss in menopausal women? If so, can this be an alternative therapy for estrogen and HRT? The original study was designed to compare the effects of nitroglycerine with HRT and the SERM raloxifene. However, as a result of data available in the early 2000 from the WHI study, to minimize potential risks to study subjects the NOVEL protocol was modified to compare nitroglycerine with a group treated with inactive ointment together with calcium and vitamin D (Wimalawansa *et al.*, 2007).

If the results of the NOVEL study confirm efficacy of nitroglycerine and the hypothesis that estrogen works through NO on bone, then nitroglycerine therapy could become a highly cost-effective and attractive treatment option for prevention and treatment of postmenopausal osteoporosis. The role of combination therapies (Wimalawansa, 1995, 2000a; Wimalawansa and Simmons, 1998; Wimalawansa *et al.*, 2008) may have a place in the future and needs to be investigated, especially the combination of NO donors with bisphosphonate therapy (Wimalawansa, 2007). Figure 33 illustrates mechanism

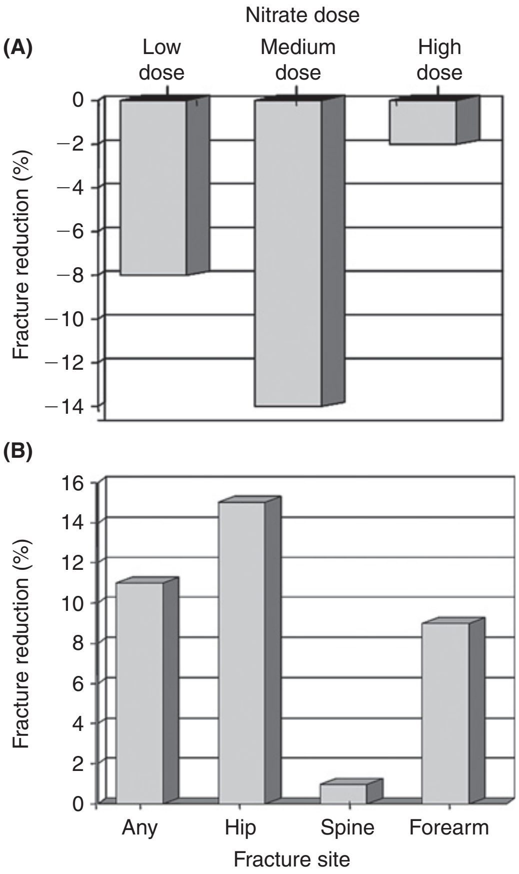


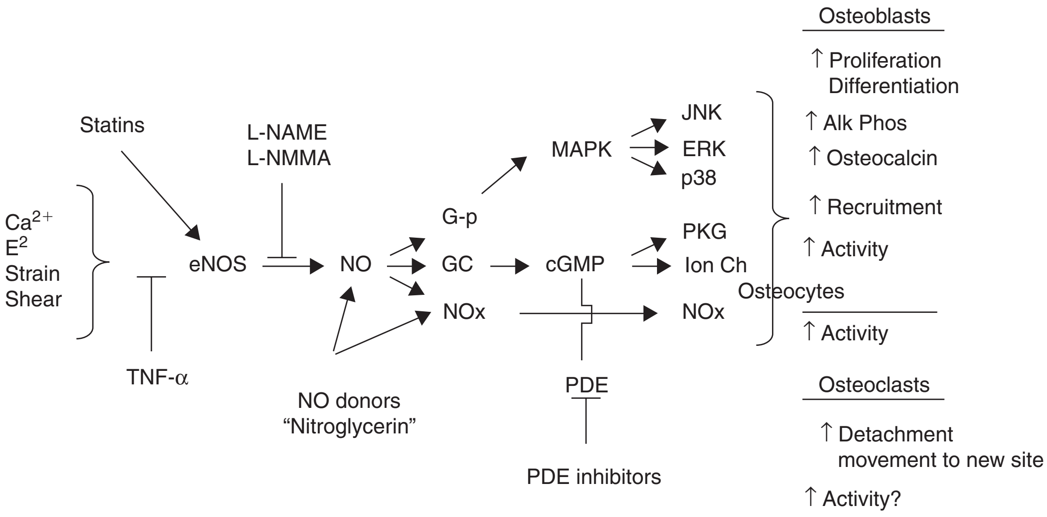
FIGURE 32 Diagrammatic representation of fracture reduction reported in a nationwide large epidemiology study in Denmark. (A) Overall effects of three broad dose categories of nitrates used, which demonstrated that the moderate dose of nitrates had the highest efficacy in fracture reduction, and at higher doses there is no effects on fracture prevention. (B) Percentage fracture reduction: any fractures, 0.89 (CI, 0.86–0.92); hip fractures, 0.85 (CI, 0.79–0.92); lumbar spine, 0.99 (CI, 0.85–1.16); and forearm, 0.91 (CI, 0.83–1.00). (Data adapted from Rejnmark *et al*., 2006).

of activation of eNOS and nNOS leading to physiological actions in bone cells.

NO and Bone Pathology

Alterations or disruption to NO synthesis within the bone environment has been implicated in the genesis of a number of diseases. Inflammatory diseases such as rheumatoid arthritis, which is characterized by articular cartilage erosion together with juxta-articular bone loss, is associated with activation of iNOS and production of high local levels of NO and NO-related species including peroxynitrite (Farrell *et al.*, 1992; Stefanovic-Racic *et al.*, 1993; Kaur and Halliwell, 1994; Grabowski *et al.*, 1996). On the basis of data using NOS inhibitors and iNOS knockout mice, activation of iNOS and increased levels of NO production contribute directly to the tissue damage associated with rheumatoid arthritis (McCartney-Francis *et al.*, 1993; Stefanovic-Racic *et al.*, 1994).

There are several other inflammatory conditions associated with activation of iNOS and in which increased production of NO or NO-derived oxidants including peroxynitrite is implicated in pathological bone loss. Increased staining for iNOS and formation of peroxynitrite in macrophages associated with bone erosions adjacent to prosthetic hip implants has also been demonstrated (Hukkanen *et al.*, 1997), suggesting the involvement of activated iNOS in the process of loosening of the implant. iNOS activation and increased NO production has been reported as a contributory factor to the extensive bone loss seen in animal models of inflammation-induced arthropathy and osteoporosis (Armour *et al.*, 1999). The deleterious effects of iNOS activation in these animal models could be minimized by administration of a NOS inhibitor.



Osteoblasts
↑ Proliferation Differentiation
↑ Alk Phos
↑ Osteocalcin
↑ Recruitment
↑ Activity
Osteocytes
↑ Activity
Osteoclasts
↑ Detachment movement to new site
↑ Activity?
Statins
L-NAME
L-NMMA
Ca^{2+}
E^2
Strain
Shear
eNOS
TNF-α
NO
NO donors "Nitroglycerin"
G-p
GC
NOx
MAPK
JNK
ERK
p38
cGMP
PKG
Ion Ch
NOx
PDE
PDE inhibitors

FIGURE 33 Diagrammatic overview of mechanism of eNOS activation and NO-mediated physiological signaling pathways in bone cells. For detailed discussion refer to main text. ERK, extra cellular signal-regulated kinase; GC, guanylate cyclase; G-p, G-proteins; Ion Ch, ion channel proteins; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NOx, nitric oxide related species; PKG, protein kinase G (MacIntyre, 2000).

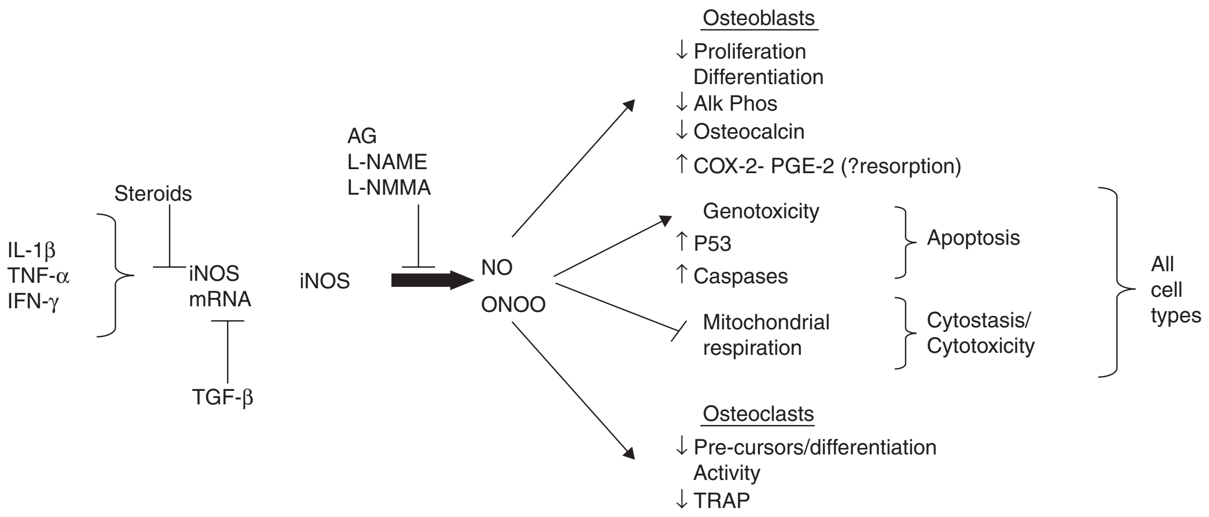


FIGURE 34 Diagrammatic overview of mechanism of iNOS activation (pathological) and these higher levels of NO-mediated signaling pathways in bone cells. ERK, extra cellular signal-regulated kinase; GC, guanylate cyclase; G-p, G-proteins; Ion Ch, ion channel proteins; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PKG, protein kinase G.

iNOS expression and staining has been demonstrated in osteoblasts, in osteoclasts, and in particular osteocytes in bone samples from osteonecrotic subjects. Interestingly, iNOS-positive cells correlated closely with the incidence of apoptotic cells and has led to the somewhat controversial suggestion that osteocyte apoptosis is induced by NO. These larger amounts of NO generated locally, together with osteoclast activation, might in fact be the underlying mechanism of the extensive bone destruction seen in inflammatory arthritis (Calder *et al.*, 2001). Figure 34 illustrates the pathological consequences following stimulation of iNOS activity on bone cells.

NO and Fracture Healing

There is evidence to show that NO is involved in various stages of fracture healing. Marked iNOS expression and activity within 24 hours after fracture in a rat model has been reported, which is consistent with the initial inflammatory phase of fracture healing (Corbett *et al.*, 1999a). In these experiments, iNOS was localized principally to endosteal osteoblasts. This expression was transient, and after 24 hours iNOS expression and activity were scarcely detectable. It is likely that this is a signal to attract osteoclasts to the site of injury and to activate the repair process starting with bone resorption; this is an essential part of the fracture healing process. During the first week after fractures there was a marked increase in expression and activity of calcium-dependent eNOS, which was localized, in particular, to cortical blood vessels and osteocytes (Corbett *et al.*, 1999a). This makes sense as the increase of blood supply that is necessary for the repair process.

Blood supply to a fracture site is critical for its proper healing. The presence of large numbers of vascular endothelial cells (Collin-Osdoby, 1994), and thus eNOS activity generating NO locally, plays a role in fracture healing (Corbett *et al.*, 1999b; Diwan *et al.*, 2000). This is supported by the demonstration of differential expression of NOS isoenzymes at the fracture sites. In addition, NO also activates other growth factors and cyclooxygenase enzymes (Salvemini *et al.*, 1993). The aforementioned is consistent with eNOS being involved in the vascular response and neovascularization that are crucial to successful fracture repair. Indeed, a further study on the same model demonstrated enhanced NO-dependent vasoreactivity around the fracture site, supporting a role for NO in the restoration of blood flow to the fractured bone (Corbett *et al.*, 1999b). Subsequent reports provided similar evidence for the involvement of NO in fracture healing (Diwan *et al.*, 2000). Of particular interest in that study was the finding that NOS inhibitors significantly impaired fracture healing, which could be reversed by local delivery of a NO donor.

Disrupted NO synthesis, whether too low or too high, is likely to be a significant factor in the postmenopausal, age-related as well as inflammation-associated bone loss associated with osteopenia. Estrogen-depletion osteoporosis induced by ovariectomy in animal models can be largely prevented or reversed by administration of NO donor compounds, notably nitroglycerin (Wimalawansa *et al.*, 1996, 2000c). This was extended to a human clinical trial showing that nitroglycerin is equally as effective as HRT in preventing or restoring postmenopausal bone loss (Wimalawansa *et al.*, 2000b).

Additional evidence for NO mediating the osteogenic effects of estrogen is provided by cyclic increases in plasma nitrite and nitrate levels (oxidation products of NO metabolism), which are highest at the mid-phase of the menstrual cycle, closely following estrogen levels

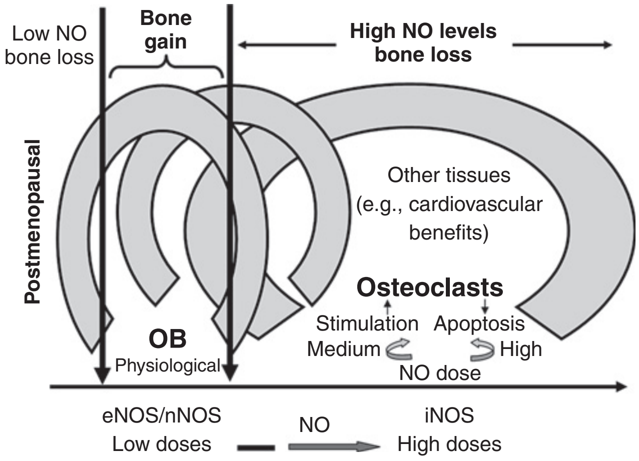


FIGURE 35 Schematic demonstration of dose-dependent effects of nitric oxide donor therapies on cellular and organ fractions. This also highlighted the relatively narrow therapeutic index for NO donor therapies in skeletal health.

(Cicinelli *et al.*, 1996). Postmenopausal subjects and amenorrheic athletes have reduced serum NO metabolites, which can be elevated following treatment with HRT (Rosselli *et al.*, 1995; Cicinelli *et al.*, 1998; Stacey *et al.*, 1998). Finally, *in vitro* studies on rodents, including eNOS knockouts, and human osteoblasts demonstrate that eNOS expression and activity are elevated by estrogen and correlate with increased osteoblast proliferation and differentiation (Armour and Ralston, 1998; O'Shaughnessy *et al.*, 2000; Aguirre *et al.*, 2001; Armour *et al.*, 2001).

Therapeutic Implications

It has been shown that NO has an estrogen-like effect and that nitric oxide can in part replace the beneficial effect of estrogen in bone (Wimalawansa, 1996; Wimalawansa *et al.*, 2000a; Armour and Ralston, 1998; Aguirre *et al.*, 2001; Armour *et al.*, 2001). Wimalawansa and coworkers (Wimalawansa *et al.*, 2000a) also demonstrated that a NO donor in humans is as effective as estrogen in inhibiting postmenopausal bone loss. Clearly this has important therapeutic implications in the treatment and prevention of osteoporosis. Further, it strongly suggests that a NO donor in combination with an osteoclast inhibitor—for example, calcitonin or bisphosphonates—can be useful in the treatment of osteoporosis. Nitroglycerine has a relatively narrow therapeutic window for treatment of osteoporosis, as illustrated in Figs. 21, 32, 35. At low doses NO promotes skeletal health, whereas at high doses (e.g., the doses of nitrates used in angina pectoris and other cardiovascular disease) it is likely to promote bone loss.

If NO or a NO donor can be administered directly to the fracture sites, it could conceivably augment fracture healing (Corbett *et al.*, 1999a, 1999b; Diwan *et al.*, 2000).

There are, however, potential limitations on the use of NO donors. Cellular desensitization following prolonged use of organic-nitrate compounds such as nitroglycerin is a particular problem that has been encountered in the treatment of ischemic heart disease and requires increased dosages to reproduce the beneficial effects. However, this can be easily overcome by providing NO therapy intermittently, such as daily or less than daily administration (Wimalawansa *et al.*, 2000a; Wimalawansa, 2007).

OTHER VASOACTIVE AGENTS

These include the members of the calcitonin family, the prostaglandins, endothelin, natriuretic peptides and VEGF (Wimalawansa, 1996, 1997; Inoue *et al.*, 1995). The calcitonin family acts as osteoclast inhibitors (Zaidi *et al.*, 1991), whereas NO enhances the angiogenesis of avascular tissues (Ziche, 2000). Calcitonin is the most potent, whereas the other members of the group all share predominant vasoactive actions with weaker osteoclast inhibitory effects. Only calcitonin has been thoroughly studied for its action on bone, and this is well described (Wimalawansa and Cooper, 1997; Wimalawansa, 1989; Zaidi *et al.*, 1991). Osteoclasts each have about six million calcitonin receptors, making it the prime target for its actions. Briefly, calcitonin acts directly on the osteoclast to produce a marked inhibition primarily via activation of adenylate cyclase, but also involving an increase in intracellular calcium (Wimalawansa, 1991; Wimalawansa and MacIntyre, 1991).

Endothelin is a potent vasoconstrictor, influence bone metabolism by impairing blood supply to bones and also by inhibiting osteoclast cells (Alam *et al.*, 1992). It also inhibits osteoblast-dependent skeletal mineralization (Hiruma *et al.*, 1998) and thereby regulates bone cell metabolism (Kasperk *et al.*, 1996; Yasoda *et al.*, 1998; Alam *et al.*, 1992; Nelson *et al.*, 1999).

The prostaglandins have a complex but very important set of actions of bone. Interestingly, NO is know to activate cyclooxygenase and also increase prostaglandin synthesis (Salvemini *et al.*, 1993) and may be important in bone cell function (Hughes *et al.*, 1999). Endothelin has not been studied extensively in bone, but has been reported to have a weaker osteoclast inhibitory effect (Alam *et al.*, 1992; Zaidi *et al.*, 1993). The effects of endothelins on osteoblast function appear to be contradictory and, perhaps, species-specific. Endothelins inhibit osteoblast differentiation in rodent osteoblast cultures (Hiruma *et al.*, 1998; Inoue *et al.*, 2000), whereas in human cultures endothelins promote osteoblast differentiation and mineralization (Kasperk *et al.*, 1996; Nelson *et al.*, 1999).

C-type natriuretic peptide is significant in the physiological control of endochondral ossification and in osteoblast proliferation differentiation (Suda *et al.*, 1996; Yasoda *et al.*, 1998; Mericq *et al.*, 2000; Inoue *et al.*, 2000). This peptide also regulate bone growth via cGMP (Mericq *et al.*, 2000) and osteoblast functions (Suda *et al.*, 1996). In some respects the actions of natriuretic peptide is not unlike those of NO. Calcitonin gene-related peptide (CGRP; a member of the calcitonin family) also has weaker antiosteoclastic effect, presumably via binding into calcitonin receptor in osteoclasts (Zaidi *et al.*, 1991; Wimalawansa and MacIntyre, 1991). Being the most potent vasodilator in humans and its wide network of distributions in the neuronal and vascular network in the bone, it is also likely to have a major effect on controlling nutrition supply to the skeleton (Wimalawansa, 1996, 1997). NO is a key factor for inductions of VEGF (Frank *et al.*, 1999). Finally, VEGF is an important vasoactive factor that plays a fundamental role in endochondral ossification (Gerber *et al.*, 1999), and angiogenesis during endochondral bone formation (Gerber *et al.*, 1999; Papapetropoulos *et al.*, 1997).

FUTURE PROSPECTS USING NO DONOR THERAPY FOR OSTEOPOROSIS

Alternatives to NO donors include delivering compounds that preserve the signaling actions of messenger molecules downstream of NO synthesis such as cGMP. Indeed, phosphodiesterase (PDE) inhibitors are able to increase bone mass by accelerating bone formation in mice (Kinoshita *et al.*, 2000). Consequently, specific PDE inhibitors may have some value in the treatment of osteoporosis and need further exploration. Other approaches for eliciting anabolic effects in the skeleton include the lipid-lowering drugs called statins (Wimalawansa *et al.*, 1997), as first reported by Wimalawansa in 2000 at the Bone and Tooth Society Meeting in London. Statins have been shown to stimulate new bone formation (Mundy *et al.*, 1999) and reduce the risk of hip fracture in elderly humans (Wang *et al.*, 2000). Interestingly, statins upregulate eNOS expression and downregulate iNOS expression ; i.e., favorable for skeletal health) at least in brain tissue and may also account for some the osteoprotective effects of these compounds. Some studies have shown an increase in BMD following the use of statins (Tanriverdi *et al.*, 2005; Kawane *et al.*, 2004; Maritz *et al.*, 2001). However, recent controlled clinical studies have failed to demonstrate any skeletal benefits of statins, e.g., atrovastatin at doses up to 80mg per day. This is probably due to an inability to deliver adequate concentrations of statins to local bone cells.

Elevated production of NO following iNOS activation is a significant factor in the progression of various inflammatory conditions including rheumatoid arthritis. Consequently, inhibition of iNOS activity appears to be an attractive target in tempering inflammation-induced tissue damage. Although there are several reports to show that when NOS inhibitors (L-NAME, L-NMMA) are administered prophylactically, these compounds were able to suppress the onset of disease in animal models (McCartney-Francis *et al.*, 1993; Stefanovic-Racic *et al.*, 1994), not all iNOS activity is detrimental. Indeed, iNOS does have protective effects such that NOS inhibitors can exacerbate some tissue injury (Wimalawansa, 2008). In part this also relates to the fact that in virtually all the studies that have been performed using NOS inhibitors, none of the compounds used are sufficiently selective in their action and can often block the beneficial effects of constitutive NO activity. None of these are safe enough to give to humans.

It is possible that in adults, a low-grade expression of iNOS activity may be useful in normal bone physiology. Thus, although modulation of NOS activity has important therapeutic implications in bone biology and pathology, much research is still required at this stage. Continued investigation of NOS gene knockouts, together with developments in the generation of more selective NOS inhibitors and NO donor compounds, should provide more information on the cellular targets and mechanisms of action of NO.

CONCLUSIONS

The predominant NOS isoform expressed by bone tissues is eNOS. iNOS is expressed in inflammatory and other pathological situations in osteoblasts, osteocytes, and in osteoclasts. eNOS is also expressed widely in vascular tissues within the bone, and consequent generation of lower levels of NO is likely to interact with bone cells. Synthesis of NO by these cells is stimulated by a variety of signals including hormones, cytokines, growth factors, fluid shear stress, and mechanical loading, resulting in a number of distinct physiological and pathophysiological responses (see Figs. 12, 20, 33, and 34). Nitric oxide donor compounds such as nitroglycerin and nitrates are safe and highly cost-effective agents. These compounds have been clinically used in humans for decades and are well tolerated; headaches are the only demonstrable adverse effects, which depend on the dose and the route of administration.

Furthermore, NO has been shown to play a role in various physiological as well as pathological conditions. The most attractive novel indication for NO donor therapy is the prevention and treatment of osteoporosis in men and women. In this regard a phase III randomized, double blind, control clinical study has just been completed (NOVEL clinical study). Recent research and developments have created the possibility of using NO donor compounds and designing new complex NO compounds capable of delivering NO directly into target tissues or to the bloodstream more efficaciously in a controlled or pulsatile manner. If a NO donor is proved to be effective in prevention of bone loss and, perhaps reduction of fracture risks, it may become a widely used, safe, and quite cost-effective therapeutic agent to combat osteoporosis. Figure 36 summarizes the current knowledge of response of BMD to

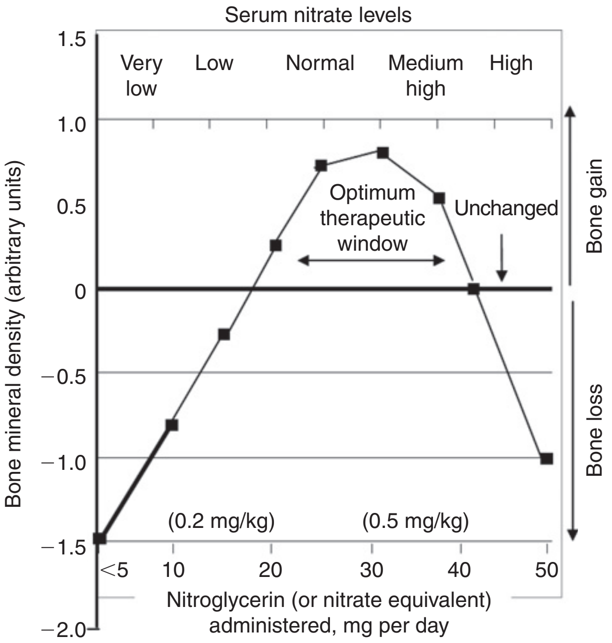


FIGURE 36 Diagrammatic representation of the BMD response to various doses of nitrates and nitroglycerine used in current clinical practice. This demonstrates the narrow therapeutic window for nitroglycerine and nitrates in general that should be used in enhancing human skeletal health (i.e., to improve BMD and decrease fractures).

various doses of nitroglycerine (or nitrate equivalents) in animals and in humans.

Physiology

At low concentrations NO, generally as a result of eNOS activity, stimulates osteoblast and osteocyte activity and keeps the osteoclast-mediated bone resorption under control. eNOS-dependent NO generation stimulates osteoblast replication and differentiation *in vitro*. Similar effects are evident *in vivo*, where NO facilitates fracture healing and is also involved as a second messenger in mechanical stress-induced bone formation. Perhaps most important, NO is significant in mediating the osteogenic effects of sex-steroid hormones, in particular estradiol (Wimalawansa, 2007). It is possible that NO also modulates osteocyte activities such as mechanical and shear-stress-induced bone formation.

Pathophysiology

Disruption of eNOS activity, as occurs following estrogen deficiency, can contribute to reduction in bone formation and enhanced bone resorption, leading to the development of osteoporosis. Inflammation-induced activation of iNOS expression and activity is also associated with the development and progression of cartilage and bone destruction that characterize inflammatory arthritis.

In summary, at physiological NO concentrations (i.e., premenopausal level), the balance of bone metabolism and bone homeostasis changes in favor of formation by stimulating osteoblast replication and differentiation, and in parallel, inhibiting osteoclast action. Lower concentrations of NO in bone tissue, as is the case in postmenopausal women, is likely to be one of the causes of bone loss that can be rectified with estrogen—or perhaps with NO replacement therapy, because the beneficial effects of estrogen in bone are mediated via NO/cGMP pathway (Wimalawansa *et al.*, 1996). High local concentrations of NO, on the other hand, whether it is endogenous (such as in rheumatoid arthritis where iNOS is induced) or exogenous (high doses of exogenous administered nitrates over a longer period of time), are likely to cause damage to bone and cartilage tissues. With this wide range of functions, there are multiple opportunities for therapeutic interventions using the NO-cGMP pathway. However, this has so far not been adequately investigated, in part because of the lack of safe NOS inhibitors, and, more important, the lack of funds to conduct research in the area. The latter is simply due to the fact that the NO donor therapies are relatively inexpensive, and thus pharmaceutical companies are not interested in developing these NO compounds as therapeutic agents for prevention and treatment of osteoporosis. As a result, though the modulation of NO synthesis or NO supplementation as a therapeutic agent for prevention and treatment of metabolic bone diseases such as osteoporosis (and arthritis) and for fracture healing, as well as for reducing the occurrence of osteoporotic fractures, seems feasible, these therapeutic interventions remain to be exploited.

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Chapter 60

Molecular Basis of PTH Overexpression

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INTRODUCTION

Over the past several years application of powerful molecular biology techniques has provided a wealth of new information and insights into the development of tumors. The purpose of this chapter is to review this new information as it relates to our understanding of parathyroid tumorigenesis. Parathyroid hyperfunction is found in several disease states including sporadic primary and secondary hyperparathyroidism and familial disorders such as the multiple endocrine neoplasia (MEN) syndromes.

Primary hyperparathyroidism is a common disorder characterized by hypercalcemia caused by an excessive secretion of parathyroid hormone (PTH). This is due to both an increased parathyroid gland mass and a resetting of the control of PTH secretion from the parathyroid cell by the ambient calcium concentration. Patients with primary hyperparathyroidism have one or more enlarged parathyroid glands with a single, benign adenoma occurring in almost 85% of cases; multiple hypercellular glands are present in about 15% of patients (Black and Utley, 1968; Castleman and Roth, 1978). In modern series, parathyroid carcinoma occurs in less than 1% of cases, and the ectopic secretion of PTH from nonparathyroid tumors is extremely rare.

The refractory state of secondary hyperparathyroidism, as seen, for example, in patients with uremia, and tertiary hyperparathyroidism (Galbraith and Quarles, 1994) are characterized by hyperfunctioning parathyroid tissue that no longer responds appropriately to physiological regulators such as ambient calcium and 1,25-dihydroxyvitamin D [1,25(OH)$_2$D].

Hyperparathyroidism may also occur as part of familial syndromes, such as multiple endocrine neoplasia types 1 and 2 (MEN1 and 2), the hereditary hyperparathyroidism and jaw tumor (HPT-JT) syndrome, and familial hypocalciuric hypercalcemia (FHH) and neonatal severe hyperparathyroidism (NSHPT).

MOLECULAR ONCOLOGY

Cancer cells contain genetic damage to key growth-regulating genes that directly contributes to the abnormal neoplastic phenotype. A cardinal feature of cancers is their clonal, or monoclonal, nature. They arise from a single precursor cell that has a selective growth advantage over normal cells and whose progeny outgrow them and ultimately make up the tumor. Typically, certain identical patterns of DNA damage are seen in each cell of such a tumor, indicating that important underlying genetic events occurred early before major proliferation or clonal expansion took place. The clonality of tumors also implies that these events occur only rarely in the large population of cells making up a tissue.

It is important to note the molecular heterogeneity underlying the development of neoplasia (Vogelstein and Kinzler, 2004). Generally, accumulating damage to several distinct genes, within the same cell, is required for the expression of the complete neoplastic phenotype. Whereas certain genes are implicated in tumors of only one or a few cell types, other genes may be involved in many different types of tumor. However, in most cases, no single gene will be both a necessary and a sufficient neoplastic agent. More commonly, the emergence of a particular tumor type may relate to disruption of specific biochemical pathways, which can be achieved by different combinations of mutated genes resulting in similar cellular and clinical consequences.

Clonal DNA damage in two groups of normal cellular genes contributes to the development of neoplasia. These are protooncogenes and tumor suppressor genes. Protooncogenes are often involved in the physiological

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control of cellular growth, proliferation, or differentiation. Conversion of a protooncogene to an "oncogene" is caused by a deregulation of the expression of its protein product or by formation of an intrinsically abnormal product. The products of tumor-suppressor genes normally restrain cellular proliferation and their gene inactivation contributes to neoplasia. Protooncogenes can be activated by a variety of mechanisms, including chromosome translocations or inversions, point mutations, proviral insertions, or gene amplification. Inactivation of tumor-suppressor genes can occur by point mutation or deletion, for example.

CLONALITY OF PARATHYROID TUMORS

The issue of the clonal status of parathyroid tumors was examined using an X-chromosome inactivation method, evaluating DNA polymorphisms, and by the direct demonstration of monoclonal DNA alterations in parathyroid adenomas (Arnold *et al.,* 1988). It was determined that most, if not all parathyroid adenomas are monoclonal (Arnold *et al.,* 1988; Arnold and Kim, 1989; Friedman *et al.,* 1989; Bystrom *et al.,* 1990; Orndal *et al.,* 1990), emphasizing that they are true neoplastic outgrowths of a single abnormal cell. This is consistent with the general experience that surgical removal of such tumors is curative of the disease. As would be expected, parathyroid carcinomas are also monoclonal (Cryns *et al.,* 1994b). In addition, monoclonal parathyroid tumors are seen in familial MEN1 (Friedman *et al.,* 1989; Thakker *et al.,* 1989; Bystrom *et al.,* 1990), in nonfamilial, sporadic, primary parathyroid hyperplasia (Arnold *et al.,* 1995), and in the refractory secondary or tertiary parathyroid hyperplasia of uremia (Arnold *et al.,* 1995; Falchetti *et al.,* 1993). Therefore, even in parathyroid hyperplasia, which begins with a stimulus for generalized, polyclonal, parathyroid cell proliferation affecting all of a patient's glands, monoclonal tumors can arise, at least in some of the glands. Such tumors may be more autonomous and exhibit a more marked dysregulation of PTH secretory control than the hyperplastic, polyclonal glands within the same patient.

An important goal is to identify the specific protooncogenes and tumor suppressor genes that are clonally activated or inactivated, respectively, in parathyroid tumors. Several successes in approaching this aim have already been achieved.

GENETIC DERANGEMENTS IN BENIGN PARATHYROID TUMORS

DNA rearrangements are some of the best characterized clonal oncogenic abnormalities, and they frequently involve juxtaposition of cellular protooncogenes with regulatory sequences of other genes. This then results in overexpression or deregulated expression of the protooncogene, converting it to an oncogene. To date the only oncogene solidly implicated in parathyroid neoplasia is *cyclin D1/PRAD1.*

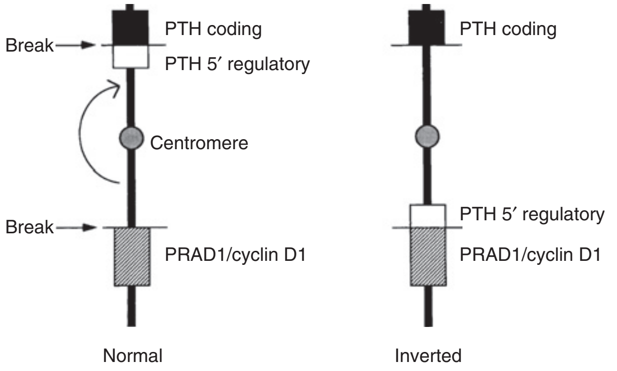

FIGURE 1 Schematic diagram of rearrangement of chromosome 11 in parathyroid tumors. In a subset of parathyroid adenomas a pericentrometric inversion of chromosome 11 is the most likely cause of the observed rearrangement involving the *PTH* gene and the *PRAD1* gene. Each tumor has another copy of chromosome 11 which bears a normal *PTH* gene. Reproduced with permission from Arnold (1993).

Cyclin D1/PRAD1

A subset of parathyroid adenomas contains a tumor-specific DNA rearrangement that separates the 5′ regulatory region of the *PTH* gene from its protein coding exons (Fig. 1). These tumor cells still possess one intact copy of the *PTH* gene, which accounts for expression of PTH by the tumor. The non-*PTH* gene sequences adjacent to the breakpoint were originally cloned from a genomic DNA library (Arnold *et al.,* 1989) and revealed a then-novel putative oncogene first called *PRAD1*, located on chromosome band 11q13. Currently, *PRAD1* is most often called *cyclin D1* or *CCND1* and is now fully established as a key human oncogene. The normal chromosomal *cyclin D1* gene contains five exons and four introns spanning approximately 15 kb and is transcribed in a centromeric to telomeric direction (Motokura and Arnold, 1993). In the cases of *PTH/cyclin D1* rearrangements characterized to date, the 11q13 breakpoints have occurred from 1 to 15 kb upstream of *cyclin D1* exon 1 with the *PTH* gene 5′ regulatory region and noncoding exon 1 placed upstream of the breakpoint (Friedman *et al.,* 1990; Rosenberg *et al.,* 1991) (Fig. 2). The overexpression of the *cyclin D1* gene is likely to be caused by its aberrant placement in close proximity to the strong tissue-specific enhancer elements of the *PTH* gene (Rosenberg *et al.,* 1993; Mallya *et al.,* 2005).

On the order of 5% of parathyroid adenomas have been shown to contain an activated form of the *cyclin D1* oncogene. However, rearrangement breakpoints on 11q13 associated with overexpression of *cyclin D1* in other tumors can occur more than 120 kb upstream of the gene (Williams *et al.,* 1993; Vaandrager *et al.,* 1996, 1997), and such rearrangement

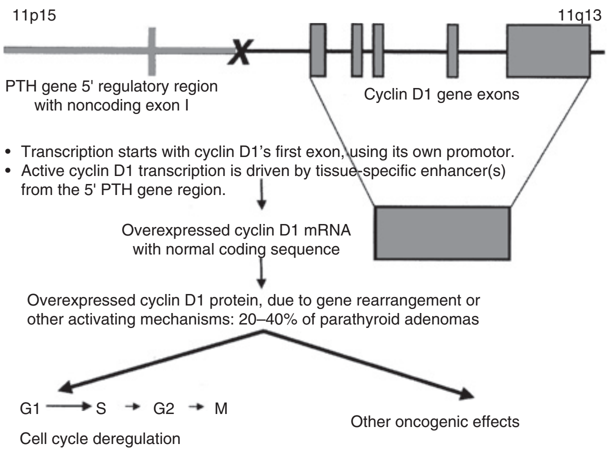
11p15
11q13
PTH gene 5' regulatory region
with noncoding exon I
Cyclin D1 gene exons
• Transcription starts with cyclin D1's first exon, using its own promotor.
• Active cyclin D1 transcription is driven by tissue-specific enhancer(s) from the 5' PTH gene region.
Overexpressed cyclin D1 mRNA with normal coding sequence
Overexpressed cyclin D1 protein, due to gene rearrangement or other activating mechanisms: 20–40% of parathyroid adenomas
G1 → S → G2 → M
Cell cycle deregulation
Other oncogenic effects

FIGURE 2 Diagram of directly observed molecular structure of the *PTH/cyclin D1 (PRAD1)* DNA rearrangement and its functional consequences. Modified with permission from Arnold (1993).

would have been missed by the Southern blot approach used in initial studies of parathyroid tumors. In addition, it seems likely that cyclin D1 expression could be deregulated in some parathyroid tumors by rearrangement with genes other than the *PTH* gene. Thus, previous studies may not have revealed the full frequency with which *cyclin D1* deregulation occurs in parathyroid neoplasia. Indeed, studies assessing the cyclin D1 protein product have revealed its overexpression in 20% to 40% of parathyroid adenomas (Hsi *et al.,* 1996; Vasef *et al.,* 1999; Tominaga *et al.,* 1999). This overexpression may occur more commonly by a *trans*-acting regulatory disturbance like that observed for *cyclin D1* overexpression in a variety of human cancer cells (Hosokawa and Arnold, 1998) rather than a clonal mutation in one gene allele. However achieved by the cell, such overexpressed cyclin D1 is a potent driver of abnormal parathyroid cell proliferation (Imanishi *et al.,* 2001; Mallya *et al.,* 2005).

Cyclin D1 is a 295-amino-acid protein homologous to various members of the cyclin family (Motokura *et al.,* 1991). Cyclins play important roles in regulation of cell cycle progression, and human cyclins have been grouped into several types based upon sequence similarities (Pestell *et al.,* 1999). Each cyclin appears to regulate the cell cycle at a specific time point by binding to and activating cyclin-dependent kinases (CDKs; Sherr, 1996). Expression of these cyclins is cell cycle phase-dependent and controlled by both transcriptional and posttranscriptional mechanisms. Cyclin D1 is a key regulator of the critical G1–S phase transition in the cell cycle. In so doing, its major cdk partners are cdk4 or cdk6, depending upon the tissue type examined. It may also be that cyclin D1 has other cellular functions, mediated through non-cdk-dependent pathways (Bernards, 1999; Arnold and Papanikolaou, 2005; Ewen and Lamb, 2004; Fu *et al.*, 2004).

Although genetic evidence for *cyclin D1*'s role as a parathyroid oncogene is incontrovertible, animal modeling has additionally provided direct experimental evidence that cyclin D1 overexpression is capable of driving parathyroid tumorigenesis. A transgenic mouse model for parathyroid neoplasia has been generated (Imanishi *et al.,* 2001) in which the *cyclin D1* gene is under the control of the regulatory region of the *PTH* gene, mimicking the rearrangement and resultant overexpression observed in human tumors. These mice develop parathyroid enlargement and increased serum calcium and PTH levels, and their study has illuminated the relationship in hyperparathyroidism between abnormal parathyroid proliferation and the biochemical phenotype (Imanishi *et al.,* 2001; Mallya *et al.,* 2005). Finally, cyclin D1 is being vigorously pursued as a target for antineoplastic therapy, primarily because of its major role in human breast cancer, lymphoma, and squamous cell cancers. If such efforts are successful, an anti-cyclin D1 agent would also be expected to have efficacy against the cyclin D1-driven subset of parathyroid tumors.

Tumor Suppressor Genes: MEN1

Inactivation of both alleles of a tumor suppressor gene, often by mutation or deletion, is required to completely deplete the gene's antineoplastic product. A common inactivation mechanism is somatic deletion of a substantial portion of chromosomal DNA that includes the relevant gene. This is revealed by a loss of heterozygosity of DNA markers in tumor DNA relative to normal DNA of the same individual. Identification of regions of chromosomes that are clonally and nonrandomly lost in parathyroid adenomas can indicate the general locations of tumor suppressor genes active in parathyroid cells. Our present state of knowledge in this regard shows that the overwhelming majority of parathyroid adenomas (and probably all) have at least one such clonal defect.

The MEN1 syndrome, which is inherited in an autosomal dominant fashion, is characterized classically by tumors of the parathyroids, pancreatic islets, and anterior pituitary. Several years ago, it was established by genetic mapping studies in families affected by MEN1 that the gene responsible is on chromosome 11*q*13 (Larsson *et al.,* 1988). Early evidence that the *MEN1* gene is a tumor suppressor gene was provided by the demonstration of somatic genetic alterations in MEN1 tumors that inactivate one allele of a gene region at 11*q*13 and so reveal the inherited *MEN1* mutation on the other allele (Fig. 3). In fact, allelic loss of polymorphic marker DNAs from this region of chromosome 11 has been found in the majority of MEN1-associated tumors including those of the parathyroid.

The *MEN1* gene was identified by positional cloning (Chandrasekharappa *et al.,* 1997; European Consortium on MEN1, 1997). More than 400 independent germline and somatic mutations scattered throughout the protein-coding

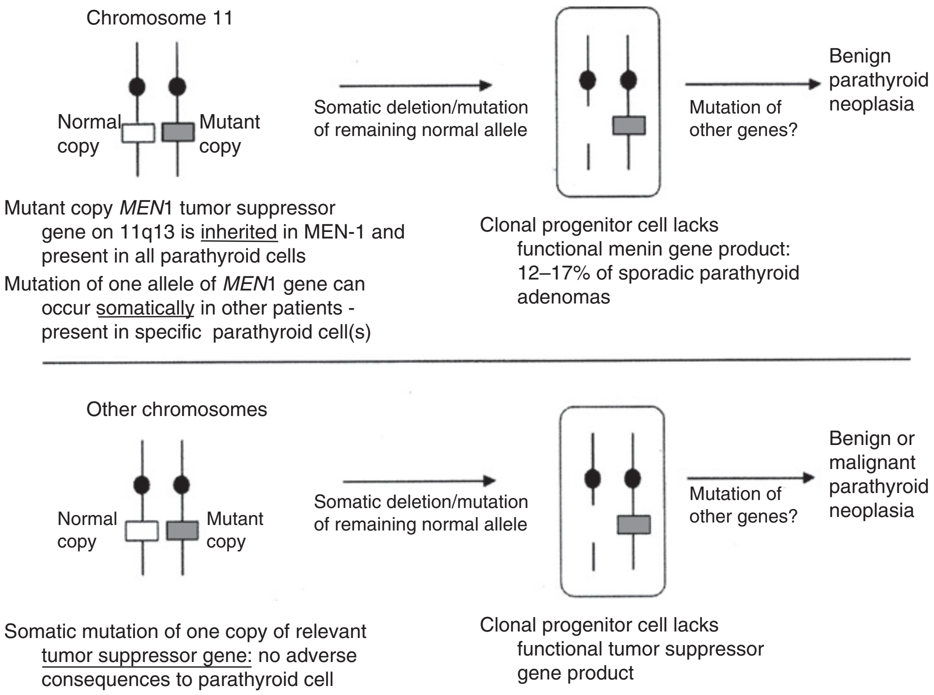
Chromosome 11
Normal copy
Mutant copy
Somatic deletion/mutation of remaining normal allele
Mutation of other genes?
Benign parathyroid neoplasia
Mutant copy MEN1 tumor suppressor gene on 11q13 is inherited in MEN-1 and present in all parathyroid cells
Mutation of one allele of MEN1 gene can occur somatically in other patients - present in specific parathyroid cell(s)
Clonal progenitor cell lacks functional menin gene product: 12–17% of sporadic parathyroid adenomas
Other chromosomes
Normal copy
Mutant copy
Somatic deletion/mutation of remaining normal allele
Mutation of other genes?
Benign or malignant parathyroid neoplasia
Somatic mutation of one copy of relevant tumor suppressor gene: no adverse consequences to parathyroid cell
Clonal progenitor cell lacks functional tumor suppressor gene product

FIGURE 3 Schematic diagram illustrating the established (for *MEN1*) and hypothesized roles of inactivation of classic tumor suppressor genes as contributory mechanisms in parathyroid neoplasia. A similar scheme applies to the role of the *HRPT2* tumor suppressor gene in the pathogenesis of parathyroid carcinoma. Modified with permission from Arnold (1993).

region have been identified. There is no correlation between genotype, meaning the specific location of mutation within the gene, and phenotype. Somatic mutations have been found to a variable extent in parathyroid adenoma as well as gastrinoma, insulinoma, lung carcinoid, and anterior pituitary tumors. Over 80% of the mutations are clearly inactivating, leading to a truncated product. This would be consistent with *MEN1* acting as a tumor suppressor gene and a lack of the menin protein caused by the loss of both alleles leading to tumor development. The human gene encodes a 610-amino-acid protein. Two nuclear localization signal sequences are present at the COOH-terminal portion of the menin protein, which is predominantly located in the nucleus (Guru *et al.,* 1998; Kaji *et al.,* 1999). The first protein shown to be a menin-interacting protein was the activator protein 1 factor, JunD, with resultant downregulation of JunD-activated transcription (Agarwal *et al.,* 1999). Because JunD is antimitogenic, in contrast to other Jun and Fos family members, and menin is a tumor suppressor, this appeared to be paradoxical. Further studies *in vitro* have suggested that the functions of JunD as a growth suppressor require interaction with menin, and that JunD is a growth promoter if menin is absent or inactive (Agarwal *et al.*, 2003). The level of menin changes throughout the cell cycle. Pituitary cells synchronized at the G1–S-phase boundary express menin at a lower level than G0–G1-synchronized cells (Kaji *et al.,* 1999). The expression of menin increases as the cell enters S phase, at which time JunD expression also increases. Cells synchronized at the G2–M phase express lower levels of menin.

Menin upregulates the cyclin-dependent kinase inhibitors, p18 and p27, important for cell-cycle regulation (Karnik *et al.*, 2005; Milne *et al.,* 2005). Menin re-expression in *Men1*-deficient cells blocks the transition from G0/G1 to S phase of the cell cycle and increases apoptosis (Hussein et al, 2007). Mice dually deficient in p18 and p27 develop a range of endocrine tumors representing those found in MEN1 and MEN2 syndromes (Franklin *et al.*, 2000).

The frequency of *MEN1* mutations in several kindreds representing a variant of MEN1 in which only parathyroid and pituitary tumors develop is very low (~10%) versus typical MEN1 kindreds (~75%). In one case of this MEN1 variant, in which *MEN1* mutation was not detected, a germline mutation of the *CDKN1B* gene encoding p27 was identified (Pellegata *et al.*, 2006). However, examination of 34 index cases from several other MEN1 variant kindreds failed to identify further mutations in p27 (Ozawa *et al.*, 2007). Hence, the main causes of this MEN1 variant remain unknown.

Menin is a component of particular histone methyltransferase complexes that control *Hox* gene expression important for early development, and cell proliferation and differentiation (Hughes *et al.*, 2004; Milne *et al.*, 2005; Yokoyama *et al.*, 2004). It may be involved in telomere biology (Lin and Elledge, 2003; Suphapeetiporn *et al.*, 2002) and DNA replication and repair (Jin *et al.*, 2003; Sukhodolets *et al.*, 2003). Menin interacts with several transcription factors including Smads, JunD, and NF-κB and modulates their activities (see Poisson *et al.*, 2003; Balogh *et al.*, 2006; Dreijerink *et al.*, 2006, for further details of menin-interacting proteins). Menin is a Smad3-interacting protein, and inactivation of menin blocks transforming factor-beta (TGF-β) and activin signaling, antagonizing their growth inhibitory properties (as well as inhibition of prolactin expression) in anterior pituitary cells (Kaji *et al.*, 2001; Lacerte *et al.*, 2004). In cultured parathyroid cells, menin inactivation achieved by menin antisense oligonucleotides leads to loss of TGF-β inhibition of cell proliferation and PTH secretion (Sowa *et al.*, 2004). Moreover, TGF-β did not affect (decrease) the proliferation and PTH production of parathyroid cells from MEN1 patients (Sowa *et al.*, 2004; Naito *et al.*, 2006). Loss of various TGF-β signaling pathway components by genetic or epigenetic means is common in cancer and is likely to be important in MEN1 tumorigenesis (Hendy *et al.*, 2005) despite the fact that, thus far, no acquired clonal mutations, insertions, or microdeletions in the Smad3 gene have been detected in a series of sporadic parathyroid adenomas and enteropancreatic tumors (Shattuck *et al.*, 2002).

Mice heterozygous for genetic ablation of the *Men1* gene develop endocrine tumors similar to human MEN1 patients (Crabtree *et al.*, 2001; Bertolino *et al.*, 2003). Parathyroid gland-specific deletion of the mouse *Men1* gene results in parathyroid neoplasia and hypercalcemic hyperparathyroidism (Libutti *et al.*, 2003). In mice (and in humans) tissue expression of menin is widespread throughout development and into adult life and occurs early in fetal life. Therefore, menin expression is not restricted to those cells and tissues that are affected in MEN1, and haploinsufficiency of menin is well tolerated in many tissues. In mice, homozygous deletion of *Men1* is embryonic lethal. The affected fetuses die at mid-gestation with defects in multiple organs. This may reflect defective TGF-β signaling or Hox gene expression.

About 20% of the menin mutations are missense and, although found throughout the entire sequence, are prevalent within amino acids 149–218 encoded by exon 3. This is in a part of the molecule thought to be important for interaction with transcriptional regulators Smad3 and JunD but not NF-κB. Although it might have been anticipated that analysis of the missense mutations would provide insight into which few of the many proteins that menin interacts with are critical for MEN1-tumorigenesis, for the most part this promise has yet to be fulfilled. To a large extent, this is because the missense menin mutants are markedly unstable and are targeted to the ubiquitin-proteasome pathway (Yaguchi *et al.*, 2004; Canaff *et al.*, 2006).

Putative Oncogenes and Tumors Suppressor Genes

Comparative genomic hybridization—a molecular cytogenetic technique in which the entire tumor genome is screened for chromosomal gains and/or losses—has identified amplified regions on several chromosomes (most consistently 7, 16, and 19; Fig. 4). These observations suggest the presence of novel parathyroid oncogenes (Palanisamy *et al.*, 1998; Agarwal *et al.*, 1998; Farnebo *et al.*, 1999; Dwight *et al.*, 2002) in these locations, which remain to be identified.

Besides loci on chromosome 11, several other regions of nonrandom clonal allelic loss have been documented in parathyroid adenomas, pointing to the location of novel tumor suppressor genes. This finding of the involvement of multiple chromosomal regions, which include 1*p* (Cryns *et al.*, 1995; Williamson *et al.*, 1997), 1*q*, 6*q*, 9*p*, and 15*q* (Tahara *et al.*, 1996a, 1996b), emphasize the molecular heterogeneity of parathyroid adenomatosis.

Comparative genomic hybridization has confirmed the chromosomal losses identified by loss of heterozygosity analysis (allelotyping) (Palanisamy *et al.*, 1998; Agarwal *et al.*, 1998; Farnebo *et al.*, 1999; see Fig. 4). One common defect uncovered to date involves allelic loss on chromosome 1*p* (Cryns *et al.*, 1995). Interestingly, chromosome 1*p* loss occurs in several other neoplasms including medullary thyroid carcinomas and pheochromocytomas which occur in association with the MEN2A inherited cancer syndrome, of which parathyroid tumors are also a part. Benign parathyroid tumors are found in 10% to 20% of MEN2A patients. This pattern of allelic loss is consistent with the existence of a tumor suppressor gene(s) on 1*p*, whose loss or inactivation is pathogenetically important. Numerous candidate genes are present in this region, but the involvement of any of them in the development of parathyroid or other types of tumors remains to be established. Some candidates on 1*p*, such as genes for the *p*18 cyclin-dependent kinase inhibitor and *RAD54*, have been excluded as parathyroid tumor suppressors (Tahara *et al.*, 1997; Carling *et al.*, 1999a). Mutations of candidate genes within the 9*p* region for the *p*16 and *p*15 cyclin-dependent kinase inhibitors do not appear to be involved in parathyroid tumorigenesis (Tahara *et al.*, 1996b). A major goal for future work is the identification of the entire constellation of oncogenes and tumor suppressor genes that contribute to the development of parathyroid adenomatosis.

It had been assumed that primary parathyroid hyperplasia and uremic refractory secondary hyperparathyroidism involved polyclonal, nonneoplastic cellular proliferation. However, the monoclonal nature of a substantial number of the "hyperplastic" tumors from such patients was demonstrated by X-chromosome inactivation analysis (Arnold *et al.*, 1995). In addition, allelic loss of chromosome 11 markers and/or *MEN1* gene inactivation has been demonstrated in only a few of these tumors (Falchetti *et al.*, 1993; Arnold *et al.*, 1995; Imanishi *et al.*, 1997, 1999a). Furthermore, cyclin D1 overexpression does not appear to be common in these uremia-associated tumors (Vasef *et al.*, 1999; Tominaga *et al.*, 1999), nor is mutation of the

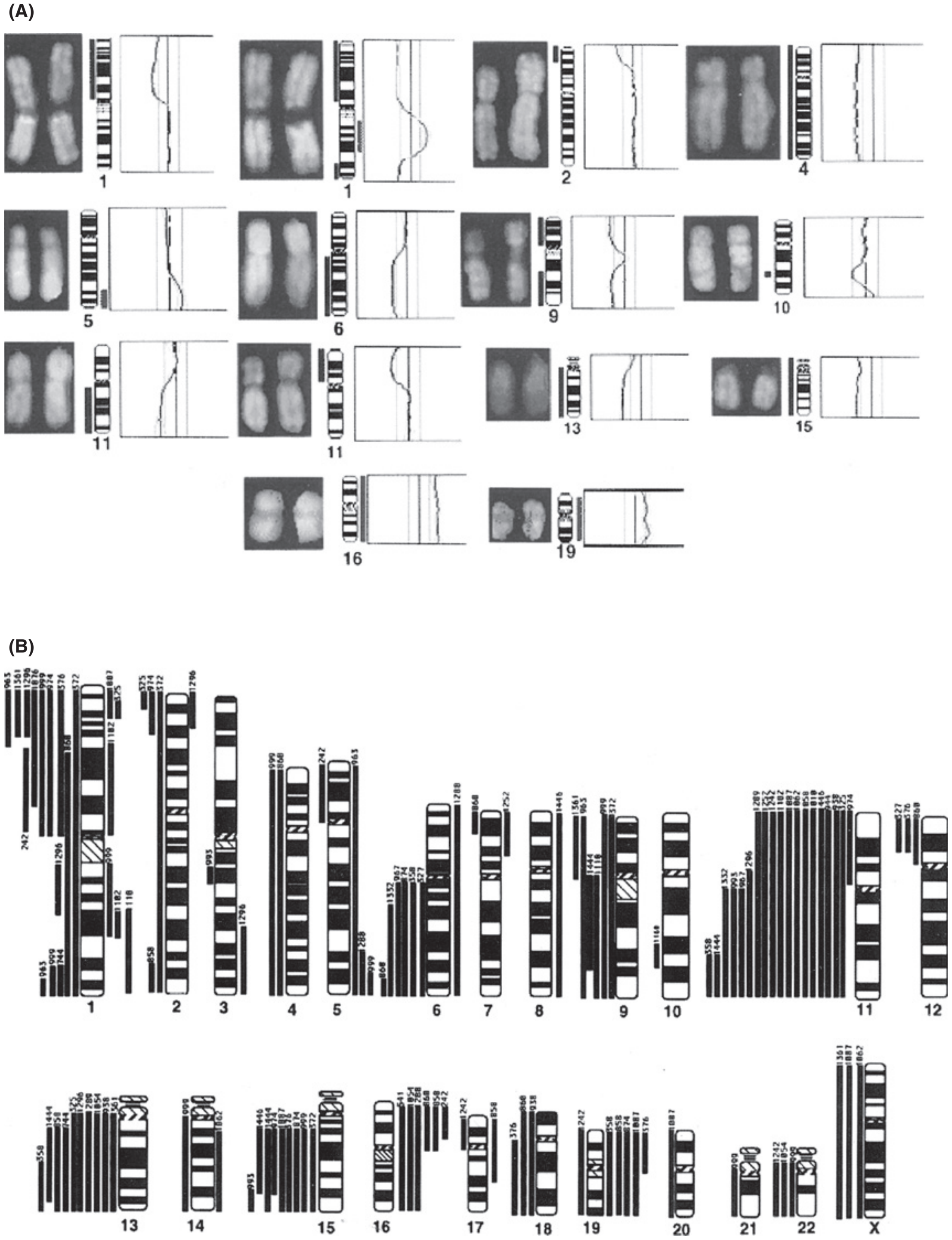

FIGURE 4 Novel chromosomal abnormalities identified by comparative genomic hybridization (CGH) in parathyroid adenomas. (**A**) Representative CGH results in parathyroid adenomas. Individual examples of fluorescent ratio profiles (*right*) and digital images (*left*) of chromosomes with recurrent gains or losses. The red vertical bar on the left side of a chromosome ideogram (*middle*) indicates the region of loss, and the green vertical bar on the right side of an ideogram indicates the region of gain. (**B**) DNA copy number changes in 53 parathyroid adenomas. Summary of all gains and losses detected by CGH. The vertical bars on the left side of the chromosome ideograms indicate losses, and those on the right side indicate gains of the corresponding chromosomal region for each individual tumor, as numbered. Adapted with permission from Palanisamy *et al*. (1998).

vitamin D receptor (*VDR*) gene, despite the low expression of this parathyroid cell antiproliferative factor in such tumors (Brown *et al.,* 2000). Neoplastic transformation of preexisting polyclonal hyperplasia involving genes not yet implicated in parathyroid tumorigenesis is thus likely to play an important role in these disorders.

Further Genetic Aspects

Some genes that play an important role in the pathogenesis of other tumor types might have been expected to be involved in the pathogenesis of benign parathyroid adenomas. However, candidate genes such as *ras* (Friedman *et al.,* 1990), *p53* (Yoshimoto *et al.,* 1992; Cryns *et al.,* 1994a; Hakim and Levine, 1994), and *RB* (Cryns *et al.,* 1994b) seem rarely, or not at all, to contribute to the development of benign adenomas. A target of the Wnt pathway, β-catenin, encoded by the *CTNNB1* gene that is implicated in other forms of tumorigenesis is a candidate for involvement in parathyroid neoplasia. Although one study reported stabilizing missense *CTNNB1* mutations in 3 of 20 parathyroid adenomas and increased β-catenin expression (Bjorklund *et al.,* 2007), this has not been found in other studies (Semba *et al.,* 2000; Ikeda *et al.,* 2002; Costa-Guda and Arnold, 2007). Therefore, the present view is that β-catenin's involvement in the initiation or early progression of parathyroid adenomatosis remains to be established (Simonds, 2007).

The development of primary hyperparathyroidism occurs with increased frequency in individuals exposed to ionizing radiation of the neck. Thus mutations in genes involved in DNA repair and recombination may contribute to parathyroid tumorigenesis irrespective of the actual involvement of irradiation. Such candidates include the *RAD51* and *RAD54* genes on chromosomes 15*q* and 1*p*, respectively, within regions that demonstrate allelic loss in parathyroid adenomas. However, no evidence for a role of somatic inactivation of these particular genes in parathyroid neoplasia has been found (Carling *et al.,* 1999a, 1999b). In some cases, those genes responsible for the rare inherited predisposition to a particular tumor also play a role in the development of the more common sporadic type. The *MEN1* gene, as detailed earlier, has proved to be such an example. Germline gain-of-function mutations in the *RET* protooncogene cause MEN2 (Eng *et al.,* 1996). This made the *RET* gene, which encodes a tyrosine kinase receptor, a candidate for involvement in nonfamilial hyperparathyroidism. However, although MEN2-type *RET* mutations have been implicated in the pathogenesis of some sporadic medullary thyroid carcinomas and pheochromocytomas, there is no evidence of these *RET* mutations in sporadic parathyroid adenomas (Padberg *et al.,* 1995; Pausova *et al.,* 1996; Williams *et al.,* 1996; Komminoth *et al.,* 1996; Kimura *et al.,* 1996). It was demonstrated that *RET* is expressed in both MEN2A parathyroid tumors and in sporadic adenomas (Pausova *et al.,* 1995, 1996; Kimura *et al.,* 1996). This suggests that parathyroid disease is an integral part of the MEN2A syndrome, but that MEN2 mutations in *RET* rarely, if ever, play a role in the pathogenesis of sporadic parathyroid tumors. Mutations within the coding region of glial cell-derived neurotrophic factor (GDNF), one of the RET ligands, do not appear to play a role in the genesis of MEN2 neoplasms or in sporadic neuroendocrine tumors such as parathyroid adenomas (Marsh *et al.,* 1997). An interesting candidate parathyroid tumor suppressor, *VDR*, is also lacking in evidence for a direct pathogenetic role by specific clonal mutation (Brown *et al.,* 2000; Samander and Arnold, 2006).

Familial hypocalciuric hypercalcemia (FHH), also known as familial benign hypercalcemia, is an autosomal dominant disorder that is characterized by enhanced parathyroid function due to reduced sensitivity to extracellular calcium (Marx *et al.,* 1981; Law et al., 1985). Mutations in the parathyroid calcium-sensing receptor (*CASR*) gene, located on chromosome 3*q*13.3-*q*21 (Janicic *et al.,* 1995a), are a primary cause of this disorder (Pollak *et al.,* 1993; Janicic *et al.,* 1995b; Hendy *et al.,* 2000; D'Souza-Li *et al.,* 2002; Pidasheva *et al.,* 2005, 2006). Moreover, individuals homozygous for such mutations present with neonatal severe hyperparathyroidism (NSHPT) with marked parathyroid hypercellularity (Pollak *et al.,* 1994), and members of some FHH kindreds atypically manifest hyperparathyroidism and surgical removal of the adenoma or hyperplastic glands is generally curative (Soei *et al.,* 1999; Carling *et al.,* 2000). Affecteds of some kindreds presenting with FIHP (with either frankly elevated serum PTH levels and/or hypercalciuria) are heterozygous for inactivating *CASR* mutations (Simonds *et al.,* 2002; Warner *et al.,* 2004). The apparent link between parathyroid calcium-sensing and proliferative pathways suggested that somatic alterations in the *CASR* gene could be tumorigenic in sporadic parathyroid tumors. However, several studies have failed to document somatic mutation of the *CASR* gene as a significant factor in parathyroid tumorigenesis (Hosokawa *et al.,* 1995; Thompson *et al.,* 1995; Degenhardt *et al.,* 1998; Cetani *et al.,* 1999). It has been reported that more than half of the parathyroid glands of patients with primary and severe uremic secondary hyperparathyroidism show reduced CASR expression (Kifor *et al.,* 1996; Farnebo *et al.,* 1997, 1998; Gogusev *et al.,* 1997; Chikatsu *et al.,* 2000). Thus, despite somatic mutation of the *CASR* gene rarely if ever contributing to the pathogenesis of sporadic parathyroid tumors, mutations in growth-deregulating genes may secondarily alter the calcium set point, perhaps in part by decreasing expression of the CASR. It can be noted that because *CASR* gene transcription is upregulated by 1,25$(OH)_2$D (Canaff and Hendy, 2002), and the clinical severity of FHH-affected individuals can be exacerbated by vitamin D deficiency (Zajickova *et al.,* 2007), reduced levels of the VDR observed in parathyroid tumors may contribute to the decreased CASR expression. A mutation in a gene involved in calcium set-point control might secondarily stimulate proliferation until the serum calcium concentration surpasses the abnormal set point (Parfitt 1994). Data indicating that the growth rate of parathyroid tumors is generally low, but must have been higher earlier in their development (Parfitt *et al.,* 1994), are consistent with this hypothesis. Interestingly, calcium regulatory abnormalities found in transgenic mice with cyclin D1-driven hyperparathyroidism show that mutations in "set-point" genes need not be the primary instigators in this disease (Imanishi *et al.,* 2001; Mallya *et al.*, 2005).

The syndrome of hereditary hyperparathyroidism and jaw tumors (HPT-JT) is an uncommon autosomal dominant disorder inherited with incomplete penetrance and characterized by early-onset recurrent parathyroid tumors (90%) and fibrous ossifying tumors of the mandibula or maxilla (30%). Less frequent renal lesions (10%) include Wilms' tumor, hamartomas, and polycystic kidney disease (Jackson *et al.*, 1990; Chen *et al.*, 2003). Uterine tumors include adenosarcoma, but benign adenofibromas and leiomyomas are more common (Bradley *et al.*, 2005). Parathyroid carcinoma occurs at increased frequency (15%) in this syndrome. In contrast to the multiglandular enlargement typically found at presentation in other classical forms of inherited hyperparathyroidism, a solitary, enlarged, and often cystic gland may be found in an HPT-JT patient. Normocalcemia is often achieved after removal of the tumor; however, recurrence is well documented. In a large group of families the HPT-JT trait was linked to 1q21-32 (Szabo *et al.*, 1995; Hobbs *et al.*, 2002). The responsible gene, *HRPT2*, at 1q31.2, was identified by positional cloning and mutation analysis, and encodes a novel transcription factor, parafibromin, of 531 amino acids (Carpten *et al.*, 2002). Parafibromin is a widely expressed nuclear protein, and within the parathyroid gland it is reported to be in both the cytoplasmic and nuclear compartments (Woodward *et al.*, 2004). Functional analysis of the several potential nuclear localization signals (NLSs) in parafibromin is the subject of ongoing study (Hahn and Marsh, 2005; Bradley *et al.*, 2007; Lin *et al.*, 2007). Nuclear parafibromin has a proapoptotic activity (Lin *et al.*, 2007). Transfection experiments have demonstrated the ability of parafibromin to block cyclin D1 expression (Woodward *et al.*, 2004). Experimental overexpression of wild-type parafibromin in transformed cell lines inhibits cell proliferation and induces G1-phase cell-cycle arrest (Zhang *et al.*, 2006). Parafibromin HPT-JT mutants act in a dominant-negative manner to abolish the ability of wild-type parafibromin to inhibit cell growth (Zhang *et al.*, 2006). In mammalian cells parafibromin is part of an RNA polymerase II associated factor complex (Paf1) and has sequence homology to yeast Cdc73, a constituent of a protein multimer important for transcription initiation/elongation, histone methylation and RNA processing (Rozenblatt-Rosen *et al.*, 2005; Yart *et al.*, 2005). Parafibromin binds directly to the COOH-terminal part of β-catenin and transduces Wnt pathway signals into transcriptional initiation and elongation by RNA polymerase II (Mosimann *et al.*, 2006). However, because stimulation of Wnt pathway signals lead to cell proliferation, it is unclear how this proposed action of parafibromin fits with its role as a tumor suppressor.

Loss of heterozygosity (LOH) at chromosome 1q, particularly in carcinomas from HPT-JT kindreds, suggests that the two-hit hypothesis of Knudson applies to the *HRPT2* gene (Howell *et al.*, 2003; Shattuck *et al.*, 2003a; Cetani *et al.*, 2004). The most frequent genetic mechanism through which the second hit occurs—small intragenic mutations—differs from that of most other tumor suppressor genes (Weinstein and Simonds, 2003). Importantly, sporadic parathyroid carcinomas very commonly contain somatic mutations of the *HRPT2* gene (Howell *et al.*, 2003; Shattuck *et al.*, 2003a; Cetani *et al.*, 2004). Of note is the finding that some patients with apparently sporadic parathyroid carcinomas harbor germline mutations (Shattuck *et al.*, 2003a; also see later section on parathyroid carcinoma).

Familial isolated hyperparathyroidism (FIHP or HRPT1) has been defined as hereditary primary hyperparathyroidism without the association of other disease or tumors. Although listed as a distinct genetic entity, some cases presenting as FIHP have now been shown, by gene mutation analysis, to be variants of other monogenic diseases—FHH (Simonds *et al.*, 2002; Warner *et al.*, 2004), MEN1 (Carrasco *et al.*, 2004; Pannett *et al.*, 2003), or HPT-JT (Guarnieri *et al.*, 2007; Kelly *et al.*, 2006; Villablanca *et al.*, 2004; Simonds *et al.*, 2004). Despite the rarity of *HRPT2* mutations in FIHP, a personal or family history of parathyroid carcinoma in FIHP warrants consideration of germline *HRPT2* mutation status that will be useful for diagnosis and management. Identification of an *HRPT2* mutation can lead to detection and removal of potentially malignant parathyroid tumors (Kelly *et al.*, 2006; Guarnieri *et al.*, 2007).

For several other FIHP kindreds the causative gene is not known and the basis of familial hyperparathyroidism distinct from other described inherited disorders remains to be clarified. A genome-wide screen of several kindreds with FIHP has suggested a 1.7-Mb region of linkage on chromosome 2p13.3-14, and mutation analysis of candidate genes within this interval is ongoing (Warner *et al.*, 2006). In addition, one kindred has been described with apparent autosomal recessive inheritance of the hyperparathyroidism and large recurrent adenomas (Law *et al.*, 1983). The mode of inheritance in this family would set it apart from the other autosomal dominant disorders, although it may prove, with further study, to be autosomal dominant with decreased penetrance.

MOLECULAR PATHOGENESIS OF PARATHYROID CARCINOMA

As noted earlier, mutation of the *HRPT2* tumor suppressor gene was recently identified as a major contributor to parathyroid carcinoma. Such mutations are detectable in over

75% of parathyroid cancers (Shattuck *et al.*, 2003; Howell *et al.*, 2003; Cetani *et al.*, 2004), and because noncoding mutations are expected and would have escaped detection, most likely *HRPT2* inactivation occurs in essentially all cases. In addition, a sizable subset of patients with apparently sporadic parathyroid carcinoma bear unsuspected germline mutations in *HRPT2* (Shattuck *et al.*, 2003). Such patients may represent *de novo* examples, or lower penetrance variants, of the HPT-JT syndrome, and the potential opportunity for early diagnosis to prevent or cure parathyroid cancer in their family members has established a new role for genetic testing in this circumstance.

Because of the impressive genetic evidence for *HRPT2*'s role, parafibromin immunostaining is being evaluated as a potential clinical test for parathyroid carcinoma (Tan *et al.*, 2004; Gill *et al.*, 2006; Juhlin *et al.*, 2006; Cetani *et al.*, 2007). Several studies have reported virtually complete loss of nuclear immunostaining in most parathyroid carcinomas in comparison with positive staining in benign parathyroid tumors. However, because of technical and/or biological factors, not all studies yield such uniform results (Juhlin *et al.*, 2007), and the clinical use of parafibromin staining remains to be established. Interestingly, adenomas of kindreds with germline *HRPT2* mutations were also parafibromin negative, suggesting that allelic loss of parafibromin is a key step in progression to carcinoma.

The cyclin D1 oncoprotein, overexpressed in 20% to 40% of adenomas, was found to be overexpressed in 10 of 11 (91%) parathyroid carcinomas in one study (Vasef *et al.*, 1999) and in 2 of 3 in another (Hsi *et al.*, 1996). This observation raises the possibility that cyclin D1 may play a critical and consistent role in parathyroid cancers, and that patients with this disease might be considered for eventual inclusion in clinical trials once novel anti-cyclin D1 therapies have been developed. The tumor suppressor genes *p53* and *RB* have been examined for abnormalities in malignant, as well as benign, parathyroid tumors. Allelic loss of the *p53* gene has been found occasionally in parathyroid carcinomas, but direct mutations have not been described, and *p53*'s overall contribution to parathyroid cancer is minimal at best (Cryns *et al.*, 1994a; Hakim and Levine, 1994). In contrast, loss of chromosome region 13*q*, which contains *RB, BRCA2,* and other potential parathyroid tumor suppressors, is likely to be a key factor in the pathogenesis of many parathyroid carcinomas (Cryns *et al.*, 1994b; Dotzenrath *et al.*, 1996; Pearce *et al.*, 1996; Imanishi *et al.*, 1999b; Kytola *et al.*, 2000). However, the absence of detectable mutations in *RB* or *BRCA2* suggests that any role for these genes would be indirect rather than as classical tumor suppressors (Shattuck *et al.*, 2003b). Similarly, loss of putative tumor suppressor genes in genomic regions including 1*p*, 3*q*, 4*q*, and 21*q* may be involved as significant factors in malignant parathyroid tumors (Cryns *et al.*, 1995; Imanishi *et al.*, 1999b; Kytola *et al.*, 2000; Agarwal *et al.*, 1998). Specific chromosome regional gains, suggesting the involvement of oncogenes, have been found in parathyroid carcinomas by comparative genomic hybridization (Imanishi *et al.*, 1999b; Kytola *et al.*, 2000; Agarwal *et al.*, 1998), but the involved genomic locations show little consistency among the existing reports, and the results require confirmation by complementary methods. Identification of genetic lesions that are specific for the malignant phenotype are key in making a molecular diagnosis, which would aid in overcoming the well-known difficulties in distinguishing malignant versus benign parathyroid tumors histopathologically. Here, the new knowledge of *HRPT2* mutations and loss of parafibromin expression in parathyroid carcinoma represents a major step forward that could be enhanced by knowledge of additional genetic contributors to parathyroid malignancy.

ECTOPIC SECRETION OF PTH

The ectopic secretion of PTH by nonparathyroid tumors is an extremely rare cause of hyperparathyroidism. The use of modern immunometric assays for PTH that show no cross-reactivity with PTHrP, the major cause of the hypercalcemia of malignancy, combined, in some cases, with molecular analysis using specific human PTH gene probes (Hendy *et al.*, 1981; Vasicek *et al.*, 1983), has suggested or confirmed the occurrence of this syndrome in several cases (see VanHouten *et al.*, 2006, and references therein).

In one case of an ovarian carcinoma (Nussbaum *et al.*, 1990), the molecular basis for the aberrant expression of PTH was determined. This involved a rearrangement (Fig. 5) and amplification of the *PTH* gene, such that it was no longer under the control of upstream regulatory elements that may normally act to silence *PTH* gene expression in nonparathyroid tissue. This case provided strong documentation that the tumor was in fact the source of the high circulating PTH levels and the patient's hypercalcemia. In a case of severe hyperparathyroidism resulting from ectopic PTH production by a pancreatic malignancy, a mechanism of transactivation of the *PTH* gene was suggested by the finding of hypomethylation of the *PTH* gene promoter in tumor tissue (VanHouten *et al.*, 2006).

SUMMARY

Several advances have been achieved toward the goal of understanding the molecular basis of sporadic parathyroid tumorigenesis. The cyclin *D1/PRAD1* oncogene has been identified as a parathyroid oncogene, is overexpressed in 20% to 40% of parathyroid adenomas, and is also involved in the development of many additional tumor types. The gene responsible for MEN1 has been identified, and mutations in menin contribute in up to 20% of sporadic parathyroid adenomas. Mutations in the *RET* gene, the causal agent in MEN2, plus *CASR* and *VDR*, appear to contribute

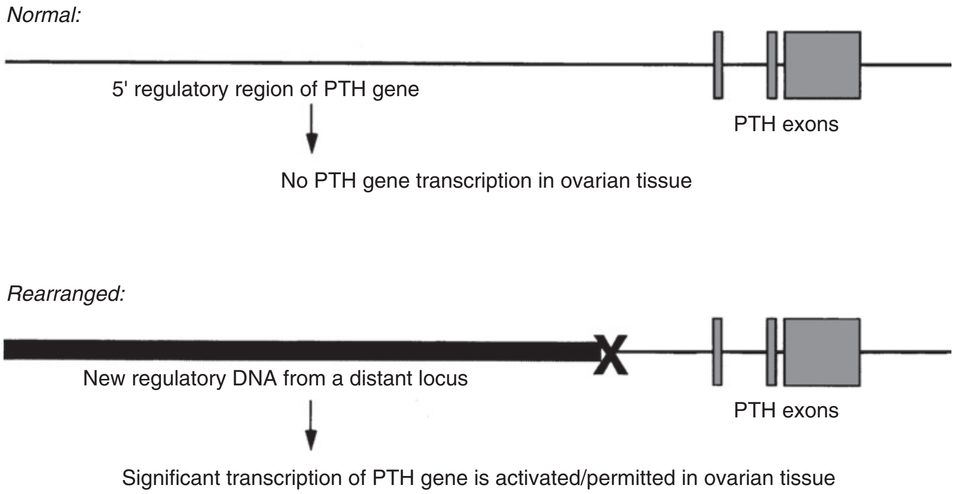
Normal:
5' regulatory region of PTH gene
PTH exons
No PTH gene transcription in ovarian tissue
Rearranged:
New regulatory DNA from a distant locus
X
PTH exons
Significant transcription of PTH gene is activated/permitted in ovarian tissue

FIGURE 5 Molecular pathology of the ectopic production of PTH by an ovarian cancer. Schematic diagram of the normal PTH gene region (top) and the rearranged, amplified PTH gene region (*bottom*) in a PTH-secreting ovarian tumor. The bold **"X"** represents the breakpoint of the DNA rearrangement. Reproduced with permission from Arnold (1993).

rarely if ever to the development of sporadic parathyroid tumors. The identification of the tumor suppressor parafibromin encoded by the *HRPT2* gene has provided important insight into parathyroid disease, especially parathyroid carcinoma. Ultimately, a description of parathyroid tumorigenesis will need to account for such features as the rarity of parathyroid carcinoma, the increased incidence of tumors after neck irradiation, and the increased frequency of hyperparathyroidism in postmenopausal women. In addition, the relationship between excessive cellular proliferation and an altered set point in the mechanism linking extracellular calcium concentration to PTH secretion requires further dissection, and recently developed animal modeling of primary hyperparathyroidism may assist in this effort.

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Chapter 61

Familial Benign Hypocalciuric Hypercalcemia and Neonatal Primary Hyperparathyroidism

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DISCOVERY AND INITIAL DESCRIPTION

Before the ready availability of inexpensive and accurate measurements of serum calcium concentration, the diagnosis of hypercalcemia was generally considered only when there were overt signs or symptoms to suggest it. Thus, most cases of hypercalcemia that were identified were severe, and patients presented with urolithiasis, nausea and vomiting, weight loss, or renal insufficiency (Albright and Reifenstein, 1948; Trigonis *et al.,* 1983). In the late 1960s, automated multichannel serum chemical analyzers became widely used in the United States and Western Europe, leading to "routine" measurement of serum calcium concentration in large numbers of adult medical patients, many having no specific symptoms. This large-scale screening of the population revealed a hitherto-unsuspected group of mildly to moderately hypercalcemic individuals, most of whom turned out to have primary hyperparathyroidism (1° HPT) (Heath *et al.,* 1980). Subsequently, the literature on mild 1° HPT has expanded enormously, and the syndrome of virtually asymptomatic 1° HPT is now recognized to be the most common form of the disease (Bilezikian *et al.,* 1991; Heath, 1991). Some forms of 1° HPT are inherited, such as in multiple endocrine neoplasia types 1 or 2A (Gagel, 1994; Metz *et al.,* 1994), the hyperparathyroidism-jaw tumor syndrome (Szabo *et al.,* 1995; Carpten *et al,* 2002), or isolated familial 1° HPT (Law *et al.,* 1983; Wassif *et al.,* 1993, 1999). However, investigators studying families in which hypercalcemia seemed to be transmitted as an autosomal dominant trait recognized about 40 years ago that some kindreds differed in important ways from those having inherited neoplastic parathyroid disease. Jackson and colleagues reported a hypercalcemic family (the WAL kindred) in which the clinical and biochemical characteristics shared certain features with 1° HPT, but in whom surgical exploration of affected individuals neither revealed parathyroid adenomas nor cured the hypercalcemia (Jackson and Boonstra, 1967). Those investigators recognized that this family might have a novel hypercalcemic syndrome (Jackson and Boonstra, 1966), but they did not give the disorder a specific name. Only in 1972 did Foley *et al.* offer the first detailed studies, clear description, and distinct name for this syndrome of benign, life-long hypercalcemia, familial benign hypercalcemia, which they postulated to be due to an abnormality of the receptor mechanism for calcium ion control and unassociated with clear-cut parathyroid hyperplasia or adenomatosis (Foley *et al.,* 1972). Subsequent studies (D. A. Heath, 1989; H. Heath, 1989; Heath, 1994; Law and Heath, 1985; Marx *et al.,* 1981a; Menko *et al.,* 1983) reinforced and extended Foley's original description, establishing the disorder as having the characteristics shown in Table I.

A related familial disorder occurring in neonates, characterized by severe hypercalcemia, bone demineralization, respiratory distress, and failure to thrive, had been described several decades earlier (Hillman *et al.,* 1964; Landon, 1932; Philips, 1948). Spiegel *et al.* were the first to recognize the relationship of this syndrome, now known as neonatal severe hyperparathyroidism (NSHPT), to familial benign hypercalcemia (Cooper *et al.,* 1986; Lillquist *et al.,*

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TABLE I Comparison of Characteristic Features of Familial Benign Hypocalciuric Hypercalcemia (FBHH), Familial Isolated Primary Hyperparathyroidism (Familial HPT), and Sporadic Primary Hyperparathyroidism (Sporadic HPT).

Variable	FBHH	Familial HPT	Sporadic HPT
Age of onset	At birth	Variable	Usually >40 years
Symptoms	Usually none	Variable	—Asymptomatic in 80% —Cortical bone loss —Urolithiasis in 20%
Serum-plasma level			
Calcium	Elevated	Elevated	Elevated
Magnesium	Normal to increased	Variable	Variable
Phosphorous	Normal to mild decrease	Normal to very low	Normal to very low
$1,25(OH)_2D$	Normal	Normal to increased	Normal to increased
Intact PTH	Normal (80–85%) Elevated (15–20%)	Elevated in >80%	Elevated in >80%
Urinary excretion			
Cyclic AMP	Normal to mildly increased	High normal to increased	High normal to increased
Calcium	Normal to low	Low to elevated	Low to elevated
Ca:Cr clearance ratio	Generally <0.01	Generally >0.03	Generally >0.02
Magnesium	Low	Normal to high	Normal to high
Other findings	Possibly increased risk of chondrocalcinosis, gallstones	Typical findings of HPT	Typical findings of HPT

1983; Marx *et al.,* 1982, 1985; Matsuo *et al.,* 1982; Page and Haddow, 1987; Spiegel *et al.,* 1977; Steinmann *et al.,* 1984). Almost a decade before the molecular basis for these two related syndromes was identified, Marx *et al.* suggested that they represented the expression of homozygous and heterozygous abnormalities in calcium-sensing, respectively (Fujimoto *et al.,* 1990; Marx *et al.,* 1982, 1985).

FAMILIAL BENIGN HYPOCALCIURIC HYPERCALCEMIA

As just noted, Foley and colleagues provided the first full clinical description of this syndrome (Foley *et al.,* 1972) as well as the name familial benign hypercalcemia (FBH)—a simple but very descriptive and reassuring name. Later, investigators at the National Institutes of Health reported the same condition as familial hypocalciuric hypercalcemia (FHH) (Marx *et al.,* 1977), in recognition of the unexpectedly low urinary excretion of calcium in affected persons. The literature is substantially divided between the use of these two names, i.e., FBH or FHH, to describe this clinical entity (D. A. Heath, 1989; H. Heath, 1989; Heath, 1994; Law and Heath, 1985; Marx *et al.,* 1981a, 1977; Menko *et al.,* 1983). A unifying term describing the key features of the syndrome, familial benign hypocalciuric hypercalcemia (FBHH), was proposed by Heath (1989) and later adopted by Strewler (1994). This unifying term will be used in this chapter.

CLINICAL AND BASIC LABORATORY CHARACTERISTICS OF FBHH

The earliest kindreds reported to have FBHH usually had one family member who had been misdiagnosed as having 1° HPT and, therefore, had undergone surgical exploration, only to have either no histologic abnormality (the commonest finding) or subtle "hyperplasia" of the parathyroids identified (Davies *et al.,* 1981; D. A. Heath, 1989; Heath, 1994; Law and Heath, 1985; Marx *et al.,* 1981a, 1977; Menko *et al.,* 1983; Paterson and Gunn, 1981; Sereni *et al.,* 1982). In any event, even subtotal parathyroidectomy usually failed to normalize serum calcium levels. Subsequent investigations of other family members often revealed someone else with failed neck exploration and/or a large number of hypercalcemic, but asymptomatic, individuals of widely varying ages within a given kindred. Approximately 9% of patients referred to the National Institutes of Health in the 1970s with failed parathyroid surgery turned out to have FBHH (Marx *et al.,* 1980). As the syndrome of FBHH has become more widely recognized, it is often diagnosed before any family members have had unnecessary parathyroid surgery. The prevalence of classical FBHH was estimated to be 1/78,000 in the west of Scotland (Hinnie *et al.*, 2001).

THE FBHH SYNDROME IS CLINICALLY BENIGN

This uncommon, globally distributed hypercalcemic syndrome is inherited in an autosomal dominant fashion and is usually characterized by lifelong asymptomatic hypercalcemia, relative hypocalciuria, and absence of classical complications of hypercalcemia, including bone disease and renal stone disease. General morbidity and mortality appeared to be essentially normal in series from the Mayo Clinic and National Institutes of Health, as well as in studies by other investigators (D. A. Heath, 1989; H. Heath, 1989; Heath, 1994; Law and Heath, 1985; Marx *et al.*, 1977, 1981a; Menko *et al.*, 1983). Furthermore, almost all affected persons probably lack symptoms of hypercalcemia (Foley *et al.*, 1972; D. A. Heath, 1989; Law and Heath, 1985; Marx *et al.*, 1977; Menko *et al.*, 1983; Pearce *et al.*, 1997; Hendy *et al.*, 2000; Rose *et al.*, 2001), although symptoms consistent with hypercalcemia have been described in some probands (Marx *et al.*, 1981a). The studies of Law and Heath (1985) suggested that selection, referral, and "coaching" biases could account for the presence of symptoms among probands; that is, the probands see physicians for various reasons that lead to biochemical screening and the incidental discovery of hypercalcemia. Probands may undergo repeated questioning about possible symptoms of hypercalcemia and be prompted inadvertently to answer in the affirmative. Thus, probands in typical FBHH kindreds may have a variety of symptoms, but affected family members detected by screening generally usually have no more symptoms than unaffected persons (see Associated Clinical Findings). However, atypical presentations such as severe hypercalcemia, hypercalciuria, nephrolithiasis and pancreatitis have been described. Indeed, genetic testing of families with some features of FBHH has led to the discovery of CaSR mutations in some families with isolated familial hyperparathyroidism (Simonds *et al.*, 2002; Warner *et al.*, 2004). In addition, some children/infants with heterozygous CaSR mutations can clearly present with symptomatic bone disease that can revert to more typical asymptomatic FBHH picture later (Pearce, *et al.* 1995b). The capacity to identify atypical FBHH families by genetic means may also bring additional unusual presentations to light as time goes by, such as stone disease in some Swedish families (Carling, *et al.*, 2000); or pancreatitis (Felderbauer *et al.*, 2003; Felderbauer *et al.*, 2006).

ASSOCIATED CLINICAL FINDINGS

Some observers have suggested that FBHH increases the risk of acute pancreatitis (Damoiseaux *et al.*, 1985; Davies *et al.*, 1981; Falko *et al.*, 1984; Robinson and Corall, 1990; Toss *et al.*, 1989; Pearce *et al.*, 1996) but others have questioned the validity of this claim (Stuckey *et al.*, 1990). Indeed, Stuckey *et al.* evaluated 10 cases with FBHH and pancreatitis: in 8/10 cases there were potential confounders for pancreatitis such as alcohol abuse ($n = 5$) and biliary pathology ($n = 3$) (Stuckey *et al.*, 1990). Clearly, there is a potential for ascertainment bias; that is, patients presenting with acute pancreatitis commonly are examined for hypercalcemia. Thus, hypercalcemia would be found incidentally in any patient with FBHH who developed pancreatitis for any reason. *Post hoc ergo propter hoc* reasoning would then lead the physician to conclude that FBHH caused the pancreatitis. Interestingly, the CaSR is expressed in pancreatic cells, raising the possibility for its role in patients with FBHH and pancreatitis (Bruce et al., 1999; Racz *et al.*, 2002). Subsequently, a Swedish group characterized a family with FBHH and a concomitant mutation in the pancreatic secretory tripsin inhibitor gene (SPINK1), a putative modifier or causative factor in chronic pancreatitis (Felderbauer *et al.*, 2003). The same group then screened 19 families ($n = 170$ members) with a history of chronic idiopathic pancreatitis, and showed another family member to have a simultaneous mutation in the CaSR and in SPINK1 (Felderbauer *et al.*, 2006). It is thus possible that under certain conditions the hypercalcemia of FBHH predisposes to or aggravates acute pancreatitis, mutations in the CaSR possibly being a potential genetic risk factor for pancreatitis.

There is one report of increased prevalence of chondrocalcinosis in FBHH families (Marx *et al.*, 1981a); and cholithiasis appeared to occur at increased frequency in FBHH kindreds (Law and Heath, 1985). The association of symptoms associated with FBHH was systematically studied by Law and Heath who administered an interview to 15 families with well-documented FBHH: 82 individuals were hypercalcemic and 52 were normocalcemic first-degree relatives. Whereas nocturia, arthritis, gallstones, and arterial hypertension were significantly more common in the index patients than in normocalcemic individuals, only gallstones were found to occur with increased frequency in patients found solely by family screening (Law and Heath, 1985). These associations require systematic reevaluation in a greater number of FBHH kindreds.

The skeleton in patients with FBHH generally appears to be normal histologically, radiographically, and by mineral densitometry (Abugassa *et al.*, 1992; Gilbert *et al.*, 1985; D. A. Heath, 1989; H. Heath, 1989; Heath, 1994; Law and Heath, 1985; Law *et al.*, 1984c; Marx *et al.*, 1977, 1981a; Menko *et al.*, 1983; Monfort-Gourand *et al*, 1993). Some case reports have described increased bone turnover on biopsy of FBHH patients (Alexandre *et al.*, 1982; Kristiansen *et al.*, 1987; Sereni *et al.*, 1982), but the data are very limited. As previously stated, serum alkaline phosphatase levels are normal in most patients with FBHH

(D. A. Heath, 1989; Marx *et al.,* 1977, 1981a; Menko *et al.,* 1983). It is not clear if the bone in patients with FBHH is altered in a manner similar to that described by others in patients with 1° HPT who exhibit increased or preserved iliac crest trabecular bone connectivity accompanied by cortical thinning (Christiansen *et al.,* 1992; Parisien *et al.,* 1990). While FBHH does not appear to threaten skeletal integrity, it does not confer immunity to osteoporosis; certainly, we have observed elderly members of some FBHH kindreds to have had osteoporosis that was clinically indistinguishable from that expected for their ages (Heath, H., III, unpublished observations). Indeed, occasional patients may have a bone density below that of controls (Abugassa et al., 1992) or even well into the osteoporotic range (Timmers et al., 2006).

Probably because of the relative hypocalciuria in patients with FBHH, nephrolithiasis is uncommon, but has, however, been described in a few cases (see Renal Function and Ion Excretion).

BIOCHEMICAL FINDINGS IN FBHH

The biochemical picture in FBHH is also benign, but can be indistinguishable from that of mild-to-moderate 1° HPT (H. Heath, 1989). The only absolute finding is hypercalcemia, which represents a true elevation of serum and total ionized calcium; serum protein concentrations are normal, and calcium binding is normal (D. A. Heath, 1989; H. Heath, 1989; Heath, 1994; Law and Heath, 1985; Marx *et al.,* 1977, 1981a; Menko *et al.,* 1983). There is a variable degree of hypophosphatemia, and while this finding can occasionally be marked, absolutely low values for serum phosphorous are uncommon. Serum total magnesium levels are increased on average to about the upper limit of normal, being absolutely elevated in no more than half of affected individuals (Marx *et al.,* 1981a). Serum alkaline phosphatase activity has been reported as elevated in some cases (Marx *et al.,* 1981a), but is usually normal (D. A. Heath, 1989; H. Heath, 1989; Heath, 1994; Law and Heath, 1985; Menko *et al.,* 1983). Other markers of bone turnover (e.g., osteocalcin or pyridinium cross-links) have not been reported in FBHH.

PTH levels have been measured in the serum of FBHH patients (Fig. 1) by first-generation radioimmunoassay (Kent *et al.,* 1987; Marx *et al.,* 1978b), cytochemical bioassay (Allgrove *et al.,* 1984), extraction-cell-based bioassay (Rajala *et al.,* 1991), and two-site immunoradiometric (Rajala *et al.,* 1991) and immunochemiluminometric assays (Firek *et al.,* 1991). The findings from all these methods are in general agreement: most persons affected with FBHH have paradoxically normal serum PTH values, and many are in the lower half of the normal range. However, 10−20% of FBHH patients have absolutely elevated serum PTH values (Fig. 1) (Firek *et al.,* 1991; Rajala *et al.,* 1991).

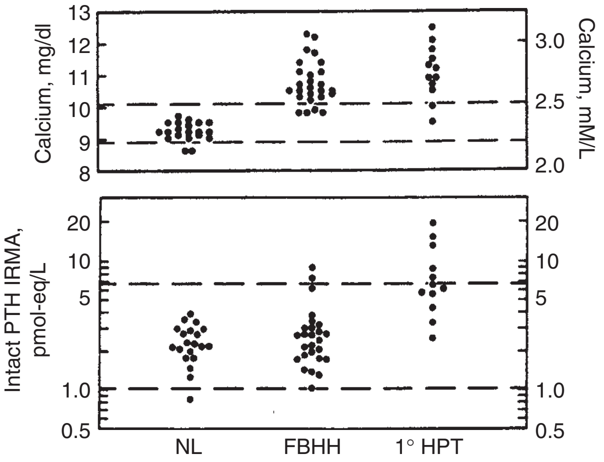


FIGURE 1 Serum calcium (top) and parathyroid hormone (PTH, bottom panel) concentrations in healthy adults (NL), patients with familial benign hypocalciuric hypercalcemia (FBHH), and patients with primary hyperparathyroidism (1 HPT) that was surgically verified. PTH was measured by a two-site immunoradiometric assay (Intact PTH, IRMA). Note the logarithmic scale for PTH. Normal ranges are indicated by horizontal dashed lines. Reprinted with permission from Rajala *et al.* (1991) *J. Bone Miner. Res.* **6,** 117−124.

In addition, one kindred coded as FBHH_{OK} had elevated serum PTH levels in affected individuals, especially the elderly (McMurtry *et al.,* 1992). Vitamin D deficiency may be the explanation for high PTH levels in some individuals with FBHH. Indeed, a recent report described a subject with FBHH, vitamin D deficiency, and frankly elevated intact PTH levels that decreased to within a range that is more characteristic of FBHH patients after vitamin D repletion (Zajickova *et al.,* 2007). In contrast, serum PTH values are supranormal in >85% of patients having 1° HPT (Firek *et al.,* 1991; Rajala *et al.,* 1991). One can imagine the ease with which mild to moderate hypercalcemia (say, 11.0 mg/dl), hypophosphatemia (perhaps 2.0−2.5 mg/dl), and slight elevation of serum intact PTH (70 pg/ml, as an example) could be mistakenly ascribed to 1° HPT in a patient who actually had FBHH.

RENAL FUNCTION AND ION EXCRETION

The characteristic urinary mineral profile in FBHH led to the term *familial hypocalciuric hypocalcemia* or FHH (Marx *et al.,* 1978a); however, most affected persons are not truly "hypocalciuric" (below the normal range), but rather excrete less calcium than expected for their degree of hypercalcemia (a finding that has been termed "relative hypocalciuria"). About 75% of affected persons excrete less than 100 mg of calcium daily (Law and Heath, 1985), and some excrete strikingly low amounts of calcium in the face of hypercalcemia; values as low as 5 mg/day have been noted. However, the physician must be aware that a few individuals with FBHH may excrete as much as 250 mg of calcium daily (Law and Heath, 1985; Marx *et al.,* 1981a; Carling *et al.,* 2000). The relatively low urinary excretion of calcium in FBHH results from avid renal tubular reabsorption of calcium, as detailed in the following section. Similarly, urinary excretion of magnesium is unexpectedly low given that the serum level is in the upper part of the normal range or, in some cases, mildly elevated (Kristiansen *et al.,* 1985, 1986; Marx *et al.,* 1978a).

The excretion of calcium in suspected FBHH cases may be expressed either as an absolute amount (mg/day) or as a unitless calcium:creatinine clearance ratio (fractional excretion of calcium), as advocated by Marx and colleagues (1980): [UCaXSCr]/[SCaXUCr], where UCa is the urinary calcium concentration, SCr the serum creatinine concentration, SCa the serum calcium concentration, and UCr the urinary creatinine concentration, all in milligrams per deciliter. Most patients having FBHH have urinary Ca:Cr clearance ratios below 0.01 (Law and Heath, 1985; Marx *et al.,* 1980, 1981a). There is considerable overlap between the absolute values for total urinary calcium excretion and Ca:Cr clearance ratios among persons having FBHH, their unaffected family members, and unrelated patients having 1° HPT, as shown in Fig. 2. The ratio is most helpful in supporting a diagnosis of FBHH in a kindred, rather than in an individual patient, because the mean Ca:Cr clearance ratio generally will be less than 0.01 when averaged across affected members of the kindred. However, in an atypical family with FBHH with a mutation in the cytoplasmic tail of the CasR, Ca:Cr clearance ratio was above 0.01 in 7 of 10 affected members, and nephrolithiasis was present in two members (Carling *et al.,* 2000).

Calcium infusions have been used to compare renal calcium handling in FBHH and hyperparathyroidism (Attie *et al.,* 1983; Davies *et al.,* 1984; Kristiansen *et al.,* 1986; Stuckey *et al.,* 1987). In response to a rising filtered load of calcium, subjects with hyperparathyroidism increase their urinary calcium excretion more than FBHH patients do. This avid tubular calcium reabsorption persists even in FBHH patients who have been rendered surgically aparathyroid, pointing to a primary renal tubular abnormality in calcium reabsorption (Attie *et al.,* 1983; Davies *et al.,* 1984; Kristiansen *et al.,* 1986; Watanabe and Sutton, 1983). Renal function is well preserved in FBHH (D. A. Heath, 1989, H. Heath, 1989; Heath, 1994; Law and Heath, 1985; Marx *et al.,* 1977, 1981a; Menko *et al.,* 1983), and there appears to be no more than the usual age-related loss of glomerular filtration rate. Moreover, the hypercalcemia of FBHH does not appear to alter renal sensitivity to vasopressin as it does in 1° HPT. Marx and colleagues demonstrated convincingly that reduced maximal urinary concentrating ability occurs in 1° HPT but not in FBHH (Marx *et al.,* 1981b). As stated above, urolithiasis is rare in patients having FBHH, but has been reported (Marx *et al.,* 1981a; Menko *et al.,* 1983; Toss *et al.,* 1989). A Swedish family with a syndrome that presented as a hybrid between 1° HPT and FBHH, with hypercalciuria that subsided after parathyroidectomy was reported by Carling *et al.* (2000).

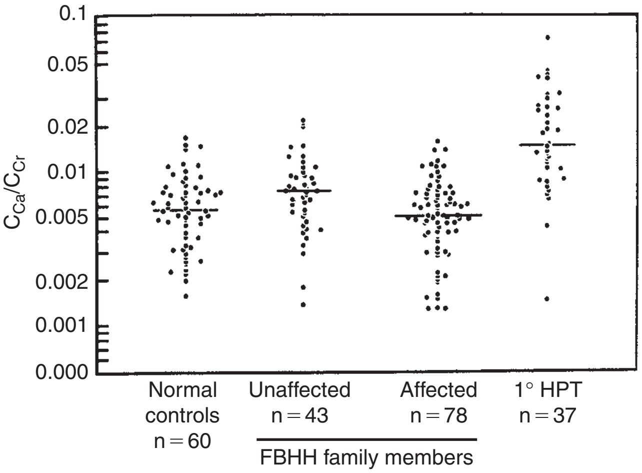


FIGURE 2 Ratios of calcium clearance to creatinine clearance in patients with familial benign hypocalciuric hypercalcemia (FBHH), unaffected family members, unrelated normal controls, and patients with surgically confirmed primary hyperparathyroidism (1° HPT). Note the logarithmic scale for C_{Ca}:C_{Cr} ratios. There is substantial overlap between FBHH and 1° HPT; however, a ratio of >0.02 is helpful in excluding FBHH. Reprinted with permission from Heath, H., III.

In summary, the clinical and laboratory picture of FBHH is generally that of incidentally discovered hypercalcemia that bears no clear relationship to any of the patient's symptoms. The biochemical picture can be very similar to that of patients with asymptomatic 1° HPT, although elevation of serum PTH levels is much less common in FBHH than in 1° HPT (Allgrove *et al.,* 1984; Firek *et al.,* 1991; Kent *et al.,* 1987; Marx *et al.,* 1978b; Rajala *et al.,* 1991). While chondrocalcinosis, gallstones, and acute pancreatitis have been reported in FBHH, the relationship of these events to the FBHH remains uncertain. As for pancreatitis, specific environmental factors or interaction with other genetic mutations may be required (Felderbauer *et al.,* 2006) Hyperparathyroid bone disease, hyposthenuria, and urolithiasis are conspicuously absent. The greatest importance of the FBHH syndrome to clinicians is that it is so easily misdiagnosed as mild to moderate 1° HPT, leading to excessive testing and to inappropriate, unnecessary, and potentially harmful exploratory cervical surgery.

PATHOPHYSIOLOGIC STUDIES IN FBHH

Extensive clinical research studies have clarified the organ-level pathogenesis of FBHH. Because the molecular basis of the predominant form of FBHH is now known (detailed later), these studies now are mainly of value for providing a clear definition of the syndrome. Notably, they also suggested basic research leads that ultimately elucidated the molecular pathogenesis of one form of the syndrome.

PARAMETERS OF SYSTEMIC PLASMA CALCIUM REGULATION

PTH Dynamics

As previously stated, the hypercalcemia of FBHH is a "true" hypercalcemia, not an artifact of calcium binding. It is stable across time and usually is reduced little or not at all by subtotal parathyroidectomy (Law and Heath, 1985; Marx *et al.*, 1981a). However, total parathyroidectomy results in permanent hypocalcemia that responds to the usual therapeutic measures for hypoparathyroidism. The hypercalcemia of FBHH is, therefore, clearly PTH-dependent in a qualitative sense, but recent data show that this is also true in a quantitative sense (Firek *et al.*, 1991). Since the first application of PTH RIAs to the study of 1° HPT, a clear, direct relationship between the degree of hypercalcemia and the elevation of serum immunoreactive PTH has been evident (Arnaud *et al.*, 1971). Studies at the Mayo Clinic (Firek *et al.*, 1991) demonstrated a similar positive correlation between serum calcium and intact PTH levels in FBHH—but with a much shallower slope than the one described for hyperparathyroidism—and there was an inverse relationship between PTH and serum inorganic phosphorus levels. Clearly, hypercalcemia and hypophosphatemia are maintained in FBHH at lower levels of ambient PTH than in 1° HPT, suggesting that inappropriately high PTH levels result from abnormal calcium-sensing at the level of not only the parathyroid gland but also the kidney. Indeed, the latter contributes to the hypercalcemia through avid calcium reabsorption thus resulting in lower than anticipated PTH levels to achieve a given degree of hypercalcemia. The reduced responsiveness of the parathyroid glands to changes in serum calcium has been documented carefully in FBHH. Induction of relative hypocalcemia by infusion of ethylenediaminetetra acetic acid (EDTA) or of relative hypercalcemia by calcium infusion causes anticipated but only partial increments and decrements of serum PTH concentrations (Auwerx *et al.*, 1984; Heath and Purnell, 1980; Khosla *et al.*, 1993). The percent decrements of PTH are greater in FBHH than in 1° HPT—in fact, indistinguishable from normal, although they occur at a higher level of serum calcium than do the corresponding reductions in PTH in normal persons—and increases are less than in 1° HPT. Systematic evaluation of PTH dynamics in FBHH patients compared to normal controls using consecutive citrate and calcium infusions indicates a shift in the calcium−PTH relationship to the right, again consistent results with altered calcium-sensing (Fig. 3, authors' personal observations, Haden *et al.*, 2000). PTH secretion responds in the usual inverse manner to changes in serum calcium concentration, but is reset so as to maintain a higher-than-normal level of plasma calcium. Interestingly, Foley *et al.* presciently interpreted their 1972 findings on FBHH as being consistent with a genetic abnormality of parathyroid calcium sensing (Foley *et al.*, 1972).

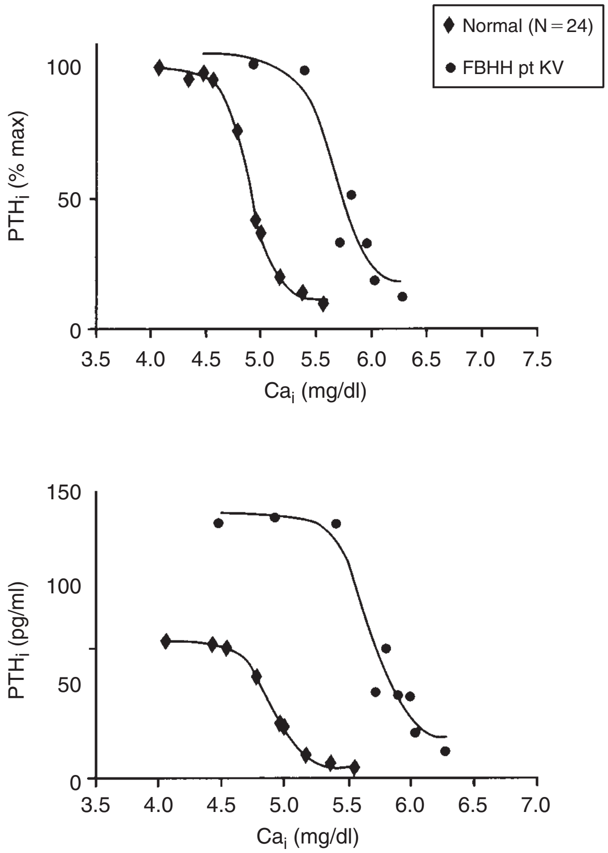


FIGURE 3 Inverse sigmoid curve between serum intact PTH and ionized calcium (Ca_i/PTH_I) in response to consecutive citrate and calcium infusions in patient KV with FBHH S. T. Haden, E. M. Brown, and G. El-Hajj Fuleihan, unpublished observation) in comparison to the Ca/PTH curve derived from 24 healthy subjects (adapted from Haden *et al.* (2000) *Clin. Endocrinol.* **52,** 329−338). There is a clear abnormality of PTH dynamics in the patient with FBHH compared to normal controls: a shift in the Ca/PTH curve to the right, increased PTH levels in response to hypocalcemia, and decreased suppression in response to hypercalcemia. The set-point, the calcium concentration at which there was 50% suppression in PTH levels, was 4.89 mg/dl in the curve derived from 24 healthy controls and 5.65 mg/dl in the patient with FBHH.

Parathyroid Glands

The histologic findings in parathyroid glands of patients having FBHH are variable, possibly because of mutation-specific effects. Most case and single-family reports have described unremarkable parathyroid tissue (Davies *et al.*, 1981; Paterson and Gunn, 1981; Sereni *et al.*, 1982, and reviewed in Law *et al.*, 1984b). These findings were corroborated by studies of large kindreds (Law and Heath, 1985; Menko *et al.*, 1983; Toss *et al.*, 1989). However, the two large histologic studies published to date focusing on parathyroid pathology yielded conflicting results. Thorgeirsson *et al.* reported that a highly variable parathyroid hyperplasia was typical in their examination of 55 parathyroid glands from 18 patients thought to have FBHH (Thorgeisson *et al.*, 1981). Conversely, Law *et al.* examined

28 parathyroid glands from 23 patients in 16 FBHH kindreds, quantifying fat-to-parathyroid-parenchymal ratios in comparison with 82 normal glands from 47 control patients, and they found no evidence to support the presence of parathyroid hyperplasia in FBHH (Law *et al.*, 1984b). The differences in the findings between these two large studies could be explained by referral bias or differences in the study populations. However, the most useful clinical point is that parathyroid histology is unremarkable or minimally abnormal in most cases of FBHH. In a few recent cases, lipohyperplasia of the parathyroid glands has been described in FBHH (Fukumoto *et al.*, 2001; Yamauchi *et al.*, 2002), although it is clearly distinctly uncommon. Of interest in this regard, however, Law *et al.* (1984b) found the percent parenchymal fat to be about 50% higher in parathyroid glands from patients with FBHH than in those with 1° HPT (30 vs. 21%, respectively). The significance of this observation is unclear at present.

Other Calciotropic Hormones

Plasma concentrations of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D are normal in FBHH patients (Davies *et al.*, 1983; Gilbert *et al.*, 1985; Kristiansen *et al.*, 1985; Law *et al.*, 1984a; Law and Heath, 1985; Lyons *et al.*, 1986), as is intestinal calcium absorption (Kristiansen *et al.*, 1985; Law and Heath 1985; Menko *et al.*, 1983). Plasma calcitonin concentrations are inappropriately normal in FBHH for the degree of calcium elevation (Kristiansen *et al.*, 1985; Law and Heath, 1985; Menko *et al.*, 1983), but they exhibit a normal response to other secretagogues (Rajala *et al.*, 1991).

The Kidney

Two careful studies of small numbers of patients support an intrinsic defect of renal tubular reabsorption of calcium in FBHH (Attie *et al.*, 1983; Davies *et al.*, 1984). These patients, who were later recognized to have FBHH, had inadvertently been rendered totally aparathyroid during surgical explorations for parathyroid tumors. Comparisons with individuals who became hypoparathyroid after removal of typical sporadic parathyroid adenomas demonstrated greater PTH-independent renal tubular reabsorption of calcium and magnesium in the FBHH patients than in the hypoparathyroid controls (Attie *et al.*, 1983).

In summary, clinical studies of the FBHH syndrome are almost uniformly consistent with a genetic disorder of calcium sensing by the parathyroid glands paired with enhanced renal tubular reabsorption of calcium. PTH secretion persists at normal or slightly elevated values in the face of hypercalcemia, but without obvious disturbances of other calcium-regulating hormone concentrations. These pathophysiologic abnormalities are most parsimoniously explained by dominant mutations of a single calcium-sensing receptor expressed both in parathyroid glands and in kidneys.

GENETIC STUDIES IN FBHH

The FBHH trait is inherited in an autosomal dominant pattern (Foley *et al.*, 1972; D. A. Heath, 1989; H. Heath, 1989; Heath 1994; Law and Heath, 1985; Marx *et al.*, 1977; 1981a; Menko *et al.*, 1983; Jackson and Boonstra, 1966; Toss *et al.*, 1989). Across numerous families, the sex distribution is essentially 1:1, and about half of all persons at risk manifest hypercalcemia. There is a considerable variation in clinical manifestation, that is, in the extent of hypercalcemia (there being almost no other clinical marker). Several groups have observed that the “hypercalcemia breeds true”; that is, there are FBHH families with relatively high and those with relatively low serum calcium concentrations (Marx *et al.*, 1981a; Rajala and Heath, 1987). There is, however, considerable variability of serum calcium values even within kindreds (Fig. 4). We have had the opportunity to follow affected women whose husbands were normocalcemic through three pregnancies and deliveries. Hypercalcemia was present in all three infants at birth, with serum calcium levels of up to 18 mg/dl observed in cord blood and during the first few days of life; subsequently, serum calcium declined by age 3−4 years to values similar to the affected mother’s (unpublished results).

The FBHH syndrome is genetically heterogeneous: in the majority of cases (over 85%) the FBHH phenotype links to a region of chromosome 3 that contains the calcium-sensing receptor sensor gene (CaSR), a disease subtype called FBHH_{3q} (Heath *et al.*, 1993; Pearce *et al.*, 1995b). In two thirds of FBHH_{3q} families, specific inactivating mutations of the CaSR were found (Fig. 5). The remainder presumably have CaSR mutations outside of the coding region, perhaps reducing expression of an otherwise normal CaSR. In a smaller number of families the linkage of the trait was to markers on the short arm of chromosome 19 (Heath *et al.*, 1993), thus termed FBHH_{19p} (Heath, 1994; Heath *et al.*, 1996; Strewler, 1994). Trump and colleagues carried out genetic linkage studies in the FBHH kindreds described by Whyte *et al.* (Trump *et al.*, 1995), and found linkage neither to chromosome 3*q* nor to chromosome 19*p* markers, establishing that there are three genetically distinct forms of the FBHH syndrome (R. V. Thakker, 2004): FBHH_{3q} (FBHH type 1 or HHC1, OMIM 145980), FBHH_{19p} (FBHH type 2 or HHC2, OMIM 145981), and FBHH_{OK} or FBHH_{19q} (FBHH type 3 or HHC3, OMIM 600740); “OK” stands for Oklahoma, the family’s state of residence (Trump *et al.*, 1995, Lloyd *et al.*, 1999).

The Molecular Basis of FBHH_{3q}: Mutations in the CaSR

Chou *et al.* achieved the first linkage of the FBHH trait to markers on chromosome 3 (Chou *et al.*, 1992), specifically, 3*q*21−*q*24. This linkage coincided with the cloning of the bovine parathyroid calcium-sensing receptor by Brown

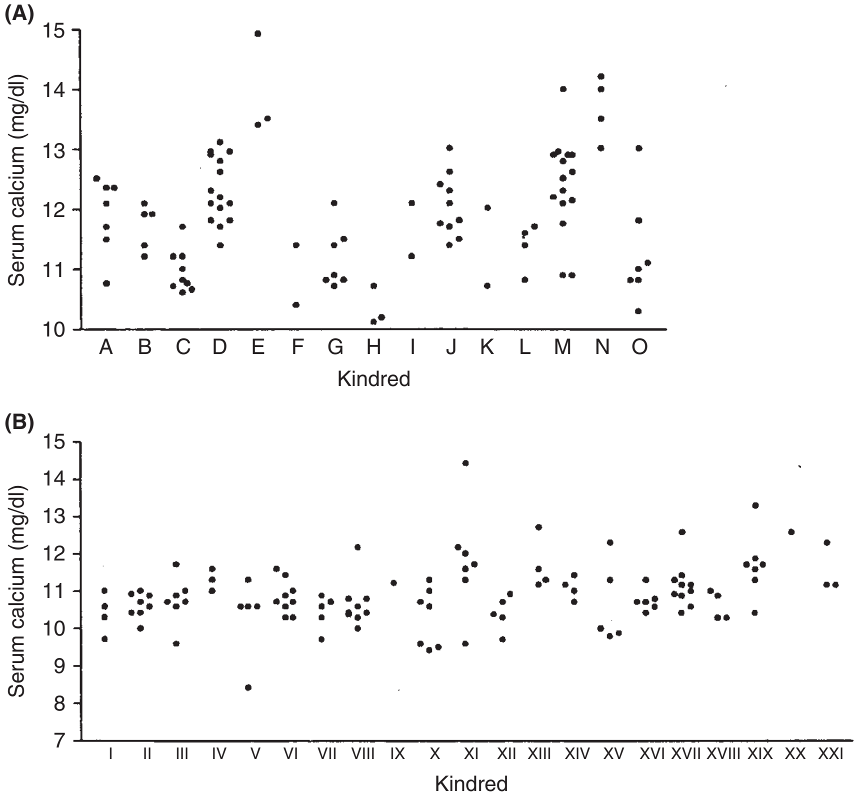


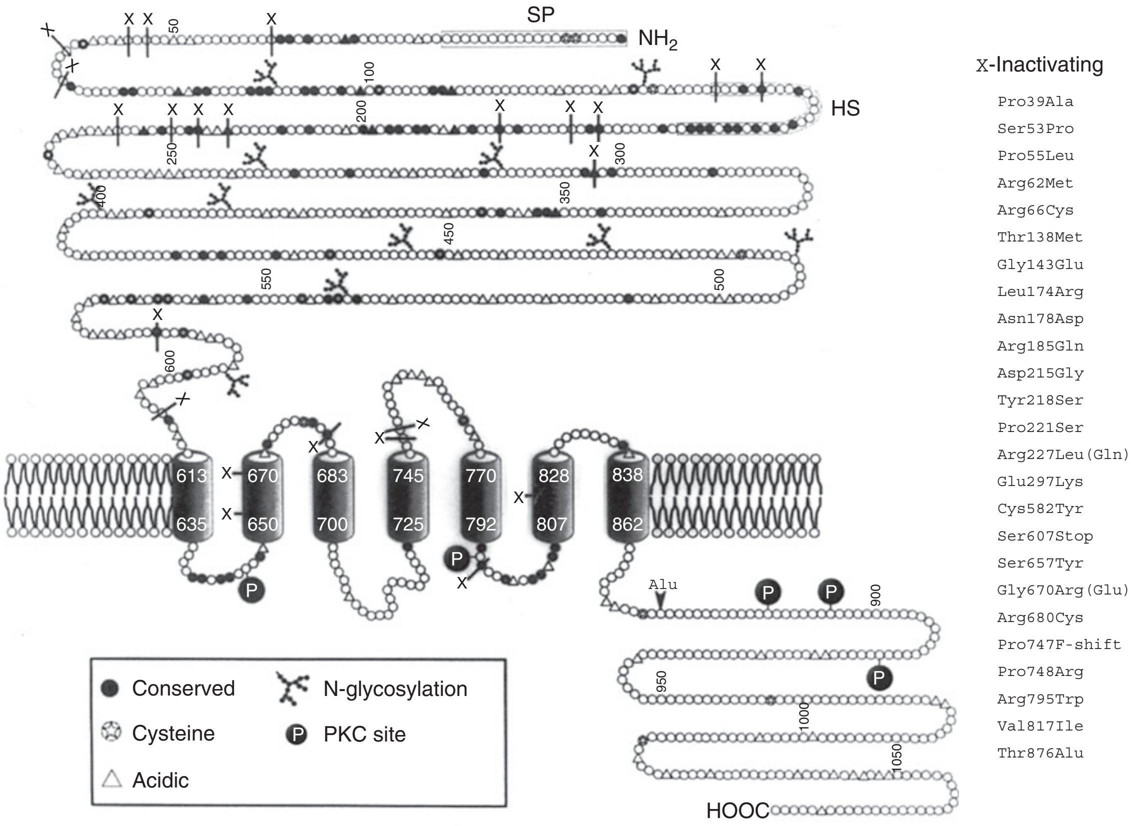
FIGURE 4 Scatter of serum calcium levels in FBHH kindreds. (A) Data from 15 kindreds reported by the National Institutes of Health group (adapted from Marx *et al.* (1981) *Medicine* **60,** 397, with permission). (B) Data from 21 kindreds reported by the Mayo Clinic group (adapted from Rajala and Heath (1987) *J. Clin. Endocrinol. Metab.* **65,** 1039, with permission). The mean ± SD calcium level in A is 12.1 ± 1.6 mg/dl and in B 10.9 ± 0.8, the mean serum calcium level from both studies is 11.4 ± 1.4 mg/dl.

et al., (1993), and the same group quickly demonstrated three distinct missense mutations in the calcium receptor genes in three separate families with $FBHH_{3q}$ (Pollak *et al.,* 1993). Numerous groups have since found various kinds of mutations in this gene that cosegregate with the $FBHH_{3q}$ trait (Aida *et al.,* 1995; Chou *et al.,* 1995; Heath *et al.,* 1996; Hendy *et al.,* 2000; Janicic *et al.,* 1995; Pearce *et al.,* 1995b; see later). For an up-to-date summary of the mutations identified to date, (see http://www.casrdb.mcgill.ca).

The CaSR exhibits a modest degree of homology with the metabotropic glutamate receptors (mGluRs) and other members of the structurally related members of the family C, G protein-coupled receptors (GPCRs), e.g., the gamma-aminobutyric acid ($GABA_B$), pheromone, taste, and odorant receptors (Brauner-Osborne, *et al.,* 2007). The CaSR has three domains: a large extracellular domain (ECD) (612 aa), a seven-transmembrane-spanning segment characteristic of the GPCR superfamily (~250 aa), and an intracytoplasmic tail (222 aa). The extracellular domain contains important Ca^{2+}-binding determinants, likely within the so-called venus fly-trap (VFT) domain in this portion of the receptor, based on homology modeling of the CaSR ECD with the known three-dimensional structure of the extracellular domain of mGluR1 (Hu and Spiegel. 2003). The transmembrane and cytoplasmic domains of the CaSR, including the C-tail, are thought to transduce structural changes in the receptor resulting from binding of Ca^{2+} into alterations in intracellular signaling systems that modulate tissue function.

The CaSR is expressed in many tissues but is found at the highest levels in the parathyroid gland, the thyroid C-cells, and various regions of the kidney, especially the cortical thick ascending limb, a segment that plays a critical role in calcium handling (Brown *et al.,* 1995b). Activation of the normal CaSR suppresses parathyroid hormone secretion, stimulates calcitonin secretion, and enhances renal calcium excretion. Therefore, reduction or loss of the normal function of one allele of the calcium receptor, in FBHH, impairs calcium-sensing, with a resultant shift in the calcium-PTH curve to the right and reduced excretion of renal calcium at any given level of serum calcium concentration.

Most of the inactivating mutations of the human CaSR gene found so far represent single amino acid substitutions, but insertion, deletion, truncation, frame shift, and splice site mutations have also been described (see summary at http://www.casrdb.mcgill.ca). To date, more than 100 different FBHH mutations of the CaSR gene are known (http://www.casrdb.mcgill.ca). Most have been described


SP
NH2
HS
HOOC
Alu
613
635
670
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807
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862
X-Inactivating
Pro39Ala
Ser53Pro
Pro55Leu
Arg62Met
Arg66Cys
Thr138Met
Gly143Glu
Leu174Arg
Asn178Asp
Arg185Gln
Asp215Gly
Tyr218Ser
Pro221Ser
Arg227Leu(Gln)
Glu297Lys
Cys582Tyr
Ser607Stop
Ser657Tyr
Gly670Arg(Glu)
Arg680Cys
Pro747F-shift
Pro748Arg
Arg795Trp
Val817Ile
Thr876Alu
Conserved
N-glycosylation
Cysteine
PKC site
Acidic

FIGURE 5 Schematic representation of the proposed structure of the extracellular Ca^{2+} calcium receptor cloned from human parathyroid gland. SP, signal peptide; HS hydrophobic substance. Also shown are 25 inactivating (missense and nonsense) mutations causing familial benign hypocalciuric hypercalcemia (FBHH). Mutations are indicated using the three-letter amino acid code, with the normal amino-acid indicated before and the FBHH mutation shown after the number of the relevant codon. Adapted from Bai *et al.* (1996), with permission. For updated information regarding mutations and polymorphisms in the CaSR please refer to *(http://www.casrdb.mcgill.ca)*

only in a single family, although three mutations (R185Q, P55L, and T138M) have been identified in two or more apparently unrelated families. These $FBHH_{3q}$ mutations have been mostly in the extracellular or transmembrane portions of the receptor (Fig. 5). Essentially all impair the capacity of Ca^{2+} to activate the mutant receptors when they are expressed heterologously, e.g., in human embryonic kidney (HEK) 293 cells. The functional abnormalities range from mild (~2-fold) reductions in the apparent affinity of Ca^{2+} for activating the receptor to complete loss of activity. Mutations in the ECD may alter the receptor's capacity to bind extracellular calcium ions by disrupting specific Ca^{2+}-binding sites (Silve *et al.,* 2005) or may cause a more generalized change of the receptor's structure and/or its ability to reach the cell surface. For example, two mutations in the signal peptide of the CaSR, which normally directs translocation of the nascent receptor polypeptide into the lumen of the endoplasmic reticulum, severely reduced receptor biogenesis and cell surface CaSR expression (Pidasheva *et al.,* 2005). Mutations in the transmembrane regions of the CaSR, in addition to producing generalized disturbances of receptor structure and/or function, may more specifically impair receptor-mediated intracellular signaling, namely, by interfering with G protein binding (Bai *et al.,* 1996). Recently kindreds have been described with mutations in the cytoplasmic tail of the receptor, one of which presented with symptoms that combine those of FBHH and hyperparathyroidism, a feature reported in some kindreds previously (Toss *et al.,* 1989; Carling *et al.,* 2000; Marx *et al.,* 1981a; Menko *et al.,* 1983). It has been postulated that this mutation may produce more severe "resistance" to Ca^{2+} in parathyroid than in kidney, therefore, resulting, in a greater hypercalciuric response to hypercalcemia than observed in most FBHH families.

The CaSR normally resides on the cell surface as a disulfide-linked dimer, involving cysteines 129 and 131 within the receptor's ECD. The variability in the severity of the hypercalcemia in FBHH may result, in part, from the capacity of mutant CaSRs to impair the function of their wild type partner within wild type-mutant heterodimers (the expected ratio of wild type homodimers, wild type-mutant heterodimers and mutant homodimers would be 1:2:1). This effect of the mutant on the wild type receptor is the so-called dominant negative effect. It has been demonstrated *in vitro* by cotransfecting wild type and mutant receptor cDNAs and comparing their function to those of the mutant and wild type receptors when transfected individually. Some mutations (e.g., Bai, *et al.,* 1997) right-shift the relationship between the extracellular calcium concentration and receptor activation for the cotransfected wild type receptor. Therefore, not only is the function of the mutant homodimer impaired (i.e., ~25% of the cell surface receptor) but also that of the heterodimer (~50%). In the families harboring such mutations, the serum calcium concentration can be unusually high, presumably reflecting the dominant negative action of the mutant receptors *in vivo* (Bai *et al.,* 1996). In contrast, a simple truncation mutation that totally abrogates production of cell surface receptor would still leave intact the ~50% of the wild type receptor arising from the normal CaSR allele. Indeed, some families with truncation mutations of the CaSR exhibit minimal or no hypercalcemia (Kobayashi *et al.,* 1997), presumably owing to the presence of a greater complement of normally functioning receptors than in cases where a dominant negative effect is operative.

In addition to the variability in serum calcium concentration in FBHH families, some kindreds may be at increased risk for pancreatitis, and in three such kindreds Pearce found three heterozygous missense mutations in the extracellular domain of the calcium receptor (Pearce *et al.,* 1996). This observation, in conjunction with that of an expression of the calcium receptor in the pancreatic duct (Bruce *et al.,* 1999; Racz *et al.,* 2002), suggests the intriguing possibility that specific mutations may indeed carry a higher risk of pancreatitis. Alternatively, mutations of the CaSR receptor may interact with mutations of the gene encoding the pancreatic secretory trypsin inhibitor (SPINK1) to aggravate the risk for pancreatitis (Felderbauer *et al*., 2006).

The role of the CaSR gene in controlling parathyroid function and the role of CaSR inactivation in causing FBHH have been elegantly supported by the mouse gene inactivation studies of Ho *et al.* (1995). Mice heterozygous for a disrupted CaSR gene have mild hypercalcemia [10.4 ± 0.55 mg/dl (mean ± SD)], nonsuppressed PTH levels, and reduced urinary calcium excretion compared to normal mice. The affected mice had normal radiographic skeletal morphology and parathyroid gland histology. Their levels of CaSR protein in parathyroid and kidney are about 50% of normal, supporting the argument made above that total loss of one CaSR allele in FBHH (i.e., by truncation) results in mild hypercalcemia that contrasts with the more severe hypercalcemia observed when a dominant negative mechanism is at play.

Thus, several mechanisms have been suggested to account for the reduced activity of the CaSR and impaired Ca^{2+}-sensing in FBHH, acting alone or in combination:

1. Inability to produce a normal complement of cell surface receptors owing to impaired biogenesis and/or accelerated degradation.
2. Decreased affinity of the receptor for extracellular calcium, despite adequate cell surface expression, owing to generalized changes in structure or specific loss of Ca^{2+}-binding motifs.
3. Failure of the cell surface CaSR to couple normally to its respective signal-transduction pathway(s), e.g., activation of appropriate G proteins.
4. A dominant-negative effect wherein the mutated receptor interferes with the function of the normal receptor.

Initially, three relatively common, apparently "benign" polymorphisms were identified in the intracellular C-tail of the CaSR encoded by exon 7 (present in up to one third of unaffected subjects) (Heath *et al.,* 1996). Subsequently, with the wealth of data available from the human genome project, more than 400 SNPs (single nucleotide polymorphisms) have been identified in the CaSR gene within coding as well as noncoding regions (Yun, *et al.,* 2007); see also *http://www.ncbi.nlm.nih.gov/SNP/_and_snp.ims.u-tokyo.ac.jp*). Those present within the coding region, however, other than the three noted above (A986S, R990G, and Q1011E), are very uncommon. The alanine (A) to serine (S) polymorphism of amino acid 986 (A986S) has been described in apparently healthy asymptomatic individuals, and associated in some studies with mild increments in serum calcium and low bone mineral density (Heath *et al.,* 1996., Cole *et al.,* 1999; Lorentzon *et al.,* 2001; Scillitani *et al.,* 2004). However, such observations have not been reproducible (Bollerslev *et al*., 2004; Harding *et al*., 2006). One study demonstrated no effect of these benign CaSR polymorphisms on receptor function when tested in vitro in kidney HEK cells (Harding *et al.,* 2006). Interestingly, a large study from Germany of over 2500 individuals with coronary artery disease revealed an association of the S allele with higher serum calcium levels both in patients and controls (Marz *et al*., 2007). Subjects with the S allele were also noted to have a 30% increased risk of coronary disease and myocardial infarction, and a 25–48% increased risk of cardiovascular and total mortality, independent of risk factors, and serum calcium (Marz *et al*., 2007).

The Molecular Bases of $FBHH_{19p}$ and $FBHH_{OK}$

Two hypercalcemic syndromes similar to $FBHH_{3q}$ have been linked to loci on chromosomes 19*p* (Heath *et al.,* 1993) and 19*q,* the latter originally known as $FBHH_{OK}$ (Trump *et al.,* 1995; Lloyd *et al.,* 1999). The $FBHH_{OK}$ syndrome has been associated with osteomalacia in one family (Trump *et al.,* 1995). At this writing, the molecular bases of the non-3*q* forms of FBHH are still unknown. However, the region mapped for $FBHH_{19p}$ (chromosome 19*p*) contains an interesting candidate gene, GNA11, which encodes a Gq protein that is expressed in human parathyroid tissue (Varrault *et al.,* 1995). It is possible that GNA11 is the

$FBHH_{19p}$ gene. The GNA11 gene product may be the Galpha11 protein acting in concert with the CaSR protein to control PTH secretion (Brown *et al.,* 1995b; Nemeth and Heath, 1995). Thus, inactivation either of one copy of the CaSR gene or of the GNA11 gene might confer the FBHH syndrome.

Autoimmune FBHH

Four cases in two families of autoimmune FBHH with biochemical features similar to those of genetic forms of FBHH have been described (Kifor *et al.,* 2003; Pallais *et al.,* 2004). All four of the patients had mild, PTH-dependent hypercalcemia, with PTH levels in the mid-normal range. Three of the four had relative hypocalciuria. All four subjects had other autoimmune disorders, three with thyroiditis and one with sprue. In those subjects, FBHH was due to the presence of anti-CaSR antibodies that interfered with calcium-mediated suppression of PTH and CaSR-mediated stimultation of mitogen-activated protein kinase (MAPK) and phospholipase C (PLC) activities. More recently, Makita *et al.* (2007) described another patient with the clinical presentation of FBHH and a blocking antibody to the receptor. Interestingly, this antibody, while inhibiting CaSR-induced activation of MAPK, potentiated the activation of PLC, suggesting a potentially important role for MAPK in CaSR-mediated inhibition of PTH secretion.

A CLINICAL STRATEGY FOR FBHH

The differential diagnosis of hypercalcemia is not the dilemma it once was; in patients with established hypercalcemia, a single measurement of serum intact PTH by an immunometric assay will allow a correct diagnosis of 1° HPT at least 80% of the time. However, many patients with 1° HPT are asymptomatic or oligosymptomatic, many have serum intact PTH concentrations within population normal ranges, and some have surprisingly low urinary calcium excretion (Marx *et al.,* 1977). A given patient, then, may present with asymptomatic mild to moderate hypercalcemia, minimal or no hypophosphatemia, urinary calcium excretion below 100 mg/day (Law and Heath, 1985), low Ca:Cr clearance ratio, and mild elevation of serum intact PTH, with no family history of hypercalcemia. The physician has no simple or inexpensive way of determining if this patient has a small parathyroid tumor or one of the variants of FBHH. What is the clinician to do? Genetic linkage studies are not practical, because of family sampling problems, cost, the large number of possible mutations, and the genetic heterogeneity of the syndrome, including the existence of FBHH variants whose molecular etiologies are not yet understood. Direct DNA screening studies of the CaSR gene are informative, if abnormal, but even $FBHH_{3q}$ families do not have known mutations of the CaSR gene coding region in a substantial minority (about a third) of cases. In any case, most clinical laboratories cannot provide appropriate molecular testing for CaSR mutations. Rapid advances in DNA testing technology may change this situation soon, however. For now, the strategy for most clinicians to follow must be based on inference and clinical judgment.

For hypercalcemic patients over the age of 40, with no familial history of hypercalcemia, a clinical and biochemical picture consistent with 1° HPT is overwhelmingly likely to represent just that. The data of Marx *et al.* suggest odds of >1000:1 in favor of 1° HPT in this situation (Marx *et al.,* 1980). Obtaining a measurement of 24-hr urinary calcium could be helpful, as hypercalciuria virtually excludes the diagnosis of FBHH, but normocalciuria or even low urinary calcium excretion does not exclude 1° HPT, particularly in elderly women. Furthermore, some families with FBHH and hypercalciuria have been described (Carling *et al.,* 2000; Simonds *et al.,* 2002). A conservative approach, if the urinary calcium were very low, the serum PTH normal, and the patient clinically well, would be to sample available first-degree relatives for occult hypercalcemia. One's suspicion for FBHH would be heightened if the patient were young (under age 40, especially if under 30), if there were a family history of hypercalcemia, if anyone in the family had undergone unsuccessful neck exploration for hypercalcemia, if neonatal hyperparathyroidism had occurred in the family, and if the urinary excretion of calcium were particularly low (say, less than 50 mg/day) in the patient and other hypercalcemic relatives. In such case, one should defer surgical therapy and observe. The key point is that FBHH does not require treatment in the vast majority of cases. The goal in the clinic must be to identify FBHH before any family member has surgery, but this is regrettably not always going to be possible with our current state of knowledge. Fortunately, in most cases, watchful waiting is a reasonable course, at least for a few years, whether the underlying condition is uncomplicated 1° HPT or FBHH (Silverberg *et al.,* 1999). One recent report documented the efficacy of the calcimimetic cinacalcet in normalizing the serum calcium concentration in a patient with hypercalcemia and a heterozygous mutation in the CaSR (Timmers *et al.,* 2006). Although this therapy is not approved for therapeutic use in patients with FBHH, it may nevertheless be useful in elucidating the etiology of nonspecific symptoms in such patients or, alternatively, as a therapy in atypical cases with unusually severe hypercalcemia.

NEONATAL PRIMARY HYPERPARATHYROIDISM

Clinical Characteristics

Neonatal primary hyperparathyroidism can be a mild syndrome representing the neonatal expression of FBHH, or it can present as a much more dramatic disease with high morbidity and even mortality if untreated, a syndrome known as neonatal severe primary hyperparathyroidism (NSHPT). Owing to the severity of its symptoms, signs, and biochemical abnormalities, NSHPT was described several decades before FBHH. It manifests at birth, with severe hypercalcemia, hypotonia, osteitis fibrosa cystica, respiratory difficulty, failure to thrive, parathyroid hyperplasia, and, in general, markedly elevated serum levels of PTH (Fujita *et al.,* 1983; Marx *et al.,* 1982; Page and Haddow, 1987; Sereni *et al.,* 1982; Steinmann *et al.,* 1984). Additional clinical features include chest wall deformities, sometimes resulting in a flail chest syndrome secondary to multiple rib fractures, dysmorphic facies, and anovaginal and rectovaginal fistulas (Marx *et al.,* 1982; Spiegel *et al.,* 1977; Steinmann *et al.,* 1984). The hypercalcemia is usually severe, ranging from 14 to 20 mg/dl [one patient had a serum calcium concentration of 30.8 mg/dl (Corbeel *et al.,* 1968)], PTH levels are elevated by 5- to 10-fold (Fujimoto *et al.,* 1990), and examination of the parathyroid glands reveals four gland hyperplasia (Cole, 1997; Cooper *et al.,* 1986; Fujimoto *et al.,* 1990; Lutz *et al.,* 1986; Marx *et al.,* 1982; Matsuo *et al.,* 1982). Some affected infants have died from NSHPT, others have survived after total or subtotal parathyroidectomy, and, in more recent series, a substantial percentage of cases survived without parathyroid surgery. Indeed, some neonates with neonatal hyperparathyroidism who originally presented with respiratory compromise and demineralization have been reported to improve (Harris and D'Ercole, 1989; Orwoll *et al.,* 1982; Page and Haddow, 1987; Wilkinson and James, 1993). Rarely, individuals with homozygous inactivating mutations of the CaSR, known to cause NSHPT, have been identified serendipitously for the first time in childhood or even adulthood with moderate to severe hypercalcemia (with serum calcium levels of 14−20 mg/dl), albeit compatible with a relatively asymptomatic state, suggesting residual activity of the two inherited mutated receptors (Aida *et al.,* 1995; Chikatsu *et al.,* 1999; Miyashiro *et al.*, 2004). The surprising lack of apparent symptoms and complications of hypercalcemia such as impaired renal function or a urinary concentrating defect in these individuals support the notion that the CaR mediates at least some of the symptoms of hypercalcemia as well as associated effects on the kidney. Marked phenotypic variation was noted in four affected individuals from one kindred carrying the same homozygous point mutation encoding an early stop codon (Waller *et al.*, 2004). Whereas two patients underwent parathyroidectomy in early childhood, the other two survived into adolescence without parathyroidectomy (Waller *et al.,* 2004). The explanation for such phenotypic variation remains elusive.

In neonatal hyperparathyroidism, the serum calcium level is usually less elevated than in NSHPT and patients have no evidence of bone disease, the latter being a prominent feature of NSHPT. This probably reflects the fact that these neonates carry only one abnormal copy of the FBHH gene, whereas the severity of hypercalcemia and bone disease in NSHPT results from the inheritance of a double dose of the abnormal gene.

Genetic Basis of NSHPT

NSHPT may occur as a sporadic disorder, but has also been described in kindreds having FBHH (Fujita *et al.,* 1983; Marx *et al.,* 1982; Page and Haddow, 1987; Sereni *et al.,* 1982; Steinmann *et al.,* 1984). When NSHPT has occurred in FBHH families, in some cases both parents have been hypercalcemic, suggesting the possibility that NSHPT is the clinical manifestation of homozygosity for an FBHH mutation (Chou *et al.,* 1995; Janicic *et al.,* 1995; Pollak *et al.,* 1993); although in occasional cases, as stated above, patients presented for the first time in childhood or even adulthood (Aida *et al.,* 1995; Chikatsu *et al.,* 1999; Miyashiro *et al.*, 2004).

Thus, NSHPT can result from any of the following genetic alterations in the CaSR:

1. Homozygous form of FBHH: the neonate from a consanguineous marriage inherits a double dose of FBHH mutation, one from each heterozygous parent (Marx *et al.,* 1981, 1982, 1985; Pollak *et al.,* 1994).
2. Compound heterozygous form of FBHH: the neonate is the offspring of two individuals with two different mutations in the CaSR (Kobayashi *et al.,* 1997).
3. Heterozygous form of NHPT: when the neonate occurs sporadically or comes from an FBHH family with only one parent affected (Harris and D'Ercole, 1989; Orwoll *et al.,* 1982; Page and Haddow, 1987; Powel *et al.,* 1993; Spiegel *et al.,* 1977; Wilkinson and James, 1993). Several potential explanations may be offered for that observation. The parents are both carriers of a CaSR mutation but only one is recognized, the offspring may experience a negative dominant effect of the abnormal gene on the normal one (Bai *et al.,* 1997), or the off-spring carries *de novo* heterozygous mutations (Bai *et al.,* 1997; Pearce *et al.*, 1995b).

It is also possible that in some cases the gestation of an FBHH fetus in an unaffected mother induces secondary hyperparathyroidism in the former because of exposure of the fetal parathyroid gland to the relative hypocalcemia of the maternal-fetal circulation.

Mice Models of NSHPT

Mice homozygous for a disrupted CaSR gene have more substantial hypercalcemia (14.8 ± 1.0 mg/dl), higher PTH levels, and increased parathyroid size compared to mice heterozygous for the mutated gene (Ho *et al.,* 1995). The homozygotes grow poorly, have multiple bony abnormalities and usually die within 4 weeks (Ho *et al.,* 1995).

Homozygous null mutation mice also have reduced skeletal radiodensity, kyphoscoliosis, and bowing of long bones, reminiscent of the skeletal anomalies in human NSHPT (Fujita *et al.,* 1983; Marx *et al.,* 1982; Page and Haddow, 1987; Sereni *et al.,* 1982; Steinmann *et al.,* 1984). These important data fulfill "Koch's postulates" for $FBHH_{3q}$ and make it almost certain that inactivating mutation of the human CaSR gene is the cause of FBHH and NSHPT in many, if not most, kindreds.

Management of Neonatal Hyperparathyroidism

In older reports, cases that were managed medically had a much worse prognosis than did those who underwent total parathyroidectomy (Cooper *et al.,* 1986; Fujimoto *et al.,* 1990; Hillman *et al.,* 1964; Marx *et al.,* 1982; Matsuo *et al.,* 1982; Spiegel *et al.,* 1977). Since 1980, milder forms of neonatal hyperparathyroidism have been noted that may improve with medical therapy alone. At this point it would be wise to aggressively support any neonate presenting with NSHPT or neonatal hyperparathyroidism with hydration, mechanical ventilation (if needed due to chest wall abnormalities), and rely on surgery only in those who fail to improve with supportive measures. In some cases, a bisphosphonate may be useful as a temporizing measure, presumably because of the severe hyperparathyroid bone disease (Waller, *et al.* 2004). It is also possible that the calcimimetic CaR activators, such as cinacalcet, may be of use, as this drug has recently been shown to be effective in ameliorating the hypercalcemia in a case of FBHH that was more severe than the norm (Timmers, *et al.,* 2006). For this drug to be active in this setting, however, there would have to be some residual activity of the mutant CaR(s), which would enable them to be sensitized to extracellular calcium by the calcimimetic. The surgical intervention of choice would be total parathyroidectomy with immediate or delayed autotransplantation. Bony remineralization generally occurs within a matter of months(s) after successful parathyroid surgery (Cooper *et al.,* 1986; Fujimoto *et al.,* 1990; Lutz *et al.,* 1986). Any person known to have FBHH should ask his/her spouse to have a measurement of serum calcium performed; if the other spouse is hypercalcemic and both appear to have FBHH, then a pediatrician knowledgeable about NSHPT should be in attendance at the birth of any children they might have. Consanguinity of the couple obviously would heighten concern about NSHPT, because there would be a 25% chance of their child having the disorder.

CaSR-based therapeutics are well into clinical trials (Silverberg *et al.,* 1997; Wada *et al.,* 1999). They may be useful tools not only in our understanding of the role of the CaSR in several organs, but also in the medical management of disorders of abnormal calcium-sensing. In NSHPT, as long as there is some residual function of the CaSR, calcium receptor agonists would presumably be helpful. They could also be used in the more severe variants of FBHH in the neonatal period as well as in adulthood—for instance in the most recent FBHH syndrome described (Carling *et al.,* 2000), as well as in autoimmune FBHH.

CONCLUSIONS

Study of the FBHH syndrome has led to fascinating, important advances in our understanding of the regulation of systemic calcium metabolism (Brown *et al.,* 1995a; Pearce *et al.,* 1995a). What appeared at first to be a single, homogeneous hypercalcemic syndrome represents at least three genetically distinct disorders: $FBHH_{3q}$ (probably always a disorder of the parathyroid CaSR), $FBHH_{19p}$ (which may result from mutation of a gene encoding another component of the calcium receptor activation pathway, a $G_{s\alpha}$ protein gene), and $FBHH_{3q}$ (which accounts for the overwhelming majority of cases to date). It is possible that FBHH may also map to as-yet-unidentified genes that encode for additional CaSR or for proteins that alter the function of these sensors. $FBHH_{3q}$ is the clinical manifestation of heterozygous reduction or loss of CaSR function in the parathyroid glands and renal tubules, whereas NSHPT is usually the phenotype of homozygous loss or reduction of CaSR function. On the other end of the spectrum, activating mutation in the CaSR causes undersecretion of PTH, otherwise known as autosomal dominant hypocalcemia or hypercalciuric hypocalcemia (**see Chapter 65 Genetic Regulation of the Parathyroid Gland Development**). Further insights into the structure−function relationship of the CaSR will undoubtedly enhance our understanding of the wide spectrum of the phenotypic expressions of FBHH and NSHPT. Furthermore, as-yet-unexplained phenotypic features of NSHPT may be providing subtle hints in this "experiment of nature" of currently unknown roles for the CaSR in nonclassical calcium-targeted organs. When the physician suspects FBHH in an individual patient, current knowledge permits only presumptive nongenetic diagnosis of the syndrome, but continuing research may soon lead to DNA-based screening studies not only for diagnosis of the FBHH syndrome, but also for determining the specific molecular basis, in individual patients. The goal of avoiding needless surgery in FBHH would then be achievable.

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Chapter 62

Multiple Endocrine Neoplasia Type 1

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INTRODUCTION

Multiple endocrine neoplasia type 1 (MEN1) syndrome is a rare disorder presenting with varying combinations among its three main endocrine tumors (parathyroid entero-pancreatic, and pituitary), but it includes a varying combination of more than 20 endocrine and nonendocrine tumors (Gagel and Marx, 2007). In fact, other endocrine and nonendocrine neoplasms such as foregut carcinoids, lipomas, and skin tumors are also common in MEN1. Furthermore, other endocrine and nonendocrine tissues can be affected but with a lower frequency (Table I). Current definition of MEN1 operationally consists of a case with two of these three principal MEN1-related endocrine tumors. Many tumors in MEN1 are benign, even if some enteropancreatic neuroendocrine tumors (mainly gastrinomas) and foregut carcinoids are often malignant. Though often regarded as a treatable endocrinopathy, MEN1 can also be regarded as a cancer syndrome lacking effective prevention or cure for associated malignancies. About one third of MEN1 carriers die eventually from an MEN1-related cancer (Wilkinson *et al.*, 1993; Doherty *et al.*, 1998). This is a lethality similar to that of untreated MEN2. MEN1 exhibits an autosomal dominant pattern of inheritance. Familial MEN1 syndrome is defined as MEN1 syndrome in an individual who has at least one first-degree relative with at least one of the three main endocrine tumors. Sporadic cases associated with mutation of the *MEN1* gene represent about 10% of cases. MEN1 has received attention out of proportion to its rarity because it is judged a promising model for insights into common tumors. The tumor develops after inactivation of both *MEN1* gene copies at chromosome 11*q*12–*q*13, with acquisition of a homozygous recessive state at the tissue level, a process of subchromosomal rearrangement that led to initial localization of the *MEN1* gene and also that assisted later in *MEN1* gene discovery by positional cloning (Larsson *et al.*, 1988; Bystrom *et al.*, 1990; Chandrasekarappa *et al.*, 1997).

The syndrome was originally described as an autopsy finding in a patient with acromegaly and four enlarged parathyroid glands (Erdheim, 1903). More than 20 years later, two groups reported a patient with the triad of parathyroid, pancreatic, and pituitary adenomas (Cushing and Davidoff, 1927). In 1953, Underdahl *et al.*, published an early review of related syndromes with a total of 14 cases (Underdahl *et al.*, 1953). However, it remained for Wermer to recognize transmission in a large family and to suggest that an autosomal dominant gene caused the trait with high penetrance (Wermer, 1954). This insight is commemorated by referring to MEN1 also as Wermer syndrome.

The clinical features of the disorder were then clarified greatly after 1960, when radioimmunoassays for the various

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TABLE I MEN1-Associated Tumors

Typical (or More Frequent) Tumors	Less Prominent Tumors	Rare Tumors
Endocrine		
Parathyroid adenoma (~90%)	Foregut-derived carcinoid (5%)	Adrenal medulla
Anterior pituitary (~25%) (prevalently PRLoma) (20%)	Adrenal cortical (~25%) (mainly nonfunctioning)	
Neuroendocrine cells of GI tract (gastrinoma, insulinoma, VIPoma, glucagonoma, PPoma) (~50%)		Ductal adenocarcinoma of the pancreas
Nonendocrine		
Nonsecreting of GI tract (20%) and of pituitary (10%)	Lipoma (visceral and cutaneous) (~30%)	Ependymoma
	Skin tumor [facial angiofibroma (85%), truncal collagenoma (70%)]	Leiomyoma of esophagus or uterus Meningioma

GI, *gastrointestinal*; *Pancreatic Polypetidetumor*, PPoma; PRL*oma*; *Vasoactive Intestinal Peptide* VIPoma.

products of the affected endocrine tumors were developed (Yalow, 1978; Malarkey, 1979; Nussbaum and Potts, 1991). Progress in the management of MEN1 paralleled knowledge of the pathogenesis of common-variety endocrine tumors and included improving surgical and pharmacological interventions (Albright and Ellsworth, 1990; Bevan *et al.*, 1992; Schmid *et al.*, 1961).

CLINICAL ASPECTS

MEN1 Variants

Because of the constantly growing clinical and genetic knowledge about this syndrome, the MEN1-associated endocrinopathies, and their sporadic counterparts, it has become possible to recognize peculiar MEN1 variants (differing clinical expressions from one gene) and phenocopies (similar phenotype but not from the same gene; Marx, 2001).

Because primary hyperparathyroidism (PHPT) is the earliest and most frequent expression of MEN1, familial isolated hyperparathyroidism (FIHP; Kassem *et al.*, 1994) in one-fifth of families with isolated hyperparathyroidism can be a prelude to typical MEN1 or an atypical expression of MEN1 (Marx *et al.*, 1982). However, in a much larger and more "elderly" family, isolated hyperparathyroidism (FIHP) could be a distinctive variant from *MEN1* mutation or an expression from a different gene (Kassem *et al.*, 2000; Miedlich *et al.*, 2001). FIHP is characterized by parathyroid adenoma or hyperplasia without other associated endocrinopathies in two or more individuals in one family. Germline mutations in *MEN1* gene have been reported in 0% (Simonds *et al.*, 2002) to 57% of families with FIHP (Pannett *et al.*, 2003; Warner *et al.*, 2004).

A variant (or subtype), caused by *MEN1* mutation, termed the prolactinoma or "Burin" MEN1 variant shows high penetrance of hyperparathyroidism and of prolactinoma (Petty *et al.*, 1994); the prevalence of gastrinoma is lower than in typical MEN1 (Hao *et al.*, 2004). The prolactinoma MEN1 variant has been associated with *MEN1* mutation or 11*q*13 linkage in each of three tested families (Agarwal *et al.*, 1997). The *MEN1* mutations in FIHP and "Burin" MEN1 variants have not shown any informative pattern; the causes of these variants are not known. Thus, a family with an MEN1-variant and proven or likely *MEN1* mutation should remain under surveillance for other expressions of MEN1. Recently, Ozawa *et al.* (2007) reviewed an MEN1 variant defined by sporadic tumors of both the parathyroids and pituitary and with a lower prevalence of *MEN1* mutations than in familial MEN1 (7% versus 90%), suggesting different causes.

MEN1 Phenocopies

An MEN1 phenocopy is a trait that strongly resembles MEN1 in a patient or a family without identified *MEN1* mutation. It could be a case of FIHP in which *MEN1* mutation analysis failed to detect mutation; thus, it could be caused by mutation in another gene, such as the calcium-sensing receptor gene (*CASR*), accounting for familial hypocalciuric hypercalcemia (FHH) syndrome (Pollak *et al.*, 1993), and the *HRPT2* gene responsible for the hyperparathyroidism-jaw tumor syndrome (HPT-JT; Szabo *et al.*, 1994; Teh *et al.*, 1998a; Carpten *et al.*, 2002). Currently, about 15% (Simonds *et al.*, 2002; Warner *et al.*, 2004) of families with FIHP have identifiable *CASR* mutations, whereas *HRPT2* mutation is less frequent (Simonds *et al.*, 2003; Warner *et al.*, 2004).

Familial isolated pituitary tumor, generally somatotropinoma, has so far yielded no *MEN1* mutation with a collective experience of more than 100 tested families (Tanaka *et al.,* 1998; Teh *et al.,* 1998b; Tsukada *et al.,* 2001; Daly *et al.*, 2007). Although positive linkage to 11q13 has been reported in each of about four large kindreds with isolated familial somatotrophinoma (Gadelha *et al.,* 2000; Luccio-Camelo *et al.,* 2004), mutation in the *MEN1* gene has not been detected. Consequently, the involvement of a nearby gene at 11q13 was sought.

The pituitary tumor gene at 11q13 was identified by a Finnish group (Vierimaa *et al.,* 2006); it was a previously characterized gene termed aryl hydrocarbon receptor interacting protein (*AIP*). Daly *et al.* (2007) confirmed this in a large series with germline *AIP* mutations in 11 of 73 (15%) kindreds with Familial Isolated Pituitary Adenoma (FIPA).

Recently one small family meeting the criteria of MEN1 was reported (Pellegata *et al.,* 2006). It showed no *MEN1* mutation, but affected members had a *p27* mutation linked to their MEN1 trait. The tumors observed were somatotropinoma (2), parathyroid (1), and renal angiomyolipoma (1). This seems to be a rare and important phenocopy of classic MEN1, and more families will need to be studied to understand the complete phenotype.

Differences between MEN1 Tumors and Common Tumors

The endocrine tumors of MEN1 are generally seen as a model of common-variety endocrine tumors. This is supported by the fact that the *MEN1* gene initiates a large proportion of common-variety endocrine tumors (see later discussion). However, there are also two important differences between the categories of MEN1 and common tumor.

First, any MEN1 tumor by itself can show a signature of the *MEN1* gene (i.e., gene expression) that is different from another gene that might initiate a common-variety tumor in the same tissue. This may account for the unusual location of MEN1 carcinoids (foregut as opposed to more distally). It also likely accounts for the larger size of pituitary tumor in MEN1 (Verges *et al.,* 2002); common-variety tumor of the pituitary is almost always initiated by genes other than *MEN1*. And in the parathyroid, it helps account for why parathyroid cancer is rare in MEN1. This unusual parathyroid pathology is usually promoted by mutation on both copies of the *HRPT2* gene (Shattuck *et al.,* 2003).

Second, the tumors of MEN1 are often multiple within a tissue affected at high penetrance. This is a general property of all hereditary tumors, and it reflects the fact that each cell (because of having inherited the gene mutation in all cells) in a target tissue is at high risk for tumor initiation from a second mutation of the other copy of the same gene. This accounts for parathyroid adenoma in three to four glands underlying the hyperparathyroidism of MEN1. It is also a feature in the Zollinger-Ellison syndrome of MEN1; there are numerous submucosal microgastrinomas. The multiplicity of endocrine tumors in these two tissues makes these tumors unusually resistant to standard surgical methods optimized to ablate one tumor. And many modifications to management have been developed (see later discussion). The most extreme is frequent avoidance of surgery for gastrinoma of MEN1 and treatment only with stomach acid-blocking drugs.

Primary Hyperparathyroidism in MEN1

Primary hyperparathyroidism is the most common endocrinopathy in MEN1, reaching nearly 100% by age 50 (Skarulis, 1998), and it is usually the first endocrine manifestation of MEN1 syndrome in 90% of individuals, recognized as early as age 8 in several cases (Trump *et al.,* 1996).

The clinical features of the parathyroid hyperfunction in MEN1 show similarities to those of sporadic or common-variety primary hyperparathyroidism (Bilezikian *et al.,* 1995; Kleerekoper, 1995) with a long period of asymptomatic hypercalcemia and a low morbidity. The complications of the disorder are prevented by early intervention from an experienced surgeon.

In contrast to sporadic hyperparathyroidism, hyperparathyroidism (often asymptomatic) in patients with MEN1 develops at a younger age (Betts *et al.,* 1980; Skogseid *et al.,* 1991). Its average onset age is 20 to 25 years (Trump *et al.,* 1996; Skarulis 1998), three decades earlier than typical for sporadic parathyroid adenoma. Moreover, different from sporadic primary hyperparathyroidism (Heath *et al.,* 1980), primary hyperparathyroidism in MEN1 has a similar prevalence in females and males. Finally, unlike single adenoma of sporadic primary hyperparathyroidism, primary hyperparathyroidism in MEN1 is associated with multiple and asymmetric parathyroid gland enlargement (Hellman *et al.,* 1992; Marx *et al.,* 1991). MEN1-associated hyperparathyroidism increases gastrin secretion from gastrinoma(s), precipitating and/or exacerbating symptoms of Zollinger-Ellison syndrome (ZES; Marx, 2001), and consequently a successful parathyroidectomy could decrease the severity of the ZES in MEN1 (Jensen, 1997). Still, because of excellent pharmacotherapy for ZES, ZES does not represent a sufficient indication for parathyroidectomy in MEN1.

After successful subtotal parathyroidectomy, hyperparathyroidism in MEN1 recurs progressively, reaching 50% by 8–12 years after surgery (Rizzoli *et al.,* 1985; Burgess *et al.,* 1998a). In theory, it could be due to the further growth of a neoplastic parathyroid tissue or to a new somatic mutation at 11*q*13 in the seemingly normal parathyroid remnant.

Burgess *et al.* (1999) reported a reduction of bone mass in MEN1-affected women with hyperparathyroidism by 35 years of age (44%), with an increased likelihood of skeletal fractures. If surgery is delayed or withheld, the use of bone antiresorptive agents should be considered in order to reduce hypercalcemia and limit PTH-dependent bone resorption, reducing future risk of osteoporosis. Parathyroidectomy and maintenance of a normocalcemic state resulted in improvement of both lumbar and femoral BMD.

Well-Differentiated Endocrine Tumors of the Gastro-Entero-Pancreatic Tract

The prevalence of gastro-entero-pancreatic (GEP) involvement (including tumors of the stomach, duodenum, and pancreas; Klöppel *et al.,* 2004) in MEN1-affected individuals varies in different clinical series at 30% to 75% (Marx, 2001; Skogseid *et al.,* 1991; Vasen *et al.,* 1989) and approaches 80% in surgery or autopsy series. A GEP tumor is often recognized because of clinically overt features during the fifth decade but may be detected by chemistries or by imaging much earlier (third decade) in asymptomatic *MEN1* carriers. Unlike nonfamilial endocrine tumor of the upper gastrointestinal tract, tumors in patients with MEN1 are usually multiple and develop at a younger age; MEN1-associated gastrinoma and insulinoma exhibit an average onset age 1 decade earlier than their sporadic counterparts (Skarulis, 1998), and, in particular, ZES usually occurs before age 40 years (Gibril *et al.*, 2004). Moreover, the gastrointestinal manifestations are influenced by other endocrinopathies (mainly primary hyperparathyroidism; Bone, 1990; Norton *et al.,* 1993).

Initial presentation of MEN1 as ZES occurs only in a small proportion of patients (Benya *et al.,* 1993a). The early presentation of these tumors is frequently associated with the lack of detection of the tumor by routine abdominal imaging techniques (Galiber *et al.,* 1988; Norton *et al.,* 1993).

The majority of tumors with MEN1 ZES are in the duodenum and are usually small (diameter <0.5 cm). The most frequent GEP functional endocrine tumors in MEN1 are gastrinomas (54%) and benign insulinomas (15%; Eberle and Grun, 1981; Jensen and Gardner, 1993; Metz and Jensen, 1993; Norton *et al.,* 1993). About 40% of MEN1 cases exhibit gastrinoma, and about one-fourth of all gastrinoma cases have MEN1. Moreover, 25% of individuals with MEN1 syndrome/ZES deny awareness of any family history of MEN1 syndrome (Gibril *et al.*, 2004). Most MEN1 gastrinomas are malignant; half have metastasized before diagnosis (Townsend and Thompson, 1990; Brandi *et al.*, 2001). One-third of patients with sporadic and MEN1-associated ZES eventually die from the malignant aspects of their tumor(s). Poor prognosis is associated with pancreatic (not duodenal) primary (more aggressive than duodenal gastrinomas, as suggested by their larger size and greater risk for hepatic metastasis), liver metastases (there is not a clear evidence that lymph node metastases negatively influence prognosis), ectopic Cushing syndrome, or very high gastrin level (Yu *et al.,* 1999).

The diagnosis of gastrinoma is made by elevated serum gastrin and high gastric acid output, with or without symptoms. Confirmatory tests can include secretin-stimulated gastrin levels (Wiedenmann *et al.,* 1998). A 72-hr fast protocol may be helpful in the diagnosis of insulinoma that is characterized by fasting hypoglycemia with high plasma or serum concentration of insulin and high plasma or serum concentration of C-peptide or proinsulin (Brandi *et al.*, 2001; Marx, 2001).

Pancreatic glucagonomas, vasoactive intestinal peptide tumors (VIPomas), growth hormone releasing factor tumors (GRFomas), and somatostatinomas have also been described in MEN1 (Eberle and Grun, 1981; Norton *et al.,* 1993) and usually exhibit a large diameter (>3 cm; Mignon *et al.*, 1993). However, nonfunctional[1] (NF) endocrine tumors, difficult to diagnose by both biochemical and imaging tests, are the most prevalent GEP endocrine neoplasms in MEN1, more than half being pancreatic polypeptide tumors (PPomas; Eberle and Grun, 1981; Metz and Jensen, 1993; Norton *et al.,* 1993; Jensen, 1999). These tumors may occur in nonoperated individuals as well as in the remnants that remain after resection for a GEP tract tumor. Type II tumors of gastric histamine-secreting enterochromaffin-like cells (ECLomas or gastric carcinoids) frequently accompany Zollinger-Ellison-associated hypergastrinemia (Jensen and Gardner, 1991; Maton and Dayal, 1991). It is generally believed that prolonged hypergastrinemia worsens ECLomas (Frucht *et al.,* 1991; Maton and Dayal, 1991; Bordi *et al.,* 1998). This is particularly true for patients with MEN1 (Frucht *et al.,* 1991; Jensen and Gardner, 1991; Maton and Dayal, 1991). The mean age at diagnosis of gastric carcinoids is 50 years. Currently, ECLomas are included in the well-differentiated endocrine tumors of the GEP tract. They are common in MEN1 and are usually recognized incidentally during gastric endoscopy for ZES (Benya *et al.,* 1993b; Bordi *et al.,* 1998; Gibril *et al.*, 2000).

Though sporadic carcinoid tumors are mainly in the midgut or hindgut, MEN1 carcinoids are all in the foregut (Duh *et al.*, 1987; Godwin, 1975). Foregut carcinoids in MEN1 rarely oversecrete amine or peptide hormones. MEN1 thymic carcinoid exhibits a male predominance, and cigarette smoking appears to be associated with a higher risk for thymic carcinoid (Teh *et al.,* 1997). MEN1 bronchial carcinoid is mainly in females. Bronchial carcinoids, often multicentric, may exhibit both synchronous and metasynchronous occurrence. In contrast to thymic carcinoids, most bronchial carcinoids usually behave indolently, albeit with the potential for local mass effect, metastasis, and recurrence after resection (Sachithanandan *et al.*, 2005).

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[1] Nonfunctional tumors are those that do not make a known hormone, make a hormone but do not secrete enough to cause a syndrome, or make and secrete a hormone (such as pancreatic polypeptide or calcitonin) that does not cause a syndrome.

Tumors of the Anterior Pituitary or the Adrenal in MEN1

An anterior pituitary tumor is the first clinical manifestation of MEN1 in 25% of simplex cases (i.e., a single occurrence of MEN1 syndrome in a family) and in 10% of familial cases (Carty *et al.*, 1998; Falchetti *et al.*, 2005). Verges *et al.* (2002) reported pituitary adenomas as the initial manifestation of MEN1 syndrome in 17% of individuals and significantly more frequent in women than in men (50% versus 31%, $P < 0.001$). The occurrence of anterior pituitary tumors in MEN1 syndrome ranges between 10% and 60% depending on the study and methods utilized in the various studies (Metz *et al.*, 1994; Brandi *et al.*, 2001). Between 65% (Brandi *et al.*, 2001) and 85% of pituitary tumors in MEN1 syndrome are macroadenomas, compared to only 42% in common-variety tumor (Verges *et al.*, 2002).

It was formerly reported that every type of anterior pituitary adenoma, except "true" gonadotropinoma, has been reported in MEN1 (Teh *et al.*, 1998b; Corbetta *et al.*, 1997), but Benito *et al.* (2005) reported the presence of a metastatic gonadotrophic pituitary carcinoma in a female individual with MEN1. The frequency of plurihormonal tumors is greater in MEN1 tumors, with fewer null-cell tumors when compared to sporadic isolated pituitary tumors (Scheithauer *et al.*, 1987).

The mean age at the time of diagnosis of MEN1 pituitary tumors is about 40 years (Oberg *et al.*, 1989; Vasen *et al.*, 1989; Skarulis, 1998), similar to that for sporadic isolated pituitary tumors (Scheithauer *et al.*, 1987). Prolactinoma (with or without simultaneous growth hormone [GH] oversecretion) is the most frequent pituitary tumor in MEN1, followed by GHoma (Scheithauer *et al.*, 1987). A universal consensus regarding the cancer risk of MEN1-associated pituitary tumors does not exist, and although Verges *et al.* (2002) reported that 32% of pituitary macroadenomas were invasive, dissemination of MEN1-associated pituitary tumors is rare.

Primary adrenocortical neoplasms are common in MEN1 (20–40%) and they are usually bilateral, hyperplastic, and nonfunctional (Skarulis, 1998; Skogseid *et al.*, 1992, 1995; Burgess *et al.*, 1996). The adrenal cortex tumors are occasionally associated with primary hypercortisolism, this resulting mainly from pituitary oversecretion of adrenocorticotrophic hormone (ACTH) (Maton *et al.*, 1986); hyperaldosteronism has been much more rare (Skogseid *et al.*, 1992; Beckers *et al.*, 1992; Honda *et al.*, 2004). Most of the adrenal enlargements exhibit an indolent clinical course (Metz *et al.*, 1994; Burgess *et al.*, 1996).

Pheochromocytoma is in less than 1% of cases and always unilateral in MEN1 (Brandi *et al.*, 2001). A low-penetrance tumor is almost never bilateral. Because 11*q*13 loss of heterozygosity (LOH) is associated with pheochromocytoma in MEN1, pheochromocytoma should be considered a direct result of *MEN1* gene inactivation (Cote *et al.*, 1998a). Thus, it is appropriate to measure urinary catecholamines prior to surgery to diagnose and treat a pheochromocytoma to avoid dangerous and potentially lethal blood pressure peaks during surgery.

Nonendocrine Tumors

Lipomas are frequently associated with MEN1 (20–30%; Ballard *et al.*, 1964; Marx *et al.*, 1982; Metz *et al.*, 1994; Darling *et al.*, 1997). They are often multiple and can also occur viscerally. Lesions, often multicentric, may be small or large and cosmetically disturbing. They typically do not recur after removal. Multiple facial angiofibromas were observed in 40% to 90% of MEN1 patients, with half of the cases having five or more (Hoang-Xuan and Steger, 1999; Sakurai *et al.*, 2000); truncal collagenomas were almost as common (Darling *et al.*, 1997).

Leiomyoma of the esophagus, uterus, or rectum has occurred; the frequency of leiomyoma in MEN1 has not been analyzed (Vortmeyer *et al.*, 1999; Dackiw *et al.*, 1999). They are benign neoplasms derived from smooth (nonstriated) muscle (McKeeby *et al.*, 2001; Ikota *et al.*, 2004).

TREATMENTS

Primary Hyperparathyroidism

Surgery for hyperparathyroidism in MEN1, more so than for the sporadic form, is still the preferred treatment. No general consensus has been reached on which technique could be the optimal surgical approach in MEN1-associated HPT. The decision about timing for parathyroid surgery usually takes into account the following criteria: (1) severity of symptoms or signs of HPT; (2) concentration of circulating PTH and calcium; (3) presence of MEN1-associated endocrinopathies, especially ZES; (4) patient age. Successful treatment of HPT is often followed by a decrease of the elevated circulating levels of gastrin. Persistence, late recurrence, and hypoparathyroidism are all more frequent after surgery for MEN1 than for sporadic adenoma. Several approaches address these problems.

In particular, the rapid intraoperative assay of PTH makes it possible for the operating surgeon to monitor the correction of hyperparathyroidism, to be sure when the important tumors have been removed, and to perform, when necessary, an immediate parathyroid autograft (Tonelli *et al.*, 1994, 2000).

Calcium-sensing receptor agonists, a new and novel class of drugs, have been demonstrated to act directly on the parathyroid gland, decreasing PTH release (Brown *et al.*, 1995; Peacock *et al.*, 2005) and perhaps even decreasing parathyroid tumor growth. They might acquire an important role in treatment of hyperparathyroidism, including that from MEN1.

Subtotal Parathyroidectomy

Subtotal parathyroidectomy is the surgical ablation of three parathyroid glands and part of the fourth gland, leaving no more than 50 mg of tissue from the least abnormal gland (Carling and Udelsman, 2005). Some authors suggest that the whole fourth parathyroid gland be left without biopsy when it appears to be of normal size. This kind of procedure has been proposed in order to avoid permanent hypoparathyroidism and to reduce the period of temporary postsurgical hypocalcemia. Rates of chronic hypocalcemia with these surgical approaches range from 0% to 30% (Goretzki *et al.*, 1991; Grant and Weaver, 1994; Kraimps *et al.*, 1992; O'Riordain *et al.*, 1993; Thompson and Sandelin, 1994). Frequent late recurrence of hyperparathyroidism follows successful subtotal parathyroidectomy in MEN1. The prevalence of recurrence increases proportionately with time after surgery (Hellman *et al.*, 1992; Kraimps *et al.*, 1992; Prinz *et al.*, 1981; Rizzoli *et al.*, 1985; Burgess *et al.*, 1998a). Elaraj *et al.* (2003) showed that subtotal and total parathyroidectomy resulted in longer recurrence-free intervals compared with lesser resection. Cumulative recurrence rates for procedures with a less than subtotal parathyroid resection were 8%, 31%, and 63% at 1, 5, and 10 years, respectively, whereas for more extensive parathyroid resection, subtotal or total, the cumulative recurrence rates were 0%, 20%, and 39% at 1, 5, and 10 years, respectively (Elaraj *et al.*, 2003).

Total parathyroidectomy with simultaneous autologous parathyroid graft (Wells *et al.*, 1980) is a more radical approach for MEN1-associated hyperparathyroidism. The whole parathyroid and thymic tissues are removed for the purpose of avoiding subsequent exploration for hyperparathyroidism or for thymic carcinoid. The parathyroid graft is performed by one of two main procedures. The first is the use of fresh autologous tissue kept in refrigerated saline solution and grafted immediately at the end of surgery. The advantage of this approach is that the graft is more likely to succeed. The disadvantage is not being able to verify the absence of functioning tissue remnant, normal remaining ectopic gland, or supernumerary gland. In the second procedure, implantation is performed some days or months after surgery using cryopreserved autologous tissue. With this approach the lack of circulating PTH can be evaluated after parathyroidectomy. However, the viability of parathyroid tissue may be lower after cryopreservation. In both techniques, the parathyroid gland to be transplanted is the one that macroscopically exhibits the features closest to a normal gland, preferably lacking any nodular histopathology. The tissue that is transplanted varies from 5 to 20 1-mm^3-sized fragments. The graft is generally placed within the brachioradialis muscle so that it is sometimes possible to verify its function by simple blood sampling from the ipsilateral antecubital vein.

Because normal parathyroid glands can also be found in MEN1 patients, some authors believe that four-glands surgery is not always necessary. In this way the risk of permanent hypocalcemia is avoided. If partial parathyroidectomy is performed, an acceptable approach includes removal of the pathological and the normal homolateral gland, including thymectomy, and biopsy of the contralateral apparently normal parathyroids (Dralle and Scheumann, 1994). Consequently, the risk of persistent hyperparathyroidism is reduced and the lack or presence of multiglandular parathyroid involvement is determined. Whatever the approach to parathyroidectomy, an initial neck operation in MEN1 should include transcervical thymectomy. Most of the thymus can thereby be removed along with possibly included parathyroid tissue and carcinoid tissue.

Neuroendocrine Tumors

Gastrinoma(s)

Gastric acid hypersecretion in virtually all patients with hypergastrinemia can be effectively controlled using histamine H_2-receptor antagonists or H^+, K^+-ATPase inhibitors (Brunner *et al.*, 1989a, 1989b; Jensen, 1997, 1999).

The surgical approach, if any, to be taken for gastrinoma(s) in MEN1 is controversial (Norton *et al.*, 1999) because successful outcome of surgery is rare. Some authors recommend surgery only in case of precise localization of gastrinoma, or in presence of particularly aggressive familial gastrinomas (Melvin *et al.*, 1993; Sheppard *et al.*, 1989), whereas other authors would perform surgery in all cases (Thompson *et al.*, 1993). Because MEN1 syndrome gastrinomas occur most commonly in the first and second portions of the duodenum, and less commonly the third and fourth duodenal portions and the first jejunal loop (Pipeleers-Marichal *et al.*, 1990), it is important that all these sites be examined during preoperative imaging, intraoperative exploration, and pathological examination of surgical specimens (Tonelli *et al.*, 2005).

Consequently, surgery has to follow the necessary procedures of tumor localization, such as SRS and selective infusion of secretin or calcium in selective pancreatic arteries followed by hepatic vein sampling for gastrin measurement (Imamura and Takahashi, 1993). Even though none of these procedures is completely precise for identifying sites of hypersecretion, transarterial challenge can indicate if excessive hormonal release is in the head or in the corpocaudal segment. Small duodenal gastrinomas are not easily appreciated by external palpation, by intraoperative ultrasound, or by duodenal transillumination. The best approach appears to be intraluminal digital examination of tumors after large longitudinal exposure of the duodenum. The possibility that removal of duodenal gastrinomas and peripancreatic periduodenal lymph nodes might cure hypergastrinemia has been proposed. Duodenal gastrinomas can be approached by a wide longitudinal duodenectomy and treated either by enucleation from the submucosa, if less than 5 mm in diameter, or by full-thickness excision of the duodenal wall if more than 5 mm in diameter. However, this conservative therapy is accompanied by a low percentage of cure (negativity of secretin stimulation test; Thomson *et al.*, 1993) and by neoplastic recurrence (Bartsch *et al.*, 2005). A more aggressive surgical approach is pancreatoduodenectomy. This procedure may be used in those cases where gastrin production is clearly localized in the duodenum or the head of the pancreas. The procedure results in removal of the duodenum, a frequent site of one or more gastrinomas, and removal of metastatic peripancreatic lymph nodes and of tumoral nodules from the pancreatic head. The main shortcoming of this approach is principally represented by the surgical risk for pancreatic fistula or pancreatitis. These complications can be higher than observed in surgery for ductal pancreatic neoplasia due to the increase softness of MEN1 pancreatic tissue. Mortality, following pancreatoduodenectomy in MEN1 patients, is not observed if performed in highly skilled surgical Centers (Bartsch *et al.*, 2005; Tonelli *et al.*, 2005). In other functioning neuroendocrine tumors (glucagonoma, VIPoma, PPoma, and somatostatinoma) and nonfunctioning tumors, the indications for surgery and the usual localization of the tumors is easier, because the latter are generally represented by large neoplasms. However, the need of pancreatic surgery for asymptomatic individuals with MEN1 syndrome can be decided when the patient is carefully monitored by endoscopic ultrasound and when the size of the lesion approaches 2 cm.This size criterion is recommended here. Other groups have used smaller or larger size cutoffs. This controversy will not be covered further here.

Endoscopic ultrasound can detect islet tumors in larger numbers and smaller sizes than other methods (Kann *et al.*, 2006). It is not effective in imaging duodenal microgastrinomas because of duodenal motion. It is not clear that detection of the many small, inevitable, and asymptomatic islet tumors in MEN1 is important. The roles for this technically demanding method will be explored further in the coming years.

Patients with systemic metastasis are usually excluded from surgery. Total pancreatectomy is generally not justified because of associated complications. A long-acting somatostatin analogue is now considered to be the drug of choice for controlling hormone secretion under conditions such as glucagonoma and VIPoma (Lamberts *et al.*, 1998; Maton *et al.*, 1989).

Insulinoma

Although one-third of MEN1 pancreatic islet microadenomas exhibit insulin immunoreactivity, only selected lesions larger than 5 mm are symptomatic (Klöppel *et al.*, 1986). Only rarely does insulinoma in MEN1 exhibit malignant degeneration, characterized by extrapancreatic infiltration and/or metastasis. Surgery is usually indicated for insulinoma for the following reasons: (a) Generally hypoglycemia is not easily controlled by drugs; (b) hypoglycemic syndrome is cured by resection of pancreatic macroscopic lesions (O'Riordain *et al.*, 1994); and (c) malignant degeneration is prevented. Surgery can be aided by pre- or intra-operative localization of a pancreatic nodule. According to Tonelli *et al.* (2005), the best surgical approach for an MEN1 insulinoma is intraoperative localization of nodules greater than 0.5 cm diameter by palpation or intraoperative ultrasound followed either by enucleation (removal) of these nodules or by pancreatic resection if multiple large, deep tumors are present. It has been reported that a limited resection or simple enucleation of nodules is more frequently followed by persistence or recurrence of the disease (Demeure *et al.*, 1991; Lo *et al.*, 1998; Simon *et al.*, 1998; Jordan, 1999). A reliable evaluation between different surgical techniques is still not possible because of the rarity of cases. Certainly, it is important to eliminate all the nodules macroscopically evident or greater than 5 mm (by ultrasound). Thus, the treatment of choice is removal of the main pancreatic nodules by pancreatic resection with removal of residual small tumors by enucleation. In case of corporocaudal pancreatic localization, splenic preservation is recommended. Removal of nodules in the pancreatic head is needed in about 50% of patients. This surgical procedure can cause both pancreatic fistulas and pancreatitis and, although a general consensus does not exist, intraoperative monitoring of glucose and insulin may be helpful to verify removal of the tumor (Carneiro *et al.*, 2002).

Carcinoids

Surgery remains the first choice for treatment of bronchial and thymic carcinoid tumors. Thymic carcinoid recurred in all individuals with MEN1 syndrome who were followed for more than 1 year after resection of the tumor (Gibril *et al.*, 2003). Prophylactic thymectomy should be considered at the time of neck surgery for primary hyperparathyroidism in males with MEN1 syndrome, particularly those who are smokers or have relatives with thymic carcinoid (Ferolla *et al.*, 2005).

Bronchial carcinoid in MEN1 has a low grade of malignancy. Our method is to remove a small solitary nodule with as conservative a pulmonary procedure as possible. Known metastatic disease is usually not treated surgically, but the course is usually indolent.

In contrast to the surgical approach suggested for the tumors previously mentioned, the approach to ECLomas in the setting of MEN1 is controversial, because of the multiplicity of gastric primary lesions and because of the uncertainties regarding the prospects of long-term cure as a result of partial or total gastrectomy. The treatment protocol for MEN1 ECLomas should, therefore, be lowering of gastrin levels, intensified endoscopic surveillance and therapy, and gastrectomy on appearance of macrolesions.

Somatostatin analogues have been successfully employed in the treatment of type II gastric carcinoids in three patients with MEN1 syndrome (Tomassetti *et al.,* 2000). During the treatment serum gastrin levels dramatically decreased and tumors regressed in all patients; one of the patients exhibited more than 30 foci widespread throughout the corpus and the fundus of the stomach that disappeared after 1 year of treatment. The regression of tumors was attributed to the decreasing of serum gastrin levels known to be an important factor in the pathogenesis of gastric carcinoid tumors in patients with MEN1 and ZES (Rindi *et al.,* 1996; Bordi *et al.,* 1998). Although long-acting somatostatin analogues can control the secretory hyperfunction associated with carcinoid syndrome, the risk for malignant progression of the tumor remains unchanged (Schnirer *et al.,* 2003).

Pituitary Tumors

In general, the therapy (medical, radiation, surgical) of pituitary tumors is the same as for sporadic pituitary tumors. Dopamine agonists such as cabergoline, bromocriptine, and quinagolide are the preferred treatment of prolactin (PRL)-secreting microadenomas (Bevan *et al.,* 1992). Optimal ways to prevent or monitor for dopaminergic drug-induced cardiac fibrosis have not been established (Schade *et al.,* 2007).

In particular, cabergoline can be considered the current treatment of choice because of its reduced side effects and greater potency, whereas somatostatin analogues are the medical therapy of choice for the treatment of growth hormone-secreting tumors (Beckers *et al.,* 2003).

In contrast, in nonsecreting pituitary adenomas surgical treatment is the treatment of choice, even if in 5% to 15% of cases, medical treatment with potent dopaminergic agonists or sometimes with somatostatin analogues may shrink the adenoma before surgery (Colao *et al.,* 1998). Although general agreement on this topic does not exist, Beckers *et al.* (2003) suggested that aggressive therapy is more frequently needed in MEN1-associated pituitary tumors than in sporadic tumors.

PATHOPHYSIOLOGY

MEN1 is an autosomal dominant disorder with penetrance that approaches 100% with increasing age and with a variable expression (different clinical phenotypes in affected members within an MEN1 kindred, in terms of tumor tissue type, onset age, and clinical aggressiveness) (Marx and Simonds, 2003). More precisely, the age-related penetrance for all clinical features rises above 50% by 20 years of age and above 95% by 40 years of age (Trump *et al.,* 1996; Bassett *et al.,* 1998; Skarulis, 1998). Anticipation has not been described. A prevalence of about 1 in 30,000 has been reported (Marx, 2001).

Pathogenic and pathophysiological issues include recent identification of the *MEN1*-encoded protein, named *menin*, and its still-unknown pathways in normal or tumor tissues.

Pathology/General Considerations

Pathological changes (i.e., a neoplasm or its precursors) in the target organs of the MEN1 syndrome are more extended than suggested by clinical and biochemical features of the patients. Several lesions, including most pancreatic or gastric endocrine proliferations, can be clinically silent, and others may present overt symptomatology only at advanced stages. A review of 32 autopsy case reports (Majewski and Wilson, 1979) revealed that, by the age of 30, pathological lesions are consistently found in each of the three main involved glands (parathyroids, endocrine pancreas, pituitary), initiating the concept that the MEN1 syndrome is an “all-or-none” phenomenon. Whether this concept may apply to other organs whose involvement has become apparent since that review has not been clarified. These include the tissues of the embryonic foregut (duodenum, stomach, lung, thymus), in which carcinoid tumors with potential malignant outcome may develop, as well as tissues that only rarely exhibit clinical morbidity in MEN1, such as adrenal cortex, adipose tissue, and skin (Friedman *et al.,* 1994; Padberg *et al.,* 1995; Darling *et al.,* 1997).

The sequence of events leading to neoplasia appears to be a subtle, diffuse one that follows a similar pattern in all MEN1 target organs. On a histopathological basis, the initial lesion appears to be a diffuse proliferation of the affected endocrine tissue with bilateral involvement of many paired organs and multifocal growth. Whether this change reflects polyclonal hyperplasia or *de novo* arising multiple clonal lesions, however, has not been clarified yet. A clear-cut stage of polyclonal hyperplasia has been identified only in the mouse model of MEN1 (Crabtree *et al.,* 2001, 2003); however, it suggests that a far more subtle polyclonal stage might precede all tumors in hereditary MEN1. One study has identified a stage of multifocal and hyperplastic precursors in gastrinoma from MEN1 but not from sporadic cases (Anlauf *et al.,* 2005). In that study, mono- versus polyclonality of the precursor stages was not proven.

The next step is development of multiple micro- and, eventually, macronodular lesions (Fig. 1). The transition between these lesions and true neoplasms is virtually unrecognizable on histopathological grounds. In fact, evidence from clonality studies in MEN1 parathyroids and pancreatic lesions revealed monoclonality of the same tissues that were

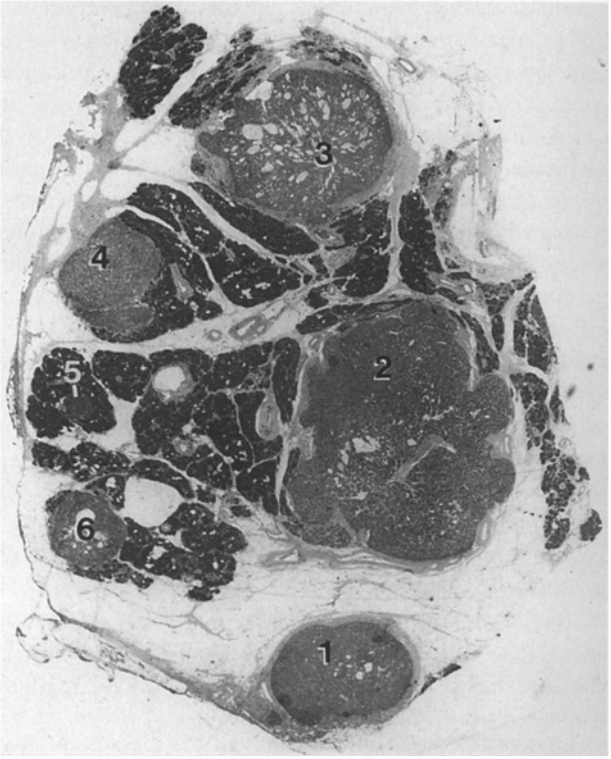

FIGURE 1 Numerous, scattered islet cell adenomas (numbered 1–6) of various size and architecture in a transverse section of the pancreas of a patient with MEN1 syndrome and ZES (H&E, 4). Reprinted with permission from Pilato *et al.* (1988).

classifiable as hyperplastic according to the established histopathological criteria (Larsson *et al.,* 1988; Friedman *et al.,* 1989). LOH analysis of multiple tumors of the same organ, including parathyroids, pancreas, duodenum, and stomach (Morelli *et al.,* 1996; Lubensky *et al.,* 1996; Debelenko *et al.,* 1997a, 1997b), consistently showed different patterns and sizes of chromosomal or subchromosomal deletions, indicating that each of multiple mono- or oligoclonal tumors is the result of an independent mutational event.

Malignancy in MEN1 syndrome is mostly confined to pancreatic, duodenal, thymic, and bronchial neuroendocrine neoplasms. Notably, malignancy in the parathyroid is very rare in MEN1 (Sato *et al.,* 2000). Gastric carcinoid tumors may also pursue a very aggressive course (Bordi *et al.,* 1997; Norton *et al.,* 2004). Gastric carcinoid tumors in multiple endocrine neoplasia-1 patients with Zollinger-Ellison syndrome can be symptomatic, demonstrate aggressive growth, and require surgical treatment (Schnirer *et al.*, 2003).

In a study of a large Tasmanian kindred, enteropancreatic malignancies were found in 14 of 69 patients (20%; Burgess *et al.,* 1998b). All but one of these patients also presented hypergastrinemia. Liver metastases are common in these cases, but their origin may be difficult to ascertain. They may derive from either pancreatic (more commonly) or duodenal gastrinoma(s). However, ECL cell carcinoids of the stomach, which are dependent on the trophic stimulus of hypergastrinemia, may also contribute (Bordi *et al.,* 1998). Immunostaining for gastrin or for the isoform 2 of the vesicular monoamino transporter (VMAT-2), the latter of which is specific for ECL cells (Rindi *et al.,* 2000), may help to establish the tissue origin of the primary tumor. As typical of endocrine tumors, histopathological definition of malignancy based on traditional criteria is often flawed. The use of immunohistochemical markers of cell proliferation, however, is useful in this regard (Bordi and Viale, 1995).

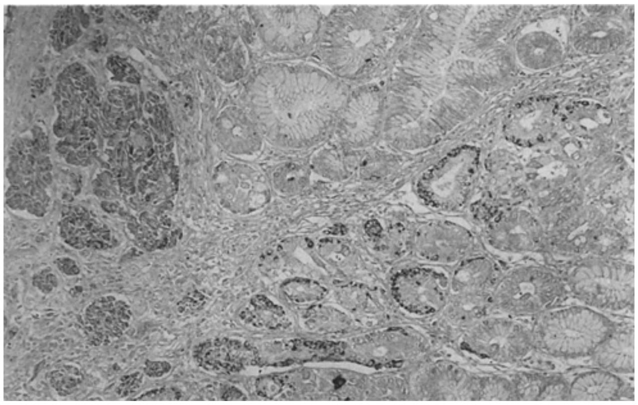

FIGURE 2 Immunostaining for basic FGF in the gastric endocrine cells of a patient with MEN1 syndrome and ZES. The peptide is expressed by both intraglandular cells showing hyperplastic pretumoral lesions (on the right) and carcinoid tumor (on the left) (immunoperoxidase, 100).

Immunohistochemistry using markers of neuroendocrine cells, such as chromogranin A, synaptophysin, and neuronspecific enolase, as well as specific hormonal products of the individual endocrine glands is useful in tumor characterization and in the assessment of preneoplastic lesions. Because of the identification in serum of an MEN1 mitogenic factor (Brandi *et al.,* 1986), structurally similar to basic fibroblast growth factor (FGF; Zimering *et al.,* 1993), immunohistochemistry, alone or in combination with molecular biology, has also been used to localize basic FGF in MEN1-related endocrine tissues. Ezzat *et al.* (1995) found high levels of basic FGF mRNA in adenomatous but not in normal pituitary glands, a finding consistent with the evidence of a pituitary origin of circulating basic FGF in MEN1 and sporadic pituitary tumor (Zimering *et al.,* 1993). Basic FGF mRNA levels correlated with *in vivo* and *in vitro* release of basic FGF but were consistently associated with lack of basic FGF immunoreactivity in tumor cells, suggesting a constitutive secretion of this peptide. However, basic FGF is elevated in serum of many patients with MEN1 and may play a pathogenetic role (Hoang-Xuan and Steger, 1999). In contrast, immunohistochemical expression of basic FGF was well documented in the gastric proliferating ECL cells (Fig. 2; Bordi *et al.,* 1994) and in pancreatic endocrine tumors (Chaudhry *et al.,* 1993) in both MEN1 and sporadic patients.

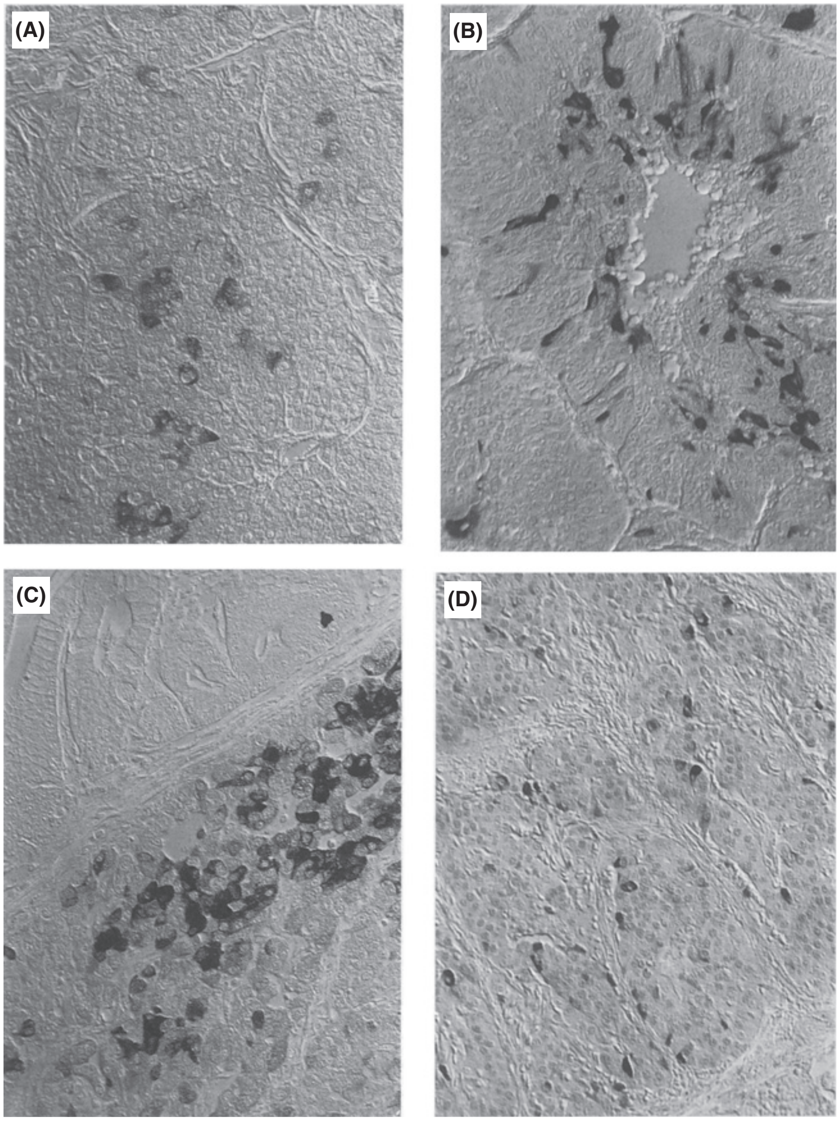

FIGURE 3 Immunohistochemical expression of α-subunit of glycoprotein hormones in different types of tumors in MEN1 patients: parathyroid (**A**), lung carcinoid (**B**), gastric carcinoid (**C**), duodenal gastrinoma (**D**) (immunoperoxidase, 170).

The expression of glycoprotein hormone α-subunit has been revealed by immunohistochemistry in all types of endocrine tumors involved in the MEN1 syndrome, including those of pituitary, pancreas, foregut, and parathyroids (Carlinfante *et al.,* 1998; Fig. 3). Such expression has been regarded as a marker of malignancy in pancreatic (Heitz *et al.,* 1983) but not in gastric (Bordi *et al.,* 1995a) or pulmonary (Bonato *et al.,* 1992) endocrine tumors. Burgess *et al.* (1998b) reported that serum levels of this protein were elevated in 71% of MEN1 patients with metastatic tumors but only in 7% of those without metastatic neoplasms ($P < 0.05$).

Pathology of the Parathyroids

It is generally assumed that in primary hyperparathyroidism of the MEN1 syndrome all parathyroid glands are pathologically involved. Size heterogeneity of parathyroid glands is, however, a consistent finding in MEN1 (Friedman *et al.,* 1994; Padberg *et al.,* 1995) with an average ratio of 9.5 between volumes of the largest and the smallest glands (Marx *et al.,* 1991). Small glands may show no detectable differences from normal glands, also when observed at the microscopic level (Harach and Jasani, 1992). When associated with asymmetric growth of one gland these findings rarely simulate adenoma (DeLellis, 1995). Parathyroid tumor within the thymus, in the thyroid gland, at the carotid bifurcation, or in the mediastinum may be responsible for surgical failures (Mallette, 1994). With four parathyroid tumors in MEN1, the odds of one being abnormally located should be about fourfold that for a solitary adenoma.

By histology, the parathyroid changes are very similar to those of non-MEN1 hyperplasia or of secondary hyperparathyroidism, although size variability is a more striking feature in MEN1 (Harach and Jasani, 1992). Both nodular and diffuse patterns of hyperplasia occur, the former being more frequently seen (DeLellis, 1995). In either pattern, chief cells are the predominant cell type and are variously associated with oxyphil or transitional oxyphil cells. Neighboring intraglandular nodules may strongly differ in their cytological and architectural features (Friedman *et al.*, 1994).

Morphological distinction between these hyperplastic nodules and true adenomas is difficult if not impossible (Friedman *et al.*, 1994; Padberg *et al.*, 1995). To date, the issue has not been clarified even by clonality studies. As a rule the size of monoclonal lesions is significantly larger than that of polyclonal lesions (Friedman *et al.*, 1989; Falchetti *et al.*, 1993). Arnold *et al.* (1995), using X-chromosome inactivation analysis in patients with either primary MEN1 and non-MEN1 parathyroid hyperplasia or uremic hyperparathyroidism, demonstrated definite monoclonal status in 67% of glands with nodular hyperplasia and in 62% of glands with diffuse hyperplasia. These results cast doubts on the long-standing assumption that multiglandular parathyroid disease is a morphologic feature specific for polyclonal parathyroid hyperplasia.

The intensity and topographic distribution of parathyroid hormone immunoreactivity show pronounced variations in MEN1 parathyroid glands (Friedman *et al.*, 1994; Harach and Jasani, 1992). As a rule, hyperplastic areas reveal less immunoreactive hormone content than contiguous normal areas (Harach and Jasani, 1992), a finding consistent with defective mechanism(s) for hormone storage.

The endothelial cells of parathyroid glands, a target of the mitogenic activity of MEN1 growth factor (Brandi, 1991; Friedman *et al.*, 1994), have been investigated by ultrastructural morphometry in a series of MEN1 patients (D'Adda *et al.*, 1993). The parathyroid endothelial component was found to be significantly more expanded in MEN1 than in uremic hyperparathyroidism, indicating its potential role in the proliferation of parathyroid tissues in MEN1.

Pathology of the Endocrine Pancreas

Pathological changes of the pancreas in MEN1 patients have been often inconsistently defined, making evaluation of the actual incidence of specific lesions and of their relationships difficult and unreliable. Two basic changes showing specific functional and clinical characteristics, multiple islet cell microadenomas (microadenomatosis) and grossly single endocrine tumors, may be found (Friedman *et al.*, 1994; Klöppel *et al.*, 1986; Padberg *et al.*, 1995; Thompson *et al.*, 1984). Terms such as *nesidioblastosis* or *islet cell hyperplasia* should be avoided owing to their difficult histological identification and uncertain clinical significance.

Microadenomatosis is the most common pancreatic islet lesion in MEN1 cases. The multiple adenomas, scattered throughout the whole pancreatic gland (see Fig. 1), may be very numerous (up to 100 in some cases) and range in size from microadenomas slightly larger than unaffected islets to occasional macroadenomas with a diameter larger than 0.5 cm. Like the multiple nodules of parathyroid hyperplasia, even contiguous pancreatic lesions may exhibit striking variations in their histological arrangement, stromal content, and cytological characteristics. The microadenomas are functionally silent and are consistently benign both histologically and clinically. They are composed exclusively of cell types normally present in the pancreas, often coexisting in single tumors but lacking the typical topographic distribution seen in normal islets. The dominant hormonal peptide in these microadenomas is not random (Pilato *et al.*, 1988), a definite predominance of glucagon-, insulin-, and PP-producing cells being most often observed (Klöppel *et al.*, 1986; Pilato *et al.*, 1988; Padberg *et al.*, 1995; Le Bodic *et al.*, 1996). In small lesions such a predominance may be the only distinctive feature with respect to unaffected islets. In spite of their most frequent association with ZES, gastrin immunoreactivity is uncommonly found in these islet multiple microadenomas, with duodenal microgastrinomas and/or their metastases being responsible for hypergastrinemia in these patients.

Perren *et al.* (2006) recently described, in addition to enteropancreatic microadenomas, small aggregates of endocrine neoplastic cells with a similar monohormonal staining pattern defined as MECCs (monohormonal endocrine cell clusters) near to neither sclerosis nor duct cells. By LOH analysis these MECCs lesions clearly revealed their monoclonal and thus neoplastic nature. Findings from this study raised the possibility that MEN1-associated endocrine tumors may originate from at least two different pancreatic compartments but mainly from islets rather than ductal cells (Perren *et al.*, 2006).

Large, macroscopically single endocrine tumors, indistinguishable from those found without MEN1, are less frequently found in MEN1 syndrome. Usually they develop on the background of islet cell microadenomatosis. Like their sporadic counterparts, these neoplasms are frequently malignant and may metastasize to the liver. A large tumor size (≥3 cm) is weakly predictive of this metastatic evolution (Cadiot *et al.*, 1999). Macroscopically single pancreatic tumors are composed of cells producing either endogenous (insulin, glucagon, PP) or ectopic (gastrin, VIP, GHRF, calcitonin, PTH-related peptide) hormones, and they often show functional activity covering the wide spectrum of clinical syndromes associated with pancreatic endocrine tumors. It has been assumed that functioning insulinomas in MEN1 are more frequently single tumors curable with surgical resection (Klöppel *et al.*, 1986; Thompson *et al.*, 1984; Tonelli *et al.*, 2005). However, others reported a frequent multicentricity of MEN1 insulinomas

(DeLellis, 1995). When the one dominant insulinoma in MEN1 is removed with confirmation of its overproduction of insulin (preoperative insulin localization, intraoperative serum glucose and insulin), the nonfunctional feature of coexistent pancreatic nodules is usually confirmed during surgery.

Pathology of the Pituitary

Pituitary tumors are found in more than 60% of MEN1 patients (Scheithauer *et al.,* 1987; Capella *et al.,* 1995) and are often appreciated only when the gland is examined microscopically (Majewski and Wilson, 1979). Despite their high penetrance in MEN1, the pituitary tumors are rarely multiple. They are usually represented by macroadenomas associated with diffuse or nodular hyperplasia of extratumoral pituitary cells (Capella *et al.,* 1995). The MEN1 tumors, like sporadic tumors, are grossly invasive in 10% to 15% of cases. By immunohistochemistry, they are most often found to produce PRL or GH, to be endocrinologically functioning, and to be plurihormonal more often than their sporadic counterparts. Between 65% (Brandi *et al.,* 2001) and 85% (Verges *et al.,* 2002) of pituitary tumors in MEN1 syndrome are macroadenomas versus a lower proportion in common tumors.

Pathology of the Foregut Carcinoid Tissues

Duodenal gastrinomas are common MEN1 tumors, being the cause of the ZES in more than 90% of MEN1 cases (Padberg *et al.,* 1995). They are multiple tumors, often so small (<1 cm diameter) that they escape detection even at intraoperative inspection and so diffusely distributed that they might require removal of the whole duodenum for adequate correction of hypergastrinemia. Immunostaining for gastrin is usually strong. Metastases are frequent and mostly restricted to regional lymph nodes (Pipeleers-Marichal *et al.,* 1990).

MEN1-associated duodenal gastrin-producing tumors may derive from hyperplastic gastrin cell proliferation as a precursor lesion. Anlauf *et al.* (2005) classified three pathological features: (a) macrotumors (>2 mm); (b) microtumors (0.25–2 mm); and (c) hyperplastic lesions.

Gastric, ECL-cell carcinoids may develop in MEN1 patients affected by ZES through a sequence of hyperplasia—dysplasia—neoplasia (Solcia *et al.,* 1988; Bordi *et al.,* 1998). In these cases hypergastrinemia acts as a potent promoter for ECL cell proliferation (Bordi *et al.,* 1995a). However, the lack of ECL-cell carcinoids in cases of sporadic ZES indicates that biallelic inactivation of the *MEN1* gene (Cadiot *et al.,* 1993; Debelenko *et al.,* 1997a) is also essential for the evolution of ECL cell hyperplasia into neoplasia (Solcia *et al.,* 1990; Bordi *et al.,* 1998). Malignant behavior is rare in these carcinoids (Rindi *et al.,* 1996). However, several cases of gastric endocrine tumors having aggressive behavior and very poor outcome have been observed (Bordi *et al.,* 1997; Schnirer *et al.,* 2003; Norton *et al.,* 2004). In one of these, multiple benign gastric carcinoids coexisted with a malignant poorly differentiated endocrine carcinoma causing the patient's death (Bordi *et al.,* 1997). 11*q*13 LOH was documented in these undifferentiated neoplasms.

Thymic and bronchial carcinoid tumors can also be regarded as an integral part of the MEN1 syndrome (Padberg *et al.,* 1995). The thymic carcinoids of MEN1 syndrome tend to be aggressive (Gibril *et al.,* 2003; Ferolla *et al.,* 2005), whereas three fourths of the latter are benign (Duh *et al.,* 1987; Teh *et al.,* 1997), although with the potential for local mass effect, metastasis, and recurrence after resection (Sachithanandan *et al.,* 2005). Surprisingly, thymic carcinoids of MEN1, including the majority that are malignant, do not exhibit 11q13 LOH (Teh *et al.,* 1997). Inactivation of the wild-type copy of the *MEN1* gene by other mechanisms needs to be explored.

Pathology of Other Organs

Various tumors of thyroid follicular cells, adrenal cortex, cutaneous and visceral adipose tissue, and skin have been described in MEN1 patients (Brandi *et al.,* 1987; Friedman *et al.,* 1994; Padberg *et al.,* 1995). These are mostly benign neoplasms. Multiple facial angiofibromas, similar to those in tuberous sclerosis, and discrete papular collagenomas of the trunk are commonly found in MEN1 patients (Darling *et al.,* 1997). Angiofibromas are benign tumors composed of blood vessels and connective tissue. These consist of acneiform papules that do not regress and that may extend across the vermilion border of the lips. Collagenomas consist of multiple, skin-colored, sometimes hypopigmented, cutaneous nodules, symmetrically arranged on the trunk, neck, and upper limbs. They are typically asymptomatic, roundish, and firm-elastic, from a few millimeters to several centimeters in diameter.

Enlargement of the adrenal glands is found in about 40% of MEN1 patients (Burgess *et al.,* 1996; Komminoth, 1997). Silent adrenal gland enlargement is likely a polyclonal or hyperplastic process (because LOH at 11q13 is not found therein), which rarely results in monoclonal neoplasm (Skogseid *et al.,* 1992). Langer *et al.* (2002) showed that the median tumor diameter at diagnosis was 3.0 cm (range 1.2–15.0 cm), with most tumors being 3 cm or smaller. The underlying pathologic lesions affect the adrenal cortex and are usually represented by diffuse or (macro)nodular hyperplasia or by adenoma, although exceptional cases of carcinomas have been reported (Komminoth, 1997). Although general agreement does not exist, some suggest surgical removal of adrenocortical tumors that exceed 3 cm in diameter because of their malignant potential (Langer *et al*., 2002).

Because of their common occurrence and lack of *MEN1* inactivation, thyroid tumors lesions do not seem to be causally related to the *MEN1* gene. In contrast, allelic loss at the *MEN1* locus has been reported in most *MEN1* tumors: dermal angiofibroma, truncal collagenoma, intracranial meningioma, esophageal leiomyoma, and lipoma (Morelli *et al.,* 1995; Pack *et al.,* 1998; Vortmeyer *et al.,* 1999). Moreover, 11*q*13 LOH was found in rare adrenal medullary tumors of MEN1 (Cote *et al.,* 1998b) but surprisingly not found in the common adrenocortical tumors of MEN1 patients (Skogseid *et al.,* 1992).

Miscellaneous Nonendocrine Malignancies in Patients with MEN1 Syndrome

A number of malignant tumors of various, nonendocrine origin, including exocrine pancreas, kidney, bladder, endometrium, and skin, have been reported in MEN1 patients (Bordi *et al.,* 1995b; Thompson *et al.,* 1984: Doherty *et al.,* 1998; Nord *et al.,* 2000). At least five cases of ductal pancreatic adenocarcinoma have been reported, suggesting an increased risk for this tumor in MEN1 (Bordi and Brandi, 1998). 11*q*13 LOH was not found in the single tumor investigated (Bordi *et al.,* 1995b). Malignant melanomas have been reported in seven typical MEN1 patients from seven MEN-1 kindreds (Nord *et al.,* 2000). However, no role of the *MEN1* gene in the development of sporadic or familial melanomas is proven (Boni *et al.,* 1999; Nord *et al.,* 2000).

BIOCHEMICAL AND RADIOLOGICAL TESTS FOR TUMORS IN LIKELY *MEN1* MUTATION CARRIERS

Periodic surveillance for tumor emergence is recommended for asymptomatic individuals with an *MEN1* disease-causing mutation and others at risk for MEN1 syndrome-associated tumors (i.e., those known to have MEN1 syndrome. About 25% of MEN1 families do not have an identifiable *MEN1* mutation. In such families, a screening program in unaffected members at 50% risk should be less comprehensive. Affected individuals may present with any of the MEN1-associated lesions in early teenage years or escape clinical symptoms for several decades (Skogseid *et al.,* 1991). A periodic investigation should be performed to look for the frequent endocrine and nonendocrine tumors associated with this syndrome in potentially affected individuals (Table II). MEN1-associated lesions may develop slowly in unaffected mutation carriers, despite earlier extensive biochemical testing. Such screening may detect the onset of a tumor about 10 years before symptoms develop, thereby providing an opportunity for earlier treatment (Bassett *et al.,* 1998). Early screening for MEN1 tumors in asymptomatic *MEN1* mutation carriers may help to reduce morbidity, but this is not proven. The overall age-related penetrance of all MEN1-related tumors is near zero below age 5 years (Stratakis *et al.,* 2000), rising quickly to more than 50% by 20 years, and more than 95% by 40 years (Metz *et al.,* 1994; Trump *et al.,* 1996; Bassett *et al.,* 1998; Skarulis, 1998). Individuals at highest risk of developing MEN1 tumors (mutant *MEN1* carriers have near 100% lifetime risk) should be screened yearly for tumors. Screening should commence in early childhood, and it should continue for life (Trump *et al.,* 1996). Because such screening for MEN1 tumors can be difficult and expensive with large numbers of available and of recommended tests, a protocol should be designed to make best use of available resources (see Table II).

Primary hyperparathyroidism is often the first clinical manifestation and the most common pathology associated with the MEN1 syndrome (Lamers and Froeling, 1979; Marx

TABLE II Survey for Tumors in an MEN1 Carrier

Tumor	Age to Begin (Years)	Biochemical Tests (Annually)	Imaging Tests (Every 3 Years)
Parathyroid adenoma	8	Calcium, PTH	None
Gastrinoma	20	Gastrin	None
Insulinoma	5	Fasting glucose	None
Other enteropancreatic	20	Chromogranin-A??[a]	$[^{111}\text{In}]$DTPA octreotide; CT/MRI
Anterior pituitary	5	Prolactin; IGF-1	MRI
Foregut carcinoid	20	Chromogranin-A??[a]	CT, endoscopy[b]

[a] *Not proven useful to screen for small tumor. Proven useful to monitor tumor burden.*

[b] *When indicated for evaluation of gastric acidity or Barrett's esophagus.*

CT, *computed tomography;* DTPA, *diethylenetriamine pentaacetic acid;* IGF-1, *insulin-like growth factor* 1; MRI, *magnetic resonance imaging.*

et al., 1986; Oberg *et al.,* 1982), being asymptomatic in 50% of hyperparathyroid cases. Accurate clinical and laboratory screening for parathyroid function promotes early biochemical diagnosis. This can help to (1) diagnose a cancer or (2) diagnose parathyroid tumor in a known carrier. A bone mineral density evaluation by dual-energy x-ray absorptiometry (DEXA), quantitative computed tomography (QCT), or ultrasound (US), both at cortical and trabecular sites can define the fracture risk in cases with hyperparathyroidism. Preoperative parathyroid tumor imaging has little role in the unoperated case. Tc-99m Sesta-MIBI scan is the most useful among many methods prior to parathyroid reoperation. However, a well-experienced neck surgeon remains the most important "clinical device" for successful initial surgery of hyperparathyroidism. Intraoperative measurement of PTH "online" is promising to test whether deleterious amounts of parathyroid tumor remain during initial or repeat surgery (Tonelli *et al.,* 2000).

Gastrinoma constitutes the second commonest manifestation but potentially the most morbid feature of MEN1 (Croisier *et al.,* 1971; Eberle and Grun, 1981). As for hyperparathyroidism, clinical and biochemical screening could promote early detection of gastrinoma and other GEP disease. Tests to consider would include basal evaluation of gastrin, insulin, C-peptide, blood glucose, proinsulin, glucagon, PP, and chromogranin-A (Skogseid *et al.,* 1987; Brandi *et al.,* 2001; see Table II). More complex or invasive tests (basal output of gastric acid, secretin or calcium stimulation for gastrin, supervised fasting for insulin) have been developed to increase sensitivity; they may be used after a screening test indicates the need for more information. Tests with abnormal levels should be repeated. False-positives include high proinsulin/insulin levels in patients developing insulin resistance or hypergastrinemia in patients with hypochlorhydria (such as from parietal cell autoimmunity or from gastric acid-blocking drugs). Somatostatin receptor scintigraphy ([^{111}In]diethylenetriamine pentaacetic acid [DTPA]-octreotide scan) is a proven method for imaging of pancreatic islet tumor; computed tomography (CT) or magnetic resonance imaging (MRI) are interchangeable here. Unfortunately, because of the small size and multiplicity of duodenal gastrinomas, the methods most sensitive for the pancreas have low sensitivity for MEN1 gastrinomas (Bansal *et al.,* 1999; Cadiot *et al.,* 1996). Endoscopic ultrasound (EUS) will image many micro-adenomas in the pancreas of MEN1. This information does not lead to intervention, and the role for EUS in MEN1 remains uncertain.

Insulinoma that had been negative to preoperative imaging is usually readily identified by intraoperative ultrasonography (Bansal *et al.,* 1999). However, since radiology of the enteropancreatic lesion is neither sensitive nor specific, surgery should be done if the biochemical diagnosis is unequivocal, even rarely without symptoms (Skogseid *et al.,* 1996; Granberg *et al.,* 1999).

Symptoms of pituitary neoplasms associated with the MEN1 syndrome depend on both the tumor volume and the hormonal secretion from the gland. Prolactin is the most frequent product of hypersecretion from the anterior pituitary in MEN1 (Croisier *et al.,* 1971; Eberle and Grun, 1981; Majewski and Wilson, 1979). Circulating prolactin measurement should be performed under basal conditions at 0 and 60 minutes in order to avoid an influence of stress on hormonal secretion. MRI is the preferred pituitary imaging method. False positives are from pregnancy or psychotropic drugs. Finally, even in cured patients, pituitary tumor screening should continue because the remaining pituitary cells may cause recurrence or the development of new adenoma.

Computed tomographic or magnetic resonance imaging of the chest are recommended for early diagnosis of thymic or bronchial carcinoids. Type II gastric ECL cell carcinoids are recognized mainly at gastric endoscopy incidental to evaluation for ZES.

DNA-BASED TESTS

MEN1 Gene Discovery

In 1997 the *MEN1* gene was identified by positional cloning (Chandrasekharappa *et al.,* 1997; Lemmens *et al.,* 1997). Combined subchromosomal mapping studies with both linkage in MEN1 kindreds (Larsson *et al.,* 1988) and microdeletion analysis in tumors (Larsson *et al.,* 1988; Friedman *et al.,* 1989; Emmert-Buck *et al.,* 1997) demonstrated that the MEN1 trait was in close linkage to *PYGM,* the gene for muscle phosphorylase, at chromosome 11*q*13. All candidates to be the *MEN1* gene were near this gene. Each candidate gene in the minimized interval was sequenced in a panel of DNAs from MEN1 probands. One candidate gene showed mutation in most of the probands, proving that it was the sought-after *MEN1* gene.

The proposed model for tumorigenesis in familial MEN1 was according to Knudson's "two hit" hypothesis (Knudson, 1993; Larsson *et al.,* 1988). An affected *MEN1* copy is inherited in the germline from the affected parent (first hit) while the wild type copy, from the healthy parent, is eliminated from one somatic cell (second hit). The second hit was deduced indirectly from loss of heterozygosity or allelic loss at the *MEN1* locus, when tumoral DNA was compared to germline DNA. These findings strongly suggested biallelic gene inactivation as the mechanism of tumorigenesis from *MEN1*.

MEN1 Sequencing in Germline DNA

Genetic diagnosis of monogenic disorders is made possible only if either cloned material from a specific gene is sequenced to allow mutation testing or the chromosomal localization of the specific gene is known, and tightly linked genetic markers have been characterized (haplotype testing). Testing the *MEN1* mutation carrier state is possible by either approach.

Most laboratories find an *MEN1* mutation in 70% to 90% of index cases in typical MEN1 families (Agarwal *et al.,* 1997; Teh *et al.,* 1998b; Marx, 2001; Ellard *et al.,* 2005; Klein *et al.,* 2005). Rare *MEN1* families have a mutation in the p27 gene (Pellegata *et al.,* 2006). The positive rate is lower (20–80%) in index cases with sporadic MEN1. The positive rate is even lower (10%) in familial isolated hyperparathyroidism (Kassem *et al.,* 2000), and the rate is nearly zero in familial pituitary tumor (Tanaka *et al.,* 1998; Gadelha *et al.,* 2000; Daly *et al.,* 2007). The likelihood of finding a mutation is correlated with the number of MEN1-related tumors and increased in the presence of a family history (Ellard *et al.,* 2005). Sporadic cases with both parathyroid and pituitary tumors have a particularly low prevalence (10%) of *MEN1* mutation (Ozawa *et al.,* 2007).

There has been no genotype-phenotype relationship, including unrevealing *MEN1* mutations in index cases for families with the prolactinoma variant of MEN1 (Agarwal *et al.,* 1997; Hao *et al.,* 2004).

MEN1-like states with unusually low prevalences of *MEN1* mutation have helped point to other genes as causes. For example, the *HRPT2* gene was shown to be one cause of familial isolated hyperparathyroidism (FIHP; Carpten *et al.,* 2002), and another unidentified gene for FIHP has been tentatively located by linkage analysis to the short arm of chromosome 2 (Warner *et al.,* 2006).

Advantages of DNA-Based Analysis

The advantages of *MEN1* mutation analysis are first that a negative test does not need to be repeated serially, unlike the biochemical screening test for ascertainment of MEN1 through tumor discovery. Second, most results of an *MEN1* mutation test influence long-range planning. Thus, a test identifying an *MEN1* mutant gene carrier may lead to earlier and more frequent biochemical screening for tumors. Screening for MEN1 tumors in asymptomatic mutation carriers may help to reduce morbidity. Lairmore *et al.* (2004), in a prospective clinical study on *MEN1* gene mutant carriers, revealed that a biochemical evidence for a neoplasm can be identified on average 10 years earlier than clinical evidence of the disease itself, providing the opportunity for earlier treatment. Moreover, in a family with a known *MEN1* mutation, the finding of a family member at no risk for tumors because of no mutation should lead to a decision for no further screening of that member.

Limitations of DNA-Based Analysis

Unfortunately, the obtained information does not usually influence an immediate intervention or longevity. This reflects the lack of a therapy or prevention of proven value for MEN1-associated malignancy, in total contrast to the situation in MEN2. Second, mutation analysis may provide a false negative for an entire family; in fact, *MEN1* mutations are not detectable in 5% to 30% of MEN1 families. Simplex MEN1 cases (i.e., a single occurrence of MEN1 syndrome not in an MEN1 family) are less likely to test positive than familial cases, in part because some of these simplex cases are caused by somatic mosaicism (Klein *et al.,* 2005). In the event that sequence analysis fails to identify a germline mutation in an individual with typical MEN1 syndrome, deletions or other gross rearrangements can be tested for by Southern blot analysis. It is estimated that between 1% and 3% of *MEN1* germline mutations are large deletions that could be detected on a Southern blot analysis or by other gene dosage procedures (i.e., PCR-based; Kishi *et al.* 1998; Bergman *et al.,* 2000; Cavaco *et al.,* 2002; Ellard *et al.,* 2005; Klein *et al.,* 2005).

In the event that a disease-causing mutation is not identified using sequence analysis or targeted mutation analysis, haplotype analysis may be utilized in certain families. In fact, if large enough for haplotype analysis, virtually any MEN1 family is likely to be positive for an 11*q*13 haplotype shared in all affected cases (Olufemi *et al.,* 1998; Larsson *et al.,* 1995; Courseaux *et al.,* 1996).

DNA-Based Analysis of Tumors

Tumor DNA has been evaluated extensively for 11*q*13 LOH and for *MEN1* mutation. These analyses have contributed importantly to research. In particular, analysis of 11*q*13 LOH contributed to narrowing the *MEN1* gene candidate interval for gene discovery (Emmert-Buck *et al.,* 1997; Chandrasekharappa *et al.,* 1997) and to understanding the clonality of certain lesions, such as parathyroid tumors and skin lesions in MEN1 (Friedman *et al.,* 1989; Pack *et al.,* 1998). *MEN1* mutation analysis confirmed a role of *MEN1* inactivation in oncogenesis of many sporadic endocrine tumors (about 30% of parathyroid adenoma, insulinoma, gastrinoma, or bronchial carcinoid; Heppner *et al.,* 1997; Boni *et al.,* 1998). On the other hand, analysis of tumor DNA for *MEN1* involvement has little application in current clinical settings. It does not give a useful predictor of tumor aggressiveness, and it does not substitute for *MEN1* mutation analyses of germline DNA.

Mutation Types in the *MEN1* Gene

About 700 different mutations, both somatic and germline, of the *MEN1* gene have been identified (Thakker, 2001; Gagel and Marx, in press). The mutations are varied and scattered throughout the coding region with no clear evidence for clustering (Fig. 4). Approximately half of mutations are unique. The other half are repeating mutations; the repeats reflect either a common founder or a mutational

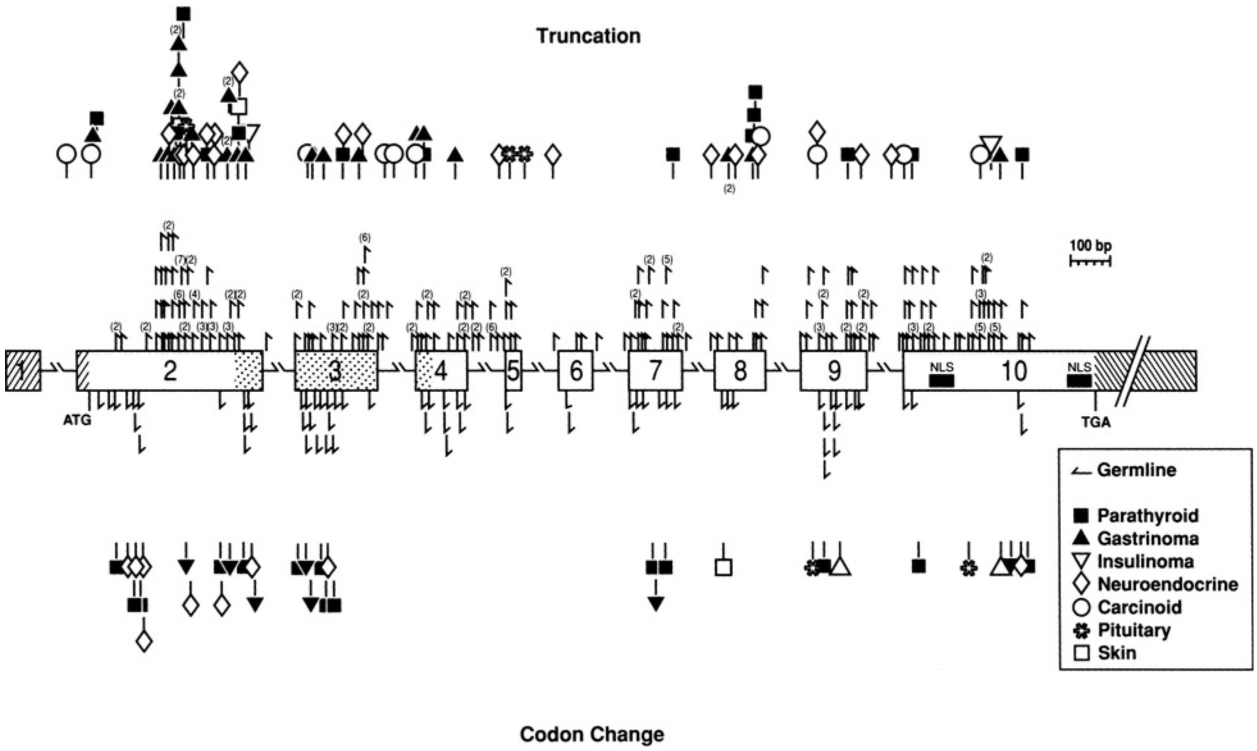

FIGURE 4 Genomic organization of the *MEN1* gene and of *MEN1* germline and somatic mutations, published as of April 2006. The gene contains 10 exons (with the first exon untranslated) and extends across 9 kb.Mutations shown above the exons cause menin truncation, and those shown below the exons cause a codon change. All unique mutations are represented; numbers in parenthesis designate multiple reports of the same mutation in presumed unrelated persons. The hatched areas indicate the untranslated regions. The location of the two nuclear localization signals (NLS), at codons 479–497 and 588–608, are indicated. Missense mutations in a region of menin (aa 139–242) (identified by stippling) prevented interaction with the API transcription factor JunD.

hotspot (Agarwal *et al.,* 1998). These potential mutational hot spots could be due to the presence in the vicinity of repeated long tracts of either single nucleotides or shorter elements. These repeated sequences may undergo misalignment during replication; a slipped strand mispairing model is the most likely mechanism to be associated with this sort of mutational hot spot (Agarwal *et al.,* 1999; Calender, 2000). All or most pathologic *MEN1* mutations are likely inactivating, as expected for a tumor suppressor gene: Approximately 60% of mutations are frameshift and 25% are nonsense. In total, some 80% of *MEN1* mutations predict protein truncation and thus an inactive or absent menin protein product. None of them have been associated to specific clinical manifestations of the disorder. Thus genotype/phenotype correlations appear to be absent in MEN1 (Teh *et al.,* 1998b; Agarwal *et al.,* 1997; Bassett *et al.,* 1998; see Fig. 4). The remaining 15% to 20% of *MEN1* gene mutations consist of missense mutations (inframe with change of one or two amino acids) potentially altering the interaction with one or more menin partners. In particular, a missense mutant of menin could favor a rapid degradation of menin that could account for a common mechanism for inactivation.

Germline mutational analysis of the *MEN1* gene in many centers failed to detect mutations in 5% to 30% of MEN1 patients. This failure could be explained if the mutation was a large deletion, causing the loss of the whole gene or a whole exon (Teh *et al.,* 1998b; Agarwal *et al.,* 1997; Bassett *et al.,* 1998). Consequently, the PCR amplification and mutational analysis shows only the normal allele. However, large deletions or other gross rearrangements can be tested for by Southern blot analysis. It is estimated that between 1% and 3% of MEN1 germline mutations are large deletions that could be detected on a Southern blot analysis or by other gene dosage procedures (i.e., PCR-based; Kishi *et al.,* 1998; Bergman *et al.,* 2000; Cavaco *et al.,* 2002; Ellard *et al.,* 2005; Klein *et al.,* 2005). Alternatively, undiscovered mutations may be within the untested parts of the gene, such as introns or the regulatory regions. Thus, even though no *MEN1* gene mutation is found in some MEN1 index cases, this does not exclude its involvement. In fact, when MEN1 families have been large enough for linkage analysis, the trait has been linked to 11*q*13 in most (Larsson *et al.,* 1992; Corseaux *et al.,* 1996; Olufemi *et al.,* 1998), suggesting cause by the *MEN1* gene in the majority.

MEN1 Gene Mutation in Endocrine Tumors

Endocrine tumors may occur either as part of MEN1 or, more commonly, as sporadic, nonfamilial tumors.

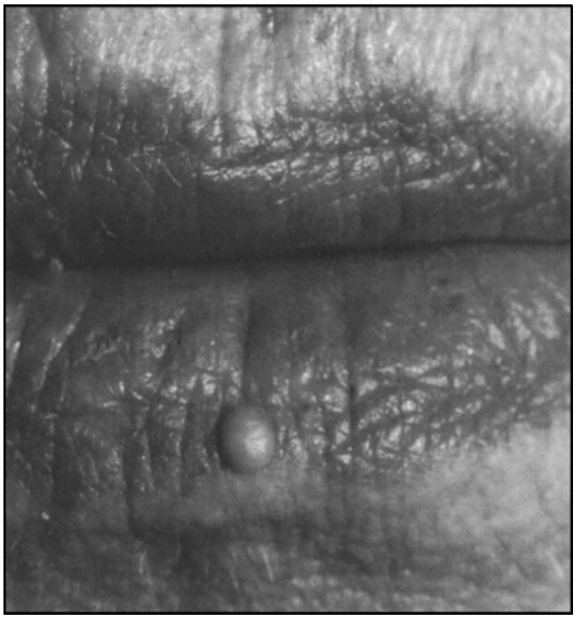

FIGURE 5 MEN1-associated angiofibroma of the lip.

Mutational analysis of MEN1-associated and sporadic endocrine tumors revealed that most tumors from MEN1 patients harbor the germline mutation together with a somatic LOH involving chromosome 11q13 (Marx *et al.*, 1998), as expected from Knudson's two-hit model and the proposed role of the *MEN1* gene as a tumor suppressor. However, LOH involving chromosome 11*q*13, which is the location of the *MEN1* gene, has also been observed in 5% to 50% of sporadic endocrine tumors, suggesting inactivation of the *MEN1* gene in the etiology of these tumors. Of those with 11*q*13 LOH, about half have shown somatic *MEN1* mutation; this includes sporadic parathyroid adenomas, sporadic gastrinomas, sporadic insulinomas, sporadic bronchial carcinoids, and sporadic anterior pituitary adenomas (Heppner *et al.*, 1997; Debelenko *et al.*, 1997c; Zhuang *et al.*, 1997). All together, these observations establish that *MEN1* is the known gene most frequently mutated in common-variety endocrine tumors.

MEN1 Gene Mutation in Nonendocrine Tumors

At least five different mesenchymal tumors in MEN1 can be caused by mutational inactivation of the *MEN1* gene. These include skin/dermal lipoma, angiofibroma (Fig. 5), and collagenoma. In each case loss of the normal allele has been proven with material from MEN1 patients (Pack *et al.*, 1998). Furthermore, sporadic lipoma and sporadic angiofibroma is occasionally associated with somatic *MEN1* mutation (Vortmeyer *et al.*, 1998; Boni *et al.*, 1998). Esophageal and uterine leiomyoma and intracranial meningioma also were associated with 11*q*13 LOH in most tested tumors of MEN1 (McKeebey *et al.*, 2001). This establishes that, like other multiple neoplasia syndromes, the tissue pattern of tumors in MEN1 does not follow a simple developmental distribution or any other recognizable distribution.

FUNCTIONS OF THE *MEN1* GENE AND ITS ENCODED MENIN IN NORMAL AND TUMOR TISSUES

The Normal *MEN1* Gene

The gene spans 9 kb and consists of 10 exons with a 1830-bp coding region (see Fig. 4) that encodes a novel 610-amino-acid protein, referred to as menin. The first exon and last part of exon 10 are not translated. A major transcript of 2.8 kb has been described in a large variety of human tissues (pancreas, thymus, adrenal glands, thyroid, testis, leukocytes, heart, brain, lung, muscle, small intestine, liver, and kidney; Lemmens *et al.* 1997). Moreover, an additional transcript of approximately 4 kb has been detected in pancreas and thymus, suggesting a tissue-specific alternative splicing (Lemmens *et al.*, 1997).

Menin: The *MEN1* Gene-Encoded Protein Product

Initial analysis of the predicted amino acid sequence encoded by the *MEN1* transcript did not reveal homologies to any other proteins, sequence motifs, signal peptides, or consensus nuclear localization signals, and thus the putative function of the protein menin could not be predicted (Guru *et al.*, 1998). Menin is widely expressed from an early developmental stage and is found in both endocrine and nonendocrine tissues.

Intracellular Localization

Studies, based on immunofluorescence, revealed that menin was located primarily in the nucleus. Furthermore, at least two independent nuclear localization signals (NLSs) were identified and located in the C-terminal quarter of the protein (Guru *et al.*, 1998). All of the truncated *MEN1* proteins that would result from the nonsense and frameshift mutations, if expressed, would lack at least one of these NLSs.

It is possible that menin-DNA interaction may play a crucial role in regulating cell proliferation by modulating the G2-M and/or G1-S phases progression (Yaguchi *et al.*, 2002; La *et al.*, 2004b). The nuclear localization of menin suggested that it may act either in the regulation of transcription, DNA replication, or in the cell cycle.

Menin Molecular Partners

Menin partners have been identified in the nuclear and cytoplasmic compartments (Poisson *et al.*, 2003); these have included a variety of transcriptional regulatory, cytoskeletal, and DNA processing and repair proteins (Agarwal *et al.*, 2005). None of the menin partners or menin pathways has yet been proven to be critical in MEN1 tumorigenesis or in menin normal physiology.

Menin-Nm23: Through interaction with a putative tumor metastasis suppressor, nm23H1/nucleoside diphosphate kinase (nm23), menin may regulate a GTPase activity (Yaguchi *et al.*, 2002).

Menin-ASK: The activator of S-phase kinase (ASK) is a component of the cell division cycle (CDC) kinase complex, crucial for cell proliferation, and it interacts with menin. Menin may inhibit the ASK-induced cell proliferation *in vivo* (Schnepp *et al.*, 2004).

Menin- Glial Fibrillary Acidic Protein (GFAP): Menin interacts with intermediate filament proteins, such as GFAP and vimentin. Menin and GFAP colocalize at the S–G_2 phase of the cell cycle in glioma cells. Such an interaction might serve as a cytoplasmic sequestering network for menin at the S and early G_2 phase of the cell cycle (Huang *et al.*, 1999). Menin could have an inhibitory role before the S phase starts, and it must be transferred to the cytoplasm to enable the S phase to proceed (Suphapeetiporn *et al.*, 2002; Lin *et al.*, 2003). Thus, the intermediate filament network could sequester menin away from the nucleus and its target genes (Lopez-Egido *et al.*, 2002).

Menin-Jun D: Menin interacts in several different test systems directly with JunD, a member of the AP1 family of transcription factors- repressing junD's transcriptional activity (Agarwal *et al.*, 1999). Studies have demonstrated that menin binds directly to the full-length form of JunD (FL-JunD) in normal conditions converting JunD to a growth suppressor, whereas junD acts as a growth promoter when it is unable to bind menin (Yazgan *et al.*, 2001; Agarwal *et al.*, 2003). JunD's conversion to an oncogene might be a component of MEN1-associated tumorigenesis.

Menin-MLL: Immunoprecipitation of menin showed that menin could be associated with several proteins in a large complex. The components of the human complex are highly homologous to the components of a yeast transcriptional complex, termed COMPASS (Hughes *et al.*, 2004). Interestingly, menin has no homologue in yeast. Menin's direct interaction in the human complex seems to be with MLL1 or MLL2. MLL1 has been studied in detail as the Mixed Lineage Leukemia protein that undergoes rearrangement as the cause in many leukemias. Menin's interaction with MLL1 in hematopoiesis or leukemogenesis seems to be as a growth promoter; in this mode, it does not account for growth suppression in the MEN1 process (Yokoyama *et al.*, 2005). However, the MLL1 complex can also act on the promoter of the *p18* and *p27* genes, where its expression of those genes results in growth suppression (Milne *et al.*, 2005).

Menin-Transforming Growth Factor β (TGFβ): The role of TGFβ in tumorigenesis is complex. It can stimulate tumorigenesis, causing tumor cell invasion and metastasis, whereas it generally causes growth inhibition in normal cells, including epithelial, endothelial, and fibroblastic cells. Activation of the TGFβ receptor stimulates transcription factors of the Smad family, which transfer its effects to the nucleus. TGFβ increases the expression of menin in a dose-dependent fashion; conversely, a reduced menin interferes with TGFβ-mediated inhibition of cell proliferation in endocrine cells (Kaji *et al.*, 2001). TGFβ exerts growth inhibitory and transcriptional responses through Smad2 and Smad3, which associate with the common mediator Smad4 after receptor-mediated phosphorylation of several substrates. Translocation of this complex into the nucleus leads to the increased expression of specific target genes. Menin has been found to physically interact with Smad3, and impaired menin function blocks the Smad3-mediated transcriptional effects of TGFβ (Kaji *et al.*, 2001). Impaired TGFβ signaling might disrupt the balanced cellular steady state, pushing the cells toward inappropriate growth and tumor formation.

Menin-Insulin-like Growth Factor Binding Protein 2 (IGFBP-2): Menin can also control proliferation through the suppression of endogenous IGFBP-2, which inhibits cell proliferation induced by IGFs and by TGFβ (La *et al.*, 2004b). Menin-mediated suppression of IGFBP-2 is, at least in part, executed through alteration of the chromatin structure of the IGFBP-2 gene promoter (La *et al.*, 2004b). La *et al.* (2006) recently showed that subtle mutations in menin NLSs compromise the ability of menin to repress expression of the IGFBP-2 gene.

Menin-Fanconi Anemia Complementation Group D2 (FANCD2) Protein: Menin interacts with *FANCD2*, one of the seven mutated genes in Fanconi anemia. FANCD2 is involved in a BRCA1-mediated DNA repair pathway. The interaction between menin and FANCD2 is enhanced by γ-irradiation and may be regulated by phosphorylation, which further enhances the function of these proteins in DNA repair (Jin *et al.*, 2003). Interestingly, past studies revealed that lymphocytes from patient with heterozygous *MEN1* mutation exhibit a premature centromere division, thus suggesting a possible role of menin in controlling DNA integrity (Sakurai *et al.*, 1999). Moreover, hypersensitivity to alkylating agents occurred in lymphocytes from patients with MEN1 (Itakura *et al.*, 2000) indicating a possible role of menin as a negative regulator of cell proliferation after one type of DNA damage (Ikeo et al., 2000).

Menin-Replication Protein A (RPA): Menin interacts with the second subunit of the RPA complex, which is required for DNA replication, recombination, and repair and is involved in the regulation of apoptosis and gene expression (Sukhodolets et al., 2003).

Menin-Nuclear Factor κB (NFκB): Menin interacts specifically with three members of the NFκB family (Heppner et al., 2001). These transcription factors are major regulators of the cellular response to stress. Menin acts as an inhibitor of NFκB-mediated transcriptional activation in a large mediator complex to repress or recruit other repressors, such as histone deacetylases.

Menin Interactions with Chromatin and Genes

Most menin-interacting proteins may imply a role for menin in the regulation of gene expression, and menin may have downstream effects on critical genes regulating normal and tumor processes. Genes regulated by menin overexpression have been identified by DNA microarray analysis in a human endocrine pancreatic cell line (BON1) transfected with vector pcDNA3 alone or with the *MEN1* gene (Stalberg et al., 2004). Menin upregulates procaspase 8 and downregulates IGFBP-2. DNA microarray comparison between independently derived strains of *Men1+/+* and *Men1–/–* mouse embryos showed the expected decreased expression of *Men1* gene itself in the menin-null cells and a decreased expression of Hoxc6, Hoxc8, and Cyt19 (Yokoyama et al., 2004). The decreased expression of the Hox genes observed in the *Men1* knockout is similar to that in the *MLL* knockout (Milne et al., 2002). Indeed, menin could be a positive regulator of MLL-associated H3K4 methyltransferase activity, required for the expression of the Hox genes. By chromatin immunoprecipitation experiments, menin and MLL were found to occupy the homeobox gene locus at Hoxc8, and menin overexpression was found to upregulate Hox gene expression (Hughes et al., 2004). On the other hand, menin knockdown produced downregulation of Hoxa9 protein expression (Yokoyama et al., 2004).

Through a general genetic screen for negative regulators of hTERT, menin was identified as a direct repressor of hTERT (Lin and Elledge, 2003).

Chromatin immunoprecipitation (ChIP) studies revealed that menin interacts with thousands of sites in chromatin. Most were in promoters, but significant numbers were elsewhere (Scacheri et al., 2006). A separate ChIP study analyzed chromatin with an independent method, termed SACO. Again hundreds of interaction sites were found, and many were in the 3′ end of genes and in introns (Agarwal et al., 2006). A specific menin binding sequence in DNA was not found in either study, suggesting that the many menin interactions with chromatin were indirect.

Menin Functions in Endocrine Tumor Tissues

Parathyroid Tumors

In the parathyroid cell from sporadic primary and secondary HPT, menin mRNA levels have been reported to be increased or not modified, whereas in MEN1-associated PHPT they are reduced (Bhuiyan et al., 2000). As mentioned earlier, TGFβ is an interacting partner of menin and it is a crucial negative regulator of both parathyroid cells proliferation and PTH secretion. Consequently, the loss of TGFβ-dependent signaling secondary to menin inactivation may substantially contribute to parathyroid tumorigenesis in MEN1, favoring proliferation and PTH secretion and production of parathyroid affected cells (Sowa et al., 2004b).

Pancreatic Tumors

Overexpression of menin in rat insulinoma cells inhibits their function and proliferation, via inhibition of promoter activity of the genes for insulin and others and increase of cell apoptosis. Menin expression significantly enables the inhibition of either the insulin promoter activity or glucose-dependent insulin secretion (Sayo et al., 2002).

More recently, it has been hypothesized that menin mediates its tumor suppressor action by regulating histone methylation, particularly of H3K4, in promoters of HOX genes and/or *p18*, *p27*, and possibly other CDK inhibitors (Milne et al., 2005; Karnik et al., 2005). Consistent with this hypothesis, H3 K4 methylation and expression of p18 and p27 were shown to be dependent on menin in pancreatic islets (Karnik et al., 2005).

Scacheri et al. (2006) identified the developmentally programmed transcription factor HLXB9, overexpressed in islets in the absence of menin, as a molecular partner normally bound by menin only in islets, and not in HeLa or HepG2 cells. These data raise the possibility for a specific bias for endocrine tumor formation in MEN1 resulting from changes in expression in distinct genes, such as *HLXB9*, specifically targeted by menin in endocrine tissues.

Pituitary Tumors

Although *MEN1* mutation has a limited role in the development and progression of sporadic pituitary tumors, its role in pituitary tumorigenesis is supported by the presence of pituitary tumors in MEN1 syndrome (Farrell and Clayton, 2000; Satta et al., 1999). Recent studies have elucidated several interactions between pituitary-specific pathways and menin. Activin, a member of the TGFβ superfamily, is a negative regulator of pituitary cell growth and of PRL, of ACTH, and of GH secretion (Hendy *et al.,* 2005). In addition, activin suppresses the transcription and expression of Pit-1, a pituitary transcription factor that has an essential role in the development and maintenance of lactotrope cells and in the regulation of PRL and GH expression (Lacerte *et al.,* 2004). The Smad pathway and menin are key regulators of activin effects, and menin is required for activin-mediated inhibition of PRL and GH expression, and this effect is mediated by Pit-1 gene expression and stability (Namihira *et al.,* 2002; Lacerte *et al.,* 2004).

Nonendocrine Functions of Menin

Roles have been suggested for menin other than in endocrine tumorigenesis. In particular, menin regulates physiological aspects in both bone development and hematopoiesis.

Bone Development

A newly recognized function of menin is the regulation of the differentiation of multipotential mesenchymal stem cells into osteoblastic cells (Sowa *et al.,* 2004). Bone morphogenetic proteins (BMPs) and TGFβ, members of the TGF superfamily, are crucial regulators of bone formation; the interactions of menin with the BMP-2 signaling pathway and Runx2 may differ depending on the osteoblast differentiation stage. Menin promotes the commitment of multipotential mesenchymal stem cells into the osteoblastic lineage through interactions with Smad1 to Smad5 and Runx2, whereas after the commitment to the osteoblast lineage the interaction of menin and Smad3 inhibits osteoblast differentiation by negatively regulating the BMP-2–Runx2 cascade (Sowa *et al.,* 2004a). Furthermore, JunD enhances both the expression of osteoblastic differentiation markers (i.e., Runx2, type 1 collagen, osteocalcin, and bone alkaline phosphatase) and bone mineralization, and menin could influence osteoblastic differentiation through JunD interactions (Naito *et al.,* 2005).

Hematopoiesis

Menin, as part of an MLL-containing complex, has been shown to interact with H3K4 methyltransferase activity of MLL, to regulate Hox gene expression *in vitro*. Menin activates Hoxa9 expression, at least in part, by binding to the Hoxa9 locus. Excision of the *MEN1* gene gradually decreased the total white blood cell count but did not significantly reduce red blood cell numbers (Chen *et al.,* 2006). Thus, menin may have a role in the maintenance of the steady-state peripheral white blood cell count (Chen *et al.,* 2006), but it remains to be delineated what determines the tissue-specific activities of menin and whether the menin–MLL–Hox pathway has a role in suppressing tumorigenesis in endocrine organs.

ANIMAL MODELS

In the mouse, it has been reported that the *Men1* gene is generally transcribed early (7 days postcoital), whereas at a later gestational stage, its expression is more readily found in tissues such as brain, thymus, and liver (Stewart *et al.,* 1998). Homozygous deletion of *Men1* is lethal *in utero*, and such embryos exhibit delayed development with defects of multiple organs, including the neural tube, heart, liver, and cranial and facial development (Crabtree *et al.,* 2001; Bertolino *et al.,* 2003). These findings strongly suggest that *Men1* gene plays a critical role in the development of multiple organs, in addition to its proposed role in the tumorigenesis of endocrine tissues in MEN1 patients. Recently, generation of homozygously deleted *Men1* gene in liver tissue of the mouse, considered not normally predisposed to developing tumors in both humans or mice with heterozygous *MEN1* mutation, revealed that menin-null livers appeared entirely normal, remaining tumor-free until late adulthood. These results indicate the need for more understanding of tissue specificity in MEN1 tumors (Scacheri *et al.,* 2004).

Inactivation of certain other tumor suppressor genes alone or in combination can cause specific endocrine tumors in mice. In particular, mice with simultaneous homozygous knockout of two genes, $p18^{INK4c}$ and $p27^{KIP1}$, develop tumors of parathyroid, pituitary, pancreas islet, and duodenum (like MEN1). They also develop C-cell cancers and pheochromocytoma (like MEN2; Franklin *et al.,* 2000). A spontaneous strain of rat shows a similar spectrum of tumors caused by homozygous mutation of *p27* (Pellegata *et al.,* 2006). And a human family was shown to express MEN1 caused by a heterozygous p27 mutation (Pellegata *et al.,* 2006). The knocked-out *p18* and *p27* genes encode members of the two cyclin-dependent kinase inhibitor families that participate in the G1 phase of the cell cycle, a phase that also includes retinoblastoma and cyclin D1.

This syndromic resemblance raises the possibility that the pathways of MEN1 and/or MEN2 interact with the cell cycling pathway and perhaps with each other.

The mouse *Menl* gene is highly homologous to *MEN1* (Guru *et al.,* 1999). Heterozygous knockout of *Men1* in the mouse results in a promising model of MEN1 (Crabtree *et al.,* 2000). Those mice develop tumors of the parathyroids, pancreatic islets, and pituitary. Unlike the ACTH-secreting pituitary intermediate lobe tumors with G1 phase tumor suppressor gene knockouts, these pituitary tumors are prolactinomas. The mouse tumors are associated with LOH at the mouse *Menl* locus. Crosses of these mice with mice showing other gene knockouts have indicated that

Men1–/+ does not synergize with Rb1– or p27=, but there is synergy in tumorigenesis with p18= (Bai *et al.,* 2006; Loffler *et al.,* 2006). This suggests that *MEN1* is in a pathway, which is linear with *p27* and *Rb1* but not with *p18*.

CONCLUSION

The discovery of the *MEN1* gene has led to changes in clinical management and to new insights about normal and abnormal functions. More studies are necessary to clearly elucidate the molecular mechanisms underlying MEN1-associated tumorigenesis.

New tools are in place to answer many questions shortly. Similarly, there are prospects for novel treatments based upon DNA, RNA, or even other small molecules.

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Chapter 58

Prostaglandins and Bone Metabolism

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INTRODUCTION

In the 37 years since prostaglandin E_2 (PGE_2) was first shown to stimulate cyclic AMP production and resorption in bone organ cultures (Klein and Raisz, 1970), ample evidence has accumulated to demonstrate that prostaglandins (PGs) and other eicosanoids have important physiologic and pathologic roles in skeletal metabolism. PGs are autocrine-paracrine (or local) factors. They are not stored but are synthesized and released as needed and rapidly metabolized in their passage through the lung. PG production is regulated by many systemic hormones and other local factors involved in bone metabolism, and PGs may function to integrate or amplify responses to these agents at the cellular level. Understanding the role of PGs in skeletal metabolism has been complicated because they act locally and transiently, they are regulated at multiple levels, and they have multiple receptors. Moreover, dual effects can be demonstrated for PGs in most test systems *in vitro*. This chapter will summarize current knowledge on the regulation of PG production in bone and the effects of PGs and other eicosanoids on bone resorption and formation.

PG PRODUCTION

Metabolic Pathways

Eicosanoids are derived from 20-carbon polyunsaturated fatty acids (PUFAs). PUFAs result from metabolism of the dietary omega (ω)6 (or n-6) and ω3 (or n-3) essential fatty acids, linoleic acid (C18:2ω6) and linolenic acid (C20:3ω3) (Min and Crawford, 2004). The shorthand for fatty acids, X:YωZ, refers to X carbon atoms and Y double bonds, whereas Z is the position of the first double bond counting from the terminal methyl (CH_3) group. There are three major families of eicosanoids (Fig. 1): (1) prostanoids (PGs and thromboxane), synthesized via the cyclooxygenase pathway; (2) leukotrienes, lipoxins, and hydroxy-fatty acids, synthesized via various lipoxygenase pathways (5-, 12-, and 15-lipoxygenase); and (3) epoxy and omega derivatives, synthesized via the cytochrome P-450-dependent epoxygenase pathway (Capdevila *et al.*, 2000; Murphy *et al.*, 2004; Smith, 1989). Although important functions are being proposed for a rapidly growing number of metabolites generated by these pathways, the prostanoids and leukotrienes are still considered the most important biologically active eicosanoids, and they are the ones most studied in bone.

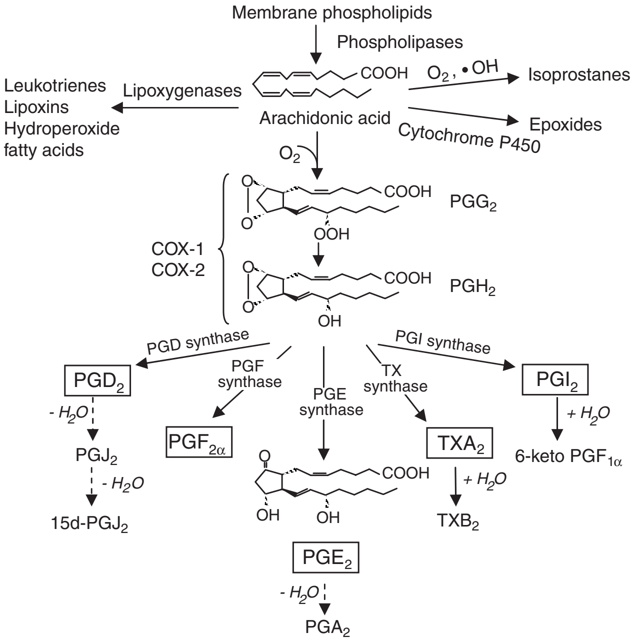
Membrane phospholipids
Phospholipases
COOH
O_2, •OH
Isoprostanes
Leukotrienes
Lipoxins
Hydroperoxide fatty acids
Lipoxygenases
Arachidonic acid
Epoxides
Cytochrome P450
O_2
COOH
PGG_2
OOH
COX-1
COX-2
COOH
PGH_2
OH
PGD synthase
PGI synthase
PGD_2
PGF synthase
TX synthase
PGI_2
PGE synthase
- H_2O
+ H_2O
PGJ_2
$PGF_{2\alpha}$
TXA_2
6-keto $PGF_{1\alpha}$
COOH
- H_2O
+ H_2O
15d-PGJ_2
OH
OH
TXB_2
PGE_2
- H_2O
PGA_2

FIGURE 1 Major families of eicosanoids derived from arachidonic acid. The family of prostanoids (prostaglandins and thromboxane) is generated via the bifunctional cyclooxygenase (COX) enzyme, previously called prostaglandin G/H synthase (PGHS), which converts arachidonic acid to PGG_2 and PGG_2 to PGH_2. Specific synthases subsequently convert PGG_2 to various prostanoids. Other major families are (1) leukotrienes, lipoxins, and hydroxy fatty acids, (2) isoprostanes, and (3) products of the cytochrome P450–dependent epoxygenase pathway.

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The lipid-soluble acid that was originally identified in seminal fluid as an activity causing muscle contraction or relaxation was named "prostaglandin" because it was thought to originate from the prostate gland (von Euler, 1936). PGs have now been shown to be produced in most tissues and cells except red blood cells. PGs derived from different substrates differ by the number of double bonds and by the substituents on the cyclopentane ring. The predominant PUFA substrates for cyclooxygenase in humans are dihomo-γ-linolenic acid (C20:3ω6), which gives rise to the 1 (1 double bond)-series of PGs; arachidonic acid (AA; C20:4ω6), which gives rise to the 2-series of PGs; and eicosapentaenoic acid (EPA; C20:5ω3), which gives rise to the 3-series of PGs (Wilson, 2004).

PGs of the 1-series, such as PGE_1, have not yet been shown to play an important role in normal physiology, but PGE_1 and synthetic analogues have been used to maintain patency of the ductus arteriosus in neonates with ductus dependent congenital heart defects (Talosi *et al.*, 2004) and to treat arterial occlusive disease and erectile dysfunction (Creutzig *et al.*, 2004; Ivey and Srivastava, 2006; Urciuoli *et al.*, 2004). Some reports have suggested that the PGE_1 analogue, misoprostol, originally developed as therapy for prevention of gastric ulceration, may prevent bone loss of estrogen depletion (Sonmez *et al.*, 1999; Yasar *et al.*, 2006). EPA, a constituent of fish oil, gives rise to the 3-series PGs, such as PGE_3. The ratio of EPA/AA in tissue phospholipids reflects the dietary ratio and in Western diets is less than 0.1, but can be increased by ingestion of fish oil (Wada *et al.*, 2007). AA is the major precursor in most mammalian systems. AA gives rise to PGs of the 2-series, the most important of the physiologically active PGs and the PGs most studied in bone, because they are highly produced by osteoblastic cells and can have marked effects on both bone resorption and formation.

The production of PGs involves three major steps, which are similar for the 1, 2, and 3 series of PGs. Steps for the 2-series of PGs, shown in Figure 1, are (1) hormone- or stress-activated mobilization of AA; (2) conversion of AA to the unstable endoperoxide intermediates, prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2); and (3) conversion of PGH_2 by terminal synthases, which have some tissue specificity, to PGE_2, PGD_2, $PGF_{2\alpha}$, prostacyclin (PGI_2), and thromboxane (TXA_2). The committed step in the conversion of AA to PGs, and the step inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs), is catalyzed by a bifunctional enzyme that converts free AA to PGG_2 in a cyclooxygenase reaction followed by reduction of PGG_2 to PGH_2 in a peroxidase reaction (Smith and Song, 2002). This enzyme, formally named prostaglandin endoperoxide H synthase or prostaglandin G/H synthase (PGHS), is popularly called cyclooxygenase (COX) in reference to its first function. The gene name is *ptgs*. All three steps are subject to transient activation, and it is not possible to make a simple conclusion as to which of these steps is "rate-limiting" for prostanoid production under all conditions because all three steps can be limiting.

PGI_2 and TXA_2 are spontaneously inactivated by hydrolytic conversion to 6-keto $PGF_{1\alpha}$ and TXB_2, respectively (see Fig. 1). In fluids containing albumin, PGE_2 and PGD_2 are slowly dehydrated within the cyclopentane ring to the cyclopentenone prostaglandins PGA_2 and PGJ_2, respectively. PGJ_2 is metabolized further to yield 15-deoxy-Δ^{12-14} PGJ_2 (15d-PGJ_2). The cyclopentenone PGs, which are not thought to act via the classical PG receptors, are active when given exogenously and can have opposite effects from some of the primary PGs (Negishi and Katoh, 2002; Straus and Glass, 2001). However, their physiological importance is unclear.

Phospholipase A_2 Enzymes

Phospholipase A_2 (PLA_2) enzymes catalyze the hydrolysis of membrane phospholipids from membrane glycerophospholipids, releasing free fatty acids, such as AA and lysophospholipids. There are multiple members of the PLA_2 superfamily, which is currently divided into 15 groups and many subgroups (Schaloske and Dennis, 2006). There are five distinct types of enzymes, and two of these, cytosolic PLA_2 ($cPLA_2$) and secreted PLA_2 ($sPLA_2$), are important for PG production (Murakami and Kudo, 2004). The $cPLA_2$s are large proteins ranging in size from 61 to 114 kDa. The most important $cPLA_2$ for PG production is thought to be $cPLA_{2\alpha}$ (85 kDa), which is selective for AA at the *sn-2* position. $cPLA_{2\alpha}$ is constitutively expressed in most cells, but its expression can be regulated to some degree. For example, PGs themselves can enhance both COX-2 and $cPLA_2$ expression (Murakami *et al.*, 1997), and IL-4 can inhibit both COX-2 and $cPLA_2$ expression (Kawaguchi *et al.*, 1996). However, the activation of $cPLA_{2\alpha}$ is essential for initiation of stimulus-coupled AA release (Murakami and Kudo, 2004). The initial step in activation is the Ca^{2+} (submicromolar concentrations)-dependent translocation of $cPLA_{2\alpha}$ from the cytosol to the nuclear/endoplasmic reticulum membrane where the COX enzymes reside. Maximal activation of $cPLA_{2\alpha}$ requires phosphorylation of two serine residues in the catalytic domain by the mitogen-activated protein kinase (MAPK) pathway. Because $cPLA_{2\alpha}$ is thought to be the major enzyme for releasing AA, it is not surprising that phenotypes of $cPLA_{2\alpha}$ knockout mice overlap the phenotypes of mice with targeted disruption of COXs in a number of respects (Murakami and Kudo, 2004). The skeletal phenotypes of these knockouts have not been reported.

Secretory PLA_2s ($sPLA_2$s) are small secreted proteins (14–18 kDa) that require millimolar Ca^{2+} concentrations for activation and do not have a distinct fatty acid selectivity. Expression of $sPLA_2$ enzymes is induced by proinflammatory agents, and they appear to play a role in inflammatory diseases and innate immunity (Schaloske and Dennis, 2006). $sPLA_2$s can act in an autocrine or paracrine manner (Murakami and Kudo, 2004). $sPLA_2$s can hydrolyze phospholipids in the outer plasma membrane of the cell of origin or neighboring cells, releasing AA that can be incorporated into cells, or $sPLA_2$s can be internalized, perhaps via caveolae/raft dependent vesicular pathways, and release AA from endoplasmic reticulum/perinuclear membranes (Murakami and Kudo, 2004). There are at least 10 mammalian $sPLA_2$s. The group IIA (GIIA) and V (GV) enzymes are thought to be major $sPLA_2$ contributors to PG production (Bingham *et al.*, 1999; Kuwata *et al.*, 1999). GV PLA_2 has been reported to be the primary $sPLA_2$ in the mouse (Sawada *et al.*, 1999). Some mouse strains, such as 129Sv and C57Bl/6, naturally lack type GIIA PLA_2 but develop normally (Kennedy *et al.*, 1995). However, they do have increased resistance to colorectal tumorigenesis (MacPhee *et al.*, 1995). It is interesting to note that many knockouts of PLA_2s or other enzymes in the PG pathway are in a C57Bl/6 or 129Sv background and, hence, they are really double knockouts (Schaloske and Dennis, 2006). It should also be noted that the MC3T3-E1 osteoblastic cell line, commonly used to study osteoblastic function and differentiation *in vitro*, was derived from C57Bl/6 mice and should therefore lack GIIA PLA_2.

Two Isoforms for COX

The two enzymes for COX, COX-1 and COX-2, are encoded by separate genes. The human COX-1 gene is located on chromosome 9 and the human COX-2 gene is located on chromosome 1. COX-1 is expressed at relatively stable levels in most tissues and is considered to be constitutive, whereas COX-2 is generally expressed at very low basal levels in most tissues but can be induced to high levels by multiple factors (Herschman, 1994; Smith *et al.*, 2000; Tanabe and Tohnai, 2002). COX-2 was initially identified as a phorbol ester-inducible primary response or immediate-early gene (TIS10) in murine 3T3 cells (Kujubu *et al.*, 1991) and a v-*src*-inducible gene product in chicken fibroblasts (Xie *et al.*, 1991). The human COX-2 gene is about 80% homologous with the chicken and murine genes (Hla and Neilson, 1992). The COX-1 gene is approximately 22 kb with 11 exons and 10 introns, and it encodes 2.8 kb mRNA (Yokoyama and Tanabe, 1989). The COX-2 gene is approximately 8 kb with 10 exons and 9 introns and encodes 4.0 to 4.6 kb mRNA (Fletcher *et al.*, 1992; Kraemer *et al.*, 1992). A major difference between COX-1 and COX-2 mRNAs is the 2 kb 3′-untranslated region (UTR) of COX-2 that contains multiple copies of AUUUA sequences, which have been associated with rapid degradation of mRNA. COX-1 and COX-2 are about 80% identical at both amino acid and nucleic acid levels, differing mainly at the N- and C-terminal ends (Herschman, 2004; Kang *et al.*, 2007; Tanabe and Tohnai, 2002). COX-2 lacks the first exon in the COX-1 gene and consequently has a shorter signal peptide region in the N-terminus. On the other hand, COX-2 contains an extra sequence in the C-terminal region encoding a 19-amino-acid insert involved in protein degradation (Mbonye *et al.*, 2006).

Both COX-1 and COX-2 proteins are homodimeric (approximately 68–72 kDa/subunit) heme-containing glycoproteins (Kang *et al.*, 2007; Kulmacz *et al.*, 2003; Mbonye *et al.*, 2006; Smith and Song, 2002). Although some early studies suggested differences in cellular locations of the two enzymes, later studies confirmed that both enzymes are located similarly in the luminal surfaces of the endoplasmic reticulum and the contiguous inner membrane of the nuclear envelope (MirAfzali *et al.*, 2006; Spencer *et al.*,1998, 1999). Unlike many membrane proteins, COX-1 and COX-2 do not have a transmembrane domain and are anchored only to one leaflet of the lipid bilayer (monotopic) through the hydrophobic surfaces of amphipathic helices (Kulmacz *et al.*, 2003; Spencer *et al.*, 1999). Recent studies have indicated that a heterodimer containing an inactive COX-2 monomer associated with a native COX-2 monomer retains full PG synthesis capability (Yuan *et al.*, 2006). Other studies have suggested that COX-1 and COX-2 monomers may heterodimerize and produce PGs (Yu *et al.*, 2006).

COX-1 and COX-2 have essentially the same catalytic mechanisms (Kulmacz *et al.*, 2003; Rouzer and Marnett, 2005; Smith and Song, 2002; van der Donk *et al.*, 2002). The cyclooxygenase (COX) and peroxidase (POX) active sites are in distinct and physically separated regions of the proteins. The initiation of COX activity is dependent on heme oxidation in the POX site by peroxidase. Heme oxidation leads to oxidation of a critical tyrosine in the COX active site. The tyrosyl radical in the COX site converts AA to an arachidonyl radical, the arachidonyl radical reacts with two molecules of oxygen to produce PGG_2, and then PGG_2 diffuses to the POX site where it is reduced to PGH_2. Studies of mice developed with a targeted knock-in of a mutation of the critical tyrosine site in the COX active site, leaving the POX activity intact, suggest that the predominant features of the COX-2 knockout mice are due to the absence of COX activity (Yu *et al.*, 2007). NSAIDs bind at the COX site, competing with AA and leaving the POX activity intact. The COX active site is about 20% larger in COX-2 compared to COX-1 and has a slightly different shape due to several differences in amino acids, making it possible to develop NSAIDs selective for one or the other COX isoforms (Kulmacz *et al.*, 2003; Rainsford, 2004; Smith *et al.*, 2000). The "classical" NSAIDs can inhibit both COX-1 and COX-2 but generally bind more tightly to COX-1 (Smith *et al.*, 2000). Currently available selective COX-2 inhibitors are tight-binding, time-dependent inhibitors of COX-2 but only rapid, competitive, and reversible inhibitors of COX-1 (Smith *et al.*, 2000).

Splice variants of both COX-1 and COX-2 genes have been identified (Diaz *et al.*, 1992; Roos and Simmons, 2005; Shaftel *et al.*, 2003). A splicing variant of COX-1, arising via the retention of intron 1, was identified in canine tissues and called COX-3 (Chandrasekharan *et al.*, 2002). COX-3 was reported to be differentially sensitive to inhibition by acetaminophen. However, later studies concluded that this variant is unlikely to be a therapeutic target of acetaminophen and probably has little or no COX activity in humans (Davies *et al.*, 2004; Kis *et al.*, 2005, 2006; Qin *et al.*, 2005; Snipes *et al.*, 2005). Interestingly, recent studies suggest that acetaminophen is a COX-2 selective inhibitor. Acetaminophen is reported to inhibit COX-2 to a degree comparable to selective COX-2 inhibitors in a study of human volunteers (Hinz *et al.*, 2007). Acetaminophen may inhibit COX-2 at the level of the POX catalytic site, with inhibitory effects being inversely proportional to the ambient peroxide levels (Aronoff *et al.*, 2006).

Why Are There Two COX Enzymes?

Despite having similar catalytic mechanisms, COX-1 and COX-2 appear to be independently functioning biosynthetic pathways (Simmons *et al.*, 2004; Smith and Langenbach, 2001). This is due in part to the differential regulation of their expression. The COX-1 promoter has relatively few identified functional regulatory elements, and COX-1 mRNA is constitutively expressed in most tissues. The COX-2 promoter, on the other hand, has multiple potential transcriptional regulatory elements typical of early response genes, and COX-2 mRNA is rapidly and transiently inducible in many tissues (Kang *et al.*, 2007). Differences in the response of COX-1 and COX-2 gene expression are exemplified by their differential responses to serum in MC3T3-E1 cells (Pilbeam *et al.*, 1993). Differential protein stability also contributes to the difference in expression. COX-1 protein has a long half life of 12 to 24 hours or more, whereas COX-2 protein has a half life of 2 to 7 hours (Kang *et al.*, 2007; Mbonye *et al.*, 2006).

Differential regulation of expression cannot account for all differences. When COX-1 is inserted under the regulatory sequences that drive COX-2 expression, COX-1 can substitute for only some, not all, of the deficits seen with COX-2 disruption (Yu *et al.*, 2007). There are clearly differences in PG production by COX-1 and COX-2. Osteoblasts from COX-2 KO mice make little PGE_2 in culture despite the constitutive expression of COX-1, unless AA is added to cultures (Chikazu *et al.*, 2005; Choudhary *et al.*, 2003; Okada *et al.*, 2000a; Xu *et al.*, 2007b). One explanation is that COX-2 is much more efficient at using low endogenous AA concentrations (below $5\,\mu M$) than is COX-1 (Swinney *et al.*, 1997). A greater cellular peroxide level may be required for COX-1 activation compared to COX-2 (Kulmacz *et al.*, 2003). Cellular glutathione peroxidase capacities and glutathione levels are high enough in many tissues to keep COX-1 in the latent state, and cells generally have unused capacity for prostanoid production. It has been suggested that having an inducible COX isoform might provide the means to increase PG production without the cellular damage that might ensue if peroxide levels were increased enough to activate COX-1 (Kulmacz *et al.*, 2003).

In many cell types, there is an early burst of PG synthesis secondary to activation of PLA_2 that releases substrate for preexisting COX-1, followed by a later or "delayed" phase of PG production secondary to induction of COX-2 (Herschman, 2004). The delayed phase can also be autoamplified by the early production of PGs, which can stimulate COX-2 expression (Murakami *et al.*, 1997). An example of the early and late phases is seen in the PG production by osteoblasts subjected to fluid shear stress (Klein-Nulend *et al.*, 1997) or to IL-1 (Harrison *et al.*, 1994). It has been proposed that some differences in PG production arise because COX-1 and COX-2 are functionally coupled to different PLA_2s (Balsinde *et al.*, 1998; Reddy and Herschman, 1997). That relationship appears complex—dependent on cell type, ligand, and relative enzyme concentration—and it is probably prudent to say that both $cPLA_2\alpha$ and some $sPLA_2$s can supply AA for both COX-1 and COX-2 (Herschman, 2004).

Because of the differential responses of COX-1 and COX-2, it was initially hypothesized that COX-2 is predominantly responsible for acute pathological PG responses, such as those associated with inflammation and pain, whereas COX-1 produces prostanoids needed for ongoing "housekeeping" functions, including maintenance of renal blood flow, platelet aggregation, and gastric cytoprotection. This hypothesis led to the development of highly selective inhibitors of COX-2 activity, such as rofecoxib and celecoxib, for treatment of pain and inflammation associated with diseases such as arthritis and periodontitis. Studies using these inhibitors have led to the realization that the initial hypothesis was too simple and that COX-2 has physiologic as well as pathologic functions in multiple tissues.

Prostaglandin E Synthases

The terminal PG synthases may be differentially distributed in tissues and were originally thought to influence PG production largely by determining the predominant type of prostanoid synthesized in a particular tissue. However, it is now known that expression of some of them can be regulated by many factors. PGE_2 is the best studied PG in bone. Prostaglandin E synthase (PGES), which converts COX-derived PGH_2 to PGE_2 occurs in multiple forms. For recent reviews see Murakami and Kudo (2006) and Park et al. (2006). The membrane-bound glutathione-dependent PGES called mPGES-1, cloned by Jakobsson (Jakobsson et al., 1999) and later by Murakami (Murakami et al., 2000), is inducible, located in the endoplasmic reticulum and perinuclear membranes, and is the predominant PGES metabolizing the PGH_2 produced by COX-2 (Forsberg et al., 2000; Murakami and Kudo, 2006; Pettersson et al., 2005). For these reasons, and because mPGES-1 is coordinately regulated by factors that regulate COX-2, mPGES-1 is said to be coupled to COX-2. A cytosolic form of PGES, cPGES, is preferentially coupled to COX-1 and thought to maintain PGE_2 production for cellular homeostasis (Tanioka et al., 2000). cPGES may be regulated by being stimulated to translocate from the cytosol to the nuclear membrane to form a complex with COX-1 (Park et al., 2006). A third PGES, called mPGES-2, is constitutively expressed and may be functionally coupled to both COX-1 and COX-2 (Watanabe et al., 1997).

Mice deficient for mPGES-1 develop normally but have reduced inflammatory and pain responses (Kamei et al., 2004; Trebino et al., 2003). Lipopolysaccharide (LPS)-induced bone resorption and bone loss are reduced in mice deficient for mPGES-1 (Inada et al., 2006). Hence, this enzyme may be a target for drug therapy to prevent inflammatory bone loss that would leave the production of other prostanoids intact. One interesting aspect of targeting mPGES-1 instead of COX-2 is that inhibition of mPGES-1 may augment prostacyclin (PGI_2) production and therefore be associated with less cardiovascular side effects than COX-2 selective inhibitors (Wang et al., 2006a).

Isoprostanes

Although synthesis of eicosanoids is predominantly dependent on enzymatic metabolism of AA by COX-1 and COX-2, nonenzymatic free radical induced peroxidation of free or membrane-bound AA can give rise to natural products that are isomers of enzymatically derived PGs, called isoprostanes (IsoPs; Montuschi et al., 2004, 2007; Morrow, 2006; Pratico et al., 2004). In contrast to PGs generated enzymatically, the side chains of IsoPs are predominantly orientated *cis* relative to the cyclopentane ring. Some PG receptors may also be receptors for IsoPs. The most studied Iso-P group is F_2-IsoPs, a group of 64 compounds isomeric in structure to COX-derived $PGF_{2\alpha}$. Studies suggest that formation of F_2-IsoPs is altered in inflammation and other clinical settings associated with oxidative stress, and measurement of F_2-IsoPs may provide a tool for studying the role of oxidative stress in human disease (Milne et al., 2007). There has been one report of negative association of urinary 8-Iso-$PGF_{2\alpha}$ levels with bone mineral density (BMD) in humans (Basu et al., 2001), but it seems likely that urinary IsoP levels will reflect general, rather than specific, pathology. $PGF_{2\alpha}$ is known to be present in human urine in significant concentrations and was presumed to be derived from COX activity. Recently, however, it was reported that levels of putative $PGF_{2\alpha}$ in urine cannot be suppressed by NSAIDs and that $PGF_{2\alpha}$ in urine is likely to be derived via the IsoP pathway (Yin et al., 2007). IsoPs themselves may also be pathophysiological mediators of disease (Montuschi et al., 2004), although some of them, the cyclopentenone IsoPs, which are structural isomers of the bioactive cyclopentenone PGs, PGA_2 and PGJ_2, may be anti-inflammatory (Musiek et al., 2005). Free radicals have been reported to be involved in bone resorption, but little is known about the importance of IsoPs in bone. 8-Iso-PGE_2 has been reported to stimulate osteoclastogenesis (Tintut et al., 2002), but this may be due to nonenzymatic conversion of 8-Iso-PGE_2 to PGE_2 (Raisz, unpublished observations).

PG RECEPTORS

G-Protein Linked Receptors

There are at least nine G-protein linked PG receptors mediating prostanoid actions; (Fig. 2). There is a G-protein-coupled receptor (GPCR) for the F and I types of PGs and for thromboxane, called FP, IP, and TP, respectively. PGD_2 has two GPCRs, DP1 and the recently identified DP2, which belongs to the chemoattractant receptor family (Kostenis and Ulven, 2006). PGE_2 effects are associated with four classes of GPCRs, called EP1, EP2, EP3, and EP4.

Much of the complexity of PGE_2 effects on skeletal tissues may be attributable to the multiple transmembrane GPCRs for PGE_2 and their ability to activate different signaling pathways (Coleman et al., 1994; Kobayashi and Narumiya, 2002). Even more complex actions are possible if these receptors can undergo nuclear compartmentalization (Gobeil et al., 2003) or heterodimerize with other GPCRs (Barnes, 2006). The EP1 receptor acts largely by increasing calcium flux. Although often said to be coupled to $G\alpha_q$, the absence of a phosphatidylinositide response has led to speculation that it is coupled to an as yet unidentified G protein (Sugimoto and Narumiya, 2007). Both the EP2 and EP4 receptors are coupled to $G\alpha_s$ and can stimulate cyclic 3,5-adenosine monophosphate (cAMP) formation, but there may be a greater cAMP response to EP2

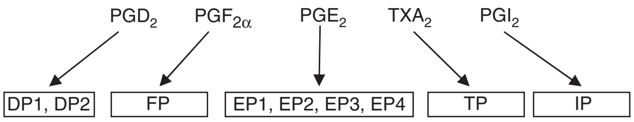

FIGURE 2 Prostaglandin and thromboxane receptors. There are at least nine G-protein-coupled receptors (GPCRs) mediating prostanoid actions. PGD_2 has two receptors, DP1 and the recently identified DP2, which belongs to the chemoattractant receptor family and is also called $CRTH_2$. PGE_2 effects are associated with four classes of GPCRs, called EP1, EP2, EP3, and EP4.

than to EP4 (Fujino *et al.*, 2005; Sakuma *et al.*, 2004). The EP_3 receptor is coupled to $G\alpha_i$ and acts largely by inhibiting cAMP production. However, there are multiple alternative transcripts of the EP_3 receptor that can act through other signal transduction pathways (An *et al.*, 1994). Mice deficient in each EP receptor subtype have been generated, and highly selective agonists for these receptors have been developed (Sugimoto and Narumiya, 2007).

EP2 and EP4 are the receptors most extensively studied in bone. Despite their importance in bone, much of what is known about EP2 and EP4 receptor signaling pathways comes from studies in non-bone tissues, designed to elucidate the role of PGE_2 in promoting tumorigenesis. These studies are summarized in several recent reviews (Castellone *et al.*, 2006; Cha and DuBois, 2007; Dorsam and Gutkind, 2007; Eisinger *et al.*, 2007; Wang and DuBois, 2006). Other data have come from studies of Chinese hamster ovary (CHO) cells or human embryonic kidney (HEK)-293 cells transfected with EP receptors (Fujino *et al.*, 2002; Nishigaki *et al.*, 1996). Potential signaling pathways by which PGE_2 may promote tumorigenesis are shown in Figure 3. EP2 and EP4 receptors stimulate $G\alpha_s$ and activate adenylyl cyclase (AC), producing cAMP and activating protein kinase A (PKA). The cAMP pathway is able to crosstalk with several other pathways that regulate cell growth, motility, migration and apoptosis. Interaction with the Wnt/β-catenin signaling pathway, resulting in increased expression of Wnt target genes, is one example (Castellone *et al.*, 2005; Shao *et al.*, 2005). Under basal conditions, β-catenin is targeted to a molecular "destruction" complex in the cytoplasm, which includes axin and glycogen synthase kinase-3β (GSK-3β). GSK-3β phosphorylates β-catenin, and phosphorylated β-catenin undergoes ubiquitin-dependent degradation. On stimulation of the EP2 or EP4 receptor, PKA may inhibit ubiquitination of β-catenin, leading to the stabilization and nuclear translocation of β-catenin (Hino *et al.*, 2005). In addition, $G\alpha_s$ subunits may bind the regulator of G-protein signaling (RGS) domain of axin, which releases GSK-3β from its complex with axin. Free Gβγ subunits can stimulate Akt through activation of phosphatidylinositol 3-kinase (PI3K), and then Akt can phosphorylate and inactivate GSK-3β, leading to the stabilization and accumulation of β-catenin. Studies in HEK-293 cells stably transfected with EP receptors suggest that the activation of T-cell factor (Tcf)/β-catenin signaling may occur primarily through a PKA-dependent pathway for EP2 and through a PI3K-dependent pathway for EP4 (Fujino *et al.*, 2002).

PGE_2 has also been shown to activate the MAPK extracellular signal-regulated kinase (ERK) pathway in various non-bone cells via the EP4 receptor (Fujino *et al.*, 2003; Pozzi *et al.*, 2004; Rao *et al.*, 2007; Qian *et al.*, 2006; Wang *et al.*, 2005). Several potential pathways leading to ERK activation have been proposed (see Fig. 3). PGE_2 can

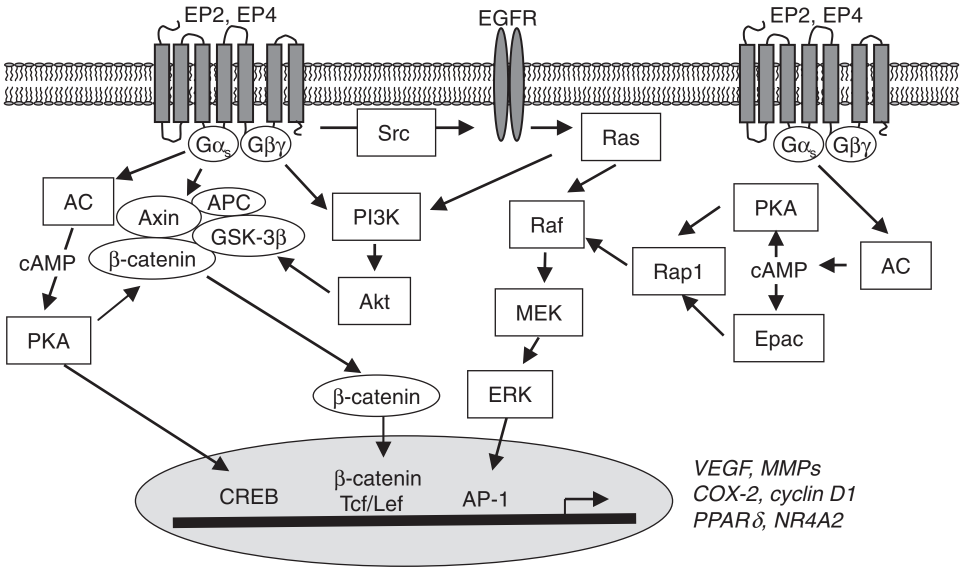

FIGURE 3 EP2 and EP4 receptor signaling pathways postulated to be involved in the promotion of tumorigenesis by PGE_2 (Cha and Dubois, 2007; Dorsam and Gutkind, 2007). EP2 and EP4 are $G\alpha_s$-coupled receptors that can activate adenylyl cyclase (AC), elevate cyclic AMP (cAMP), activate protein kinase A (PKA), and phosphorylate the cAMP response element binding (CREB) transcription factor. PGE_2 may also stimulate stabilization, nuclear translocation, and transcriptional activation of β-catenin through several pathways, including activation of the phosphatidylinositol 3-kinase (PI3K)–Akt pathway. Activation of extracellular signal-regulated kinase (ERK) leading to activation of activating protein-1 (AP-1) transcription factors, such as c-Fos and c-Jun, may occur via transactivation of epidermal growth factor receptor (EGFR) or via PKA-dependent or independent pathways. Downstream targets regulated by these pathways may include vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), peroxisome proliferator-activated receptor-δ (PPARδ), and NR4A2, a member of the Nurr1 orphan nuclear receptor family.

transactivate the epidermal growth factor receptor (EGFR) signaling pathway by a number of mechanisms, including proteolytic release of EGFR ligands, such as amphiregulin or transforming growth factor-α (TGF-α) (Buchanan *et al.*, 2003; Pai *et al.*, 2002; Shao *et al.*, 2003). Although little studied in response to PGE_2, cAMP is also capable of activating Rap1, a small guanosine triphosphatase (Gesty-Palmer *et al.*, 2006; Stork and Dillon, 2005). This activation can occur through a PKA-dependent pathway or a PKA-independent pathway mediated by the guanine exchange factor called EPAC (exchange protein directly activated by cAMP) (Fujita *et al.*, 2002; Gerdin and Eiden, 2007; Li *et al.*, 2006c). cAMP can stimulate proliferation in some cells and inhibit in others, associated with stimulation and inhibition, respectively, of ERK (Faure and Bourne, 1995; Stork and Schmitt, 2002). The ability to stimulate ERK depends on the expression of B-Raf, a MAPK kinase target of Rap1 (Fujita *et al.*, 2002; Stork and Dillon, 2005; Wang *et al.*, 2006b). In cells that do not express B-Raf, Rap1 antagonizes Ras-dependent ERK activation.

It is possible that EP2 or EP4 receptors can change signaling specificity (G-protein "switching"), similar to β-adrenergic GPCRs (Martin *et al.*, 2004). In HEK-293 cells stably transfected with EP2 or EP4 receptors, EP4 but not EP2 can couple to a pertussis toxin sensitive G-protein ($G\alpha_i$) that can activate ERK signaling and inhibit cAMP-dependent signaling (Fujino and Regan, 2006). Alternatively, GPCRs coupled to $G\alpha_s$, including the PTH/PTH-related peptide receptor, have been shown to activate ERK via G-protein independent pathways involving β-arrestin (Gesty-Palmer *et al.*, 2006; Shenoy and Lefkowitz, 2005) and c-Src (Sun *et al.*, 2007). Such G-protein independent pathways have not yet been associated with EP receptors but might explain some of the biphasic effects of PGE_2.

Some of the downstream targets identified as being upregulated by PGE_2 in studies examining the PGE_2 promotion of carcinogenesis may also be important for both normal bone metabolism and metastatic bone disease (Castellone *et al.*, 2006; Cha and DuBois, 2007). Among these targets are angiogenic factors, such as vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), COX-2 itself, and NR4A2 (a member of the Nurr1 orphan nuclear receptor family). NR4A2 has been shown to be expressed in osteoblasts and to stimulate osteoblastic differentiation (Holla *et al.*, 2006; Lee *et al.*, 2006; Pirih *et al.*, 2005).

EP receptors are expressed in both osteoblastic and osteoclastic cells. EP3 and EP4 were demonstrated in human fetal and adult osteoblasts by immunolocalization (Fortier *et al.*, 2004). Studies using Northern blot analysis and reverse transcription polymerase chain reaction (RT-PCR) showed EP1, 2, and 3 receptors in rat calvarial osteoblasts (Kasugai *et al.*, 1995) and EP 1, 2, and 4 receptors in murine osteoblastic MC3T3-E1 cells (Kasugai *et al.*, 1995; Suda *et al.*, 1996). A study in human osteosarcoma cells using real-time RT-PCR reported expression of all four EP receptors (Shoji *et al.*, 2006). EP3 and EP4 receptors have been reported in mature human osteoclasts (Fortier *et al.*, 2004; Sarrazin *et al.*, 2004). Both EP2 and EP4 receptors appear to be involved in regulation of osteoblast function, including the ability of these cells to support osteoclast formation, based on experiments with KO animals and selective agonists, which are described later. EP receptors may also be important in chondrocytes. All four EP receptors have been shown to be expressed in growth plate chondrocytes, but the EP1 receptor pathway appears to be the major EP pathway involved in regulating proliferation and differentiation of these cells (Brochhausen *et al.*, 2006; Sylvia *et al.*, 2001).

Although less studied, receptors for other prostanoids are also expressed in osteoblasts. Studies have shown IP receptor expression in MC3T3-E1 cells (Wang *et al.*, 1999) and cultured primary human osteoblastic cells (Sarrazin *et al.*, 2001). IP receptors have also been demonstrated by immunolocalization in human bone (Fortier *et al.*, 2001). Cultured human osteoblasts have been shown to produce PGD_2 and to have DP1 and DP2 receptors (Gallant *et al.*, 2005; Samadfam *et al.*, 2006). In MC3T3-E1 osteoblastic cells, PGD_2 and PGI_2 can induce COX-2 expression (Pilbeam *et al.*, 1994). Agonists for IP, DP, and TP receptors have also been shown to induce COX-2 expression in primary murine calvarial osteoblasts (Sakuma *et al.*, 2004). A functional TP receptor was demonstrated in primary human osteoblasts (Sarrazin *et al.*, 2001). The FP receptor is reported to be expressed in primary human osteoblastic cells (Sarrazin *et al.*, 2001). There is also evidence for an FP pathway in rodent clonal osteoblastic cells, which may act through a protein kinase C (PKC) pathway to induce COX-2 (Pilbeam *et al.*, 1994), inhibit collagen synthesis (Fall *et al.*, 1994), and activate the MAPK pathway (Hakeda *et al.*, 1997).

Expression of EP receptors is likely to vary with stage of differentiation and with treatment regime. In bone marrow macrophage osteoclast precursors, which express all four EP receptors, EP2 and EP4 receptor expression is downregulated with maturation (Kobayashi *et al.*, 2005b). Studies in CHO cells stably overexpressing EP2 and EP4 receptors suggest that the EP4 receptor undergoes short term agonist-induced desensitization, whereas the EP2 receptor is desensitized in response to prolonged agonist exposure (Nishigaki *et al.*, 1996).

Peroxisome Proliferator-Activated Receptors (PPARs)

PPARs are members of the nuclear receptor family of transcription factors that are important in lipid metabolism and may have a variety of effects on cellular function.

Three major subtypes, α, γ, and δ (also called β), have been described (Bishop-Bailey and Wray, 2003; Hihi *et al.*, 2002). Upon ligand binding, PPARs form heterodimeric complexes with retinoid X receptors and bind to PPAR response elements, attracting coactivators and other proteins, to mediate gene transcription.

PGs have been suggested to be endogenous ligands for PPARs. PPARγ and PPARδ are expressed in human osteoblastic cells (Maurin *et al.*, 2005), and PPARδ may be expressed by mature osteoclasts (Mano *et al.*, 2000a). PGI_2 is proposed to be an endogenous ligand for PPARδ, and PPARδ may be the receptor mediating effects of PGI_2 on embryo implantation in the mouse (Lim and Dey, 2002). However, PPARδ knockout mice do not show an implantation defect (Ide *et al.*, 2003). Studies suggest that PPARδmay be a downstream mediator of effects of PGE_2 on promoting colorectal tumor growth (Cha and DuBois, 2007; Wang *et al.*, 2004a; Wang and DuBois, 2007). This effect is postulated to occur not by binding of PGE_2 to PPARδ but via an indirect transactivation of PPARδ signaling by PGE_2. PPARδ may be a direct transcriptional target of the β-catenin/TCF pathway, which may be stimulated by PGE_2 (see Fig. 3).

PPARγ has been widely studied because it mediates the insulin-sensitizing effects of thiazolidinediones on glucose and lipid metabolism. Administration of rosiglitazone or other PPARγ agonists to rodents can result in bone loss (Li *et al.*, 2006b; Rzonca *et al.*, 2004), while deficiency of PPARγ results in increased bone mass (Cock *et al.*, 2004). PPARγ is expressed in adipocytes and is important for adipogenesis. It is also expressed in bone marrow precursors and may play a role in determining adipocyte versus osteoblastic differentiation from a common stem cell (Lecka-Czernik and Suva, 2006). Activation of PPARγ may also have anti-inflammatory actions (Zingarelli and Cook, 2005). PPARγ has a wide array of potential ligands, including AA, NSAIDs at high concentrations, and some lipoxygenase metabolites. It has been proposed that a spontaneous dehydration product of PGD_2, the cyclopentenone 15-deoxy-Δ^{12-14} PGJ_2 (15d-PGJ_2) (see Fig. 1), is the endogenous ligand mediating anti-inflammatory effects of PPARγ (Scher and Pillinger, 2005). Pharmacological doses of 15d-PGJ_2 can activate PPARγ, as well as inhibit several other signaling transduction pathways. However15d-PGJ_2 levels *in vivo* are reported to be several orders of magnitude too low to be the endogenous mediator of PPARγ activation (Bell-Parikh *et al.*, 2003; Powell, 2003).

Although PGE_2 itself is unlikely to be a ligand for PPARs, metabolites of PGE_2 may be ligands. PGE_2 can be metabolized in cells by 15-hydroxyprostaglandin dehydrogenase (PGDH) to 15-keto-PGE_2 (Tai *et al.*, 2002). PGDH is expressed in many tissues (Tai *et al.*, 2006; Yan *et al.*, 2004), including calvarial osteoblasts (Pilbeam, unpublished observations). Although 15-keto-PGE_2 is orders of magnitude less potent at the EP2 and EP4 receptors than PGE_2 itself (Nishigaki *et al.*, 1996), 15-keto-PGE_2 may function as a PPAR ligand and stimulate the differentiation of murine fibroblasts into adipocytes (Chou *et al.*, 2007). It remains to be seen if this effect occurs at levels of 15-keto-PGE_2 that occur *in vivo*.

REGULATION OF PG PRODUCTION IN OSTEOBLASTS

Stimulation of PG Production

Early studies using complement-sufficient antisera, which contain antibodies to rodent cell surface antigens, showed that this serum could increase resorption of fetal rat long bones by stimulating endogenous PG production (Raisz *et al.*, 1974). Subsequently, many agonists were found to increase bone PG production, including many of the regulators of bone metabolism. Prostanoids produced by bone include PGE_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$, the metabolite of PGI_2, as well as some PGD_2 and thromboxane (Feyen *et al.*, 1984; Gallant *et al.*, 2005; Klein-Nulend *et al.*, 1991a, 1997; Pilbeam *et al.*, 1989; Raisz *et al.*, 1979; Raisz and Martin, 1983; Voelkel *et al.*, 1980). In some studies of cultured calvarial bone and primary calvarial cells, more 6-keto-$PGF_{1\alpha}$ than PGE_2 was measured in the media (Pilbeam *et al.*, 1989). On the other hand, there was no detectable accumulation of 6-keto-$PGF_{1\alpha}$ seen in media from clonal MC3T3-E1 osteoblastic cells (Harrison *et al.*, 1994), and it is possible that endothelial cells are the main source of PGI_2 in bone organ cultures.

Among the factors that stimulate PG production in bone are proinflammatory mediators, such as interleukin-1 (IL-1; Sato *et al.*, 1986) and TNF-α (Tashjian *et al.*, 1987); multifunctional regulators of cell growth and differentiation, such as transforming growth factor (TGF)-α and -β (Hurley *et al.*, 1989; Sumitani *et al.*, 1989; Tashjian *et al.*, 1985); systemic calcium-regulating hormones, such as parathyroid hormone (PTH; Pilbeam *et al.*, 1989) and $1,25(OH)_2D_3$ (Klein-Nulend *et al.*, 1991b); and mechanical loading of bone (Lanyon, 1992; Rawlinson *et al.*, 1991; Reich and Frangos, 1993). Thyroid hormone (Klaushofer *et al.*, 1995), platelet-derived growth factor (PDGF; Tashjian *et al.*, 1982), bradykinin (Ljunggren *et al.*, 1991b), and thrombin (Ljunggren *et al.*, 1991a) can also stimulate PG production in osteoblasts.

Most commonly used osteoblastic cell models constitutively express COX-1. Prior to the identification of the inducible COX-2, many of us tried to explain marked increases in PG production by small increases in COX-1 expression coupled with ligand-stimulated AA release, despite the rather large mismatch in size of effects (Klein-Nulend *et al.*, 1991a). Following identification of COX-2 in the early 1990s (O'Banion *et al.*, 1991; Kujubu *et al.*, 1991; Xie *et al.*, 1991), it became clear that induction of COX-2 in osteoblastic cells was responsible for most acutely stimulated PG production. Multiple agonists have been shown to induce COX-2 in osteoblastic cells. Some examples are cytokines—IL-1 (Harrison *et al.*, 1994; Kawaguchi *et al.*, 1994; Min *et al.*, 1998), TNF-α (Kawaguchi *et al.*, 1996) and IL-6 (Tai *et al.*, 1997); growth factors—TGFα (Harrison *et al.*, 1994), TGF-β (Pilbeam *et al.*, 1997a), basic fibroblast growth factor (FGF-2; Kawaguchi *et al.*, 1995b), and bone morphogenetic protein (BMP-2; Chikazu *et al.*, 2005); systemic hormones—PTH (Kawaguchi *et al.*, 1994; Tetradis *et al.*, 1996, 1997) and 1,25(OH)$_2$ vitamin D$_3$ (Okada *et al.*, 2000a); calcium (Choudhary *et al.*, 2003) and strontium (Choudhary *et al.*, 2007); and fluid shear stress or mechanical loading (Klein-Nulend *et al.*, 1997; Mehrotra *et al.*, 2006a; Pavalko *et al.*, 1998; Wadhwa *et al.*, 2002a).

When COX-2 was initially identified, it was mistakenly thought to have very limited cell and tissue expression, in part because the tissue preparations used to screen for expression were obtained under conditions that did not induce COX-2. Although the expression of COX-2 in bone has not received much attention outside the bone research field, bone may be one of the highest COX-2 expressing tissues in the body. We injected mice transgenic for the COX-2 promoter (−371/+70 bp) fused to a luciferase reporter with LPS and measured COX-2 mRNA and luciferase activity in multiple tissues 4 hours later (Fig. 4; Freeman *et al.*, 1999). LPS-stimulated COX-2 mRNA expression was highest in calvarial bone and brain, and the induction of COX-2 promoter activity was greatest in calvarial bone. Hence, although PGs produced by COX-2 expression in other cell types interfacing with bone, such as macrophages and vascular endothelial cells, may influence bone cell differentiation and function, the converse may also be true—osteoblasts may be an important source of PGs that influence neighboring cells in the bone marrow and in the vascular network.

Fresh serum is a potent stimulator of COX-2 expression and PGE$_2$ production in cultured osteoblastic MC3T3-E1

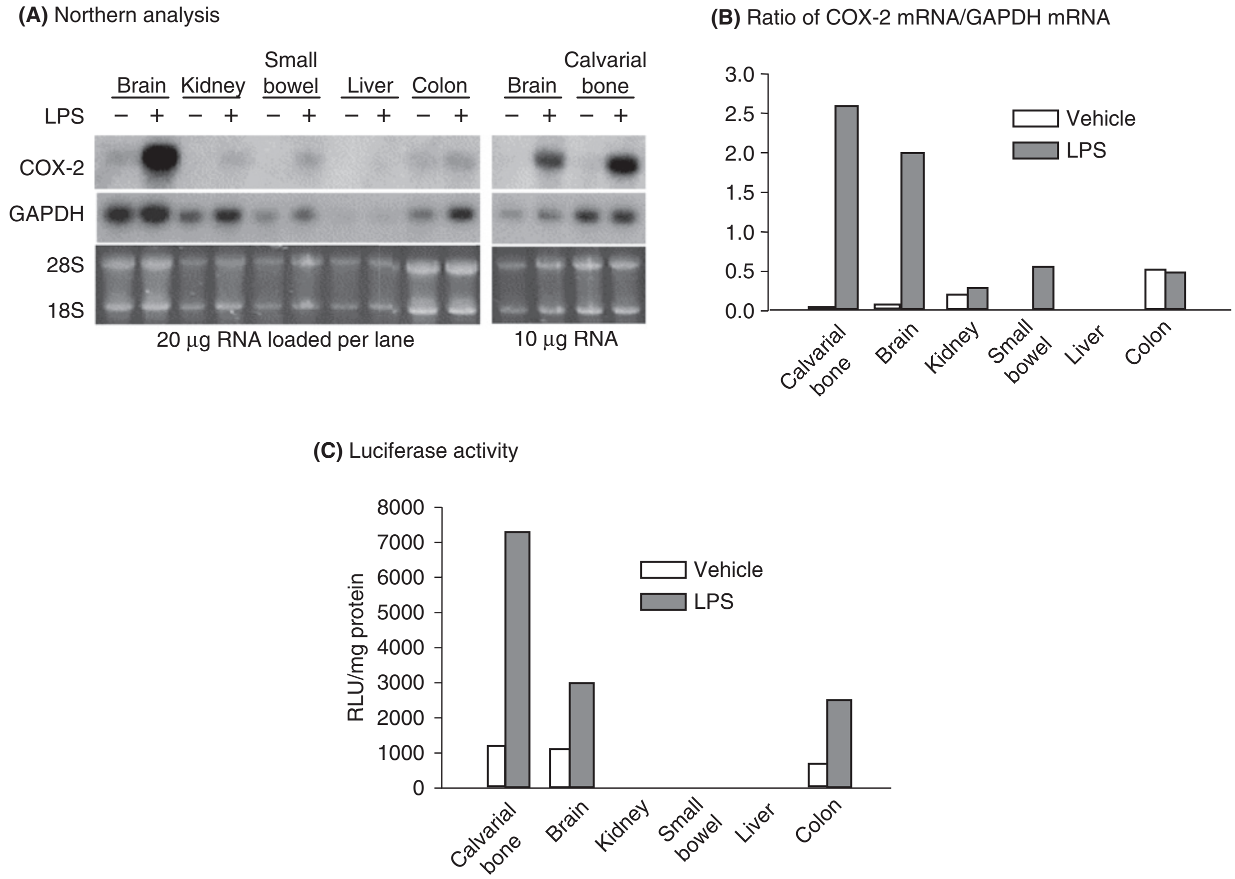

FIGURE 4 Comparison of lipopolysaccharide (LPS) induced COX-2 mRNA and promoter activity *in vivo* (Freeman *et al.*, 1999). Mice transgenic for 371 bp of the COX-2 promoter fused to a luciferase reporter were injected with LPS (IP, 4 mg/mouse) or vehicle and killed 4 hours later. RNA and luciferase measurements were made on the same tissue from a single mouse. (**A**) Northern blot analysis. (**B**) Quantification of Northern analysis. Compared to other tissues, it is more difficult to extract RNA or luciferase from mineralized calvariae. On the other hand, COX-2 mRNA is poorly expressed in some of the other tissues relative to calvariae. Hence, we ran two Northern gels and compared bone to brain, at 10 µg total RNA, and other tissues to brain, at 20 µg total RNA, on separate gels. The housekeeping gene glyceraldehyde 3–phosphate dehydrogenase (GAPDH) and ethidium bromide stained 28S and 18S ribosomal RNA on gels are shown to assess loading. (**C**) Luciferase activity. Each sample was analyzed in triplicate.

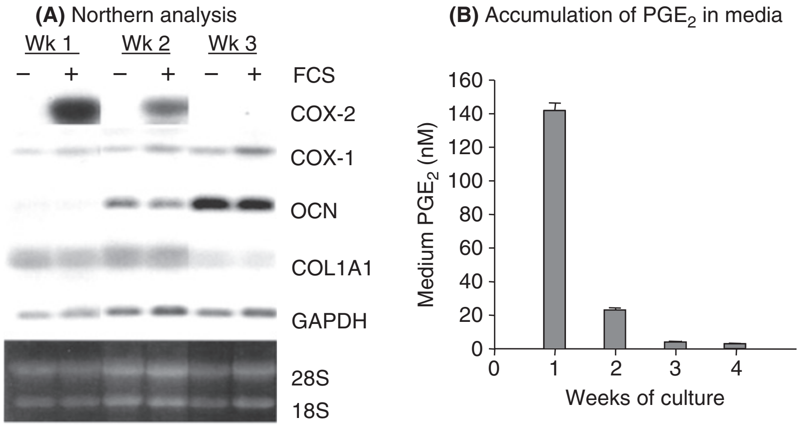

FIGURE 5 Serum-stimulated COX-2 mRNA expression and PGE_2 production in MC3T3–E1 cells (Pilbeam, unpublished data). Cells were cultured for 3 to 4 weeks with replacement of medium containing 10% heat-inactivated fetal calf serum (FCS) every 3 to 4 days. At the beginning of week 2, 50 μg/mL phosphoascorbate was added. At the end of each week of culture, cells were serum deprived for 24 hours and then treated with fresh 10% FCS for 2 hours. (**A**) Northern blot analysis. Levels of mRNA were analyzed for COX-1, COX-2, osteocalcin (OCN), and type 1 collagen (COL1A1) as markers of osteoblastic differentiation. The housekeeping gene GAPDH and ethidium bromide stained 28S and 18S ribosomal RNA on gels are shown to assess loading. (**B**) Medium PGE_2 accumulation after serum stimulation was measured by radioimmunoassay (Xu *et al.*, 2007b). PGE_2 levels in the serum-deprived cultures prior to treatment with FCS were below the detection limit of the assay (<0.1 n*M*).

cells (Pilbeam *et al.*, 1993; Fig. 5). At the end of each week in culture, cells were serum deprived for 24 hours and then treated with fresh fetal calf serum (FCS, 10%) for 2 hours. As cells became more differentiated, the serum induction of COX-2 and PGE_2 production diminished, suggesting that early osteoblastic precursors are more sensitive to this effect of serum. Compared to effects at the end of the first week, serum-stimulated PGE_2 production was reduced 85% after 2 weeks of culture and 97% after 3 weeks. The serum induction of COX-2 can occur when culture media are replaced with media containing fresh serum under routine culture conditions, and the associated PGE_2 production is likely to stimulate the osteoblastic differentiation that occurs under these conditions (see following discussion and Fig. 8).

In some osteoblastic cell cultures (Pilbeam *et al.*, 1994), in contrast to bone organ cultures (Kawaguchi *et al.*, 1994, 1996), little PGE_2 may be produced despite induction of COX-2 expression, unless serum is present or AA is added, suggesting that substrate availability is limiting. Some agonists, such as TGF-β (Pilbeam *et al.*, 1997a), extracellular calcium (Choudhary *et al.*, 2003), and PDGF (Chen *et al.*, 1997), stimulate substantial PGE_2 production in serum-free cultures of osteoblasts, probably secondary to effects on both COX-2 expression and phospholipase activation.

PGE_2 can stimulate its own production by stimulating COX-2 and $cPLA_2$ expression (Kawaguchi *et al.*, 1994; Murakami *et al.*, 1997; Pilbeam *et al.*, 1993, 1994). This autoamplification can also amplify the effects of PGE_2 agonists. For example, $PGF_{2\alpha}$, a poor stimulator of resorption, increases PGE_2 production in organ culture, and PGE_2 then enhances the effects of $PGF_{2\alpha}$ on resorption (Raisz *et al.*, 1990). Other PGs can also induce COX-2. Effects of selective agonists for the EP1-4, IP, TP, FP, and DP receptors were examined in cultured murine calvarial osteoblasts (Sakuma *et al.*, 2004). PGE_2 and the selective agonists for EP2 were the most effective stimulators of COX-2 expression, but agonists for EP4, IP, TP, and DP were also able to increase COX-2 expression or promoter activity. The PG induction of COX-2 in this system generally paralleled the PG stimulation of cAMP production and was mediated predominantly via the PKA pathway. However, in MC3T3-E1 cells, induction of COX-2 expression was greater with $PGF_{2\alpha}$ and PGD_2 than with PGE_2, indicating involvement of a phopholipase C signaling pathway (Pilbeam *et al.*, 1994; Takahashi *et al.*, 1994).

The autoamplification effect may be particularly important in cell and organ cultures, where PGs are not rapidly degraded, and may account for the biphasic or recurrent induction of COX-2 seen in some long-duration cultures. For example, in cultured neonatal calvariae treated with FGF-2, COX-2 mRNA expression peaked at 2 to 4 hours of treatment, decreased to undetectable at 24 hours, but was elevated again at 48 hours (Kawaguchi *et al.*, 1995b). The recurrent elevation of COX-2 could be blocked by inhibiting COX activity with NSAID. The induction of COX-2 secondary to PG production may involve different signaling pathways than the initial induction by the COX-2 agonist and may complicate interpretation if unrecognized. *In vivo*, this autoamplifying mechanism could contribute to prolonging the effects of short periods of impact loading or mechanical strain in skeletal tissue and to prolonging effects of cytokines in inflammatory diseases. The autoamplification effect might be self-limited by homologous desensitization. EP2 and EP4 receptors can be desensitized by PGE_2 (Nishigaki *et al.*, 1996), and PGE_2 pretreatment can downregulate the PGE_2 induction of COX-2 (Pilbeam *et al.*, 1994).

Inhibition of PG Production

Glucocorticoids (GCs) have been shown in many studies to be potent inhibitors of stimulated PG production, and their anti-inflammatory actions are thought to be due in part to this inhibition. Prior to the identification of COX-2, GCs were thought to work predominantly through interference with release of AA release. Although GCs can inhibit agonist-stimulated release of AA (Sampey *et al.*, 2000), it is now clear that the majority of GC effects on PG production in bone and other tissues are the result of inhibiting COX-2 expression (Kawaguchi *et al.*, 1994; Pilbeam *et al.*, 1993). GCs can also inhibit mPGES-1 (Korotkova *et al.*, 2005). The inhibition of COX-2 occurs by both transcriptional and post-transcriptional mechanisms (DeWitt and Meade, 1993; Lasa *et al.*, 2001; Newton *et al.*, 1998). A number of factors can induce COX-2 via MAPK signaling pathways (Choudhary *et al.*, 2003; Tsatsanis *et al.*, 2006; Wadhwa *et al.*, 2002b), and some of the effects of GCs on COX-2 expression may be mediated via the GC induction of MAPK phosphatase-1 (MKP-1), also called dual specificity phosphatase-1 (DUSP-1), which dephosphorylates and inactivates MAPKs (Abraham *et al.*, 2006). Retinoic acid is also a potent transcriptional inhibitor of COX-2 gene expression and PG production in osteoblasts, inhibiting induction of COX-2 by multiple factors (Pilbeam *et al.*, 1995). The ability of retinoic acid to inhibit COX-2 expression may explain some of its anticancer actions (Eisinger *et al.*, 2006; Kong *et al.*, 2005; Li *et al.*, 2002). It is also interesting to note that retinoic acid, similar to GCs, can induce MKP-1 (Xu *et al.*, 2002). The cytokines IL-4 and IL-13 have also been shown to inhibit COX-2 expression and PG production in bone organ and cell cultures (Kawaguchi *et al.*, 1996; Onoe *et al.*, 1996). Other agents reported to inhibit PG production in bone cells are vitamin K_2 (Hara *et al.*, 1993) and interferon-γ (Peterlik *et al.*, 1985).

Estradiol has been shown to reduce PG production in some culture models. Estradiol decreased PTH-stimulated PG production in cultured murine calvaria (Pilbeam *et al.*, 1989). This effect was not associated with decreased COX-2 mRNA expression (Pilbeam, unpublished data). Medium PGE_2 levels in cultured calvaria dissected from ovariectomized rats were increased relative to levels in calvaria from estrogen replete rats, and this increase was reversed by estradiol administration *in vivo* (Feyen and Raisz, 1987). When marrow supernatants from ovariectomized or sham-operated mice were added to murine calvarial cultures, marrow supernatants from ovariectomized mice stimulated more bone resorption than supernatants from estrogen-replete mice, and this increase was mediated by increased PG production secondary to increased COX-2 expression (Kawaguchi *et al.*, 1995a; Miyaura *et al.*, 1995). The increased COX-2 expression in these cultures was secondary to increased IL-1α activity in the supernatants and was reversed by *in vivo* treatment of the ovariectomized mice with estradiol (Kawaguchi *et al.*, 1995a). Some studies have suggested that endogenous PGs might mediate some of the bone loss due to estrogen deficiency (Gregory *et al.*, 2006). Partial reversal of bone loss in ovariectomized rats by an NSAID has been reported, although the effect was not sustained (Kimmel *et al.*, 1992; Lane *et al.*, 1990). However, it may not have been possible to produce a sustained inhibition of COX-2 activity with the nonselective NSAID at tolerated concentrations. We examined the effects of ovariectomy on COX-2 wild-type (WT) and KO mice in an outbred CD-1 background. Mice underwent ovariectomy or sham operation at 4 months of age and were killed 9 weeks later (Xu *et al.*, 2006a). Following ovariectomy, COX-2 KO mice had a significantly greater decrease (−8.2%) in total body BMD than COX-2 WT mice (−4.5%). Hence, COX-2 deficiency did not prevent the bone loss associated with ovariectomy.

As discussed earlier, NSAIDs inhibit PG production by blocking the cyclooxygenase catalytic site. Because PGs themselves can induce COX-2 expression, NSAIDs can also decrease COX-2 expression by reducing PG-mediated autoamplification. NSAIDs have been widely used to study the role of endogenous PGs. NS-398 was the first selective NSAID to become commercially available for *in vitro* and animal studies and is still widely used. The fact that NS-398 loses its selectivity at high doses is often overlooked. In rodent osteoblastic cells, NS-398 at a concentration of $0.01\,\mu M$ was selective for inhibition of COX-2 activity; however, at concentrations of 0.1 and $1\,\mu M$, NS-398 also inhibited COX-1 activity by 60% and 85%, respectively (Pilbeam *et al.*, 1997b). Hence, there was little selectivity of NS-398 at $1\,\mu M$, a commonly used dose. NSAIDs may also have effects independent of inhibiting PG production. As the result of many studies employing COX-2 selective NSAIDs to study effects of PGs on promotion of tumorigenesis, it has become apparent that high doses of these inhibitors can have multiple effects on cell growth and survival in addition to inhibiting COX-2 activity (Grosch *et al.*, 2006; Paik *et al.*, 2000).

Transcriptional Regulation of COX-2

Acute changes in PG production in osteoblasts are primarily the result of regulation of COX-2 expression. Although COX-2 expression is regulated by post-transcriptional mechanisms (Cok and Morrison, 2001), it is the transcriptional regulation that has been most studied. In osteoblasts, COX-2 frequently behaves like an immediate early gene in response to agonists, with the induction of mRNA expression being rapid, transient, and independent of new protein

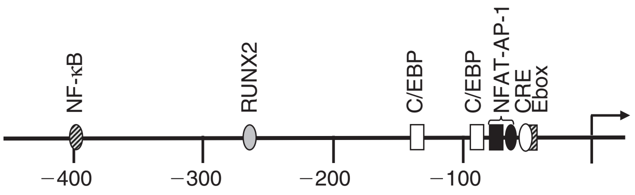

FIGURE 6 *Cis*-acting sites in the murine COX-2 promoter. Only sites thought to be important for regulating COX-2 transcription in osteoblastic cells and in osteoclast precursors (RAW264.7 murine macrophages) are shown. See text for discussion.

production. Multiple *cis*-acting promoter elements and *trans*-acting factors have been demonstrated to be involved in COX-2 induction, depending on cell type (Kang *et al.*, 2007). In murine MC3T3-E1 osteoblastic cells, 371 bp of the 5′-flanking COX-2 gene proximal to the transcription start site is adequate to mediate transcriptional induction by many agonists (Harrison *et al.*, 2000; Kawaguchi *et al.*, 1995b; Okada *et al.*, 2000c; Pilbeam *et al.*, 1997a; Tetradis *et al.*, 1997; Wadleigh and Herschman, 1999). This length of promoter does not include known NF-κB binding sites. *Cis*-acting sequences in this region shown to mediate effects on COX-2 expression in osteoblastic cells include a cAMP response element (CRE) at −57/−52 bp (ACGTCA) and an activator protein-1 (AP-1) binding site at −69/−63 bp (AGAGTCA) (Fig. 6). Both of these sites are involved in the phorbol ester (PMA) and serum induction of COX-2 promoter activity (Okada *et al.*, 2000c). The AP-1 site can inducibly bind Fos/Jun, while the CRE site constitutively binds CREB and ATF proteins. One study reported mutation of the CRE site abolished COX-2 promoter responses to serum, growth factors and cytokines in MC3T3-E1 cells (Wadleigh and Herschman, 1999). We have also seen involvement of the CRE site in COX-2 promoter responses to FGF-2, TNFα and PTH in MC3T3-E1 cells (Chikazu *et al.*, 2001; Okada *et al.*, 1998, 2000c; Tomita *et al.*, 1998) but have not found mutation of the CRE alone to abolish induction of the COX-2 promoter response by any agonist.

One or more CCAAT enhancer binding protein (C/EBP) sites located between −150 and −40 bp have been shown to mediate the IL-1 induction of COX-2 promoter activity in MC3T3-E1 cells (Harrison *et al.*, 2000; Wadleigh and Herschman, 1999; Yamamoto *et al.*, 1995). Putative C/EBP sites have been identified at −93/−85 bp (TGGGAAAG) and at −138/−130 bp (TTGCGCAAC). Joint mutation of these two sites has been reported to reduce COX-2 promoter activity in response to multiple factors, including FGF-2, PDGF, and a combination of IL-1 and TNFα (Wadleigh and Herschman, 1999). However, nuclear extracts from control and IL-1 treated MC3T3-E1 cells bound specifically only to an oligonucleotide spanning the −138/−130 bp C/EBP site (Harrison *et al.*, 2000). Another study found both the C/EBP site at −138/−130 bp and an NF-κB binding site at −401/−393 bp were required for maximal response to TNFα in MC3T3-E1 cells (Yamamoto *et al.*, 1995). However, mutation of this NF-κB site was found to have no affect on COX-2 responses to multiple agents in another study (Wadleigh and Herschman, 1999). Mutations in the C/EBP (−138/−130 bp) site, AP-1 site, and CRE also reduced the fluid shear stress stimulation of COX-2 promoter activity in MC3T3-E1 cells (Ogasawara *et al.*, 2001).

After publication of our initial study on the AP-1 site, we noticed that this AP-1 site was actually part of a nuclear factor of activated T-cells (NFAT)/AP-1 composite binding site, GGA(N)9TCA, at −77/−63 bp. We found that this composite site mediated in large part the PTH induction of COX-2 promoter activity in MC3T3-E1 cells (Chikazu *et al.*, 2001). Transcription factors of the NFAT family, originally identified as important regulators of gene expression in T cells, have now been shown to be expressed in multiple tissues (Macian, 2005). NFAT transcription factors are known to be critical for osteoclastogenesis (Hirotani *et al.*, 2004; Ikeda *et al.*, 2004; Matsuo *et al.*, 2004), and a role for them in bone formation has also been demonstrated (Koga *et al.*, 2005; Stern, 2006; Winslow *et al.*, 2006). NFAT proteins and the AP-1 heterodimer, Fos/Jun, can cooperatively bind the composite DNA site and activate or repress gene expression (Macian *et al.*, 2001). Whether or not signaling pathways lead to cooperative interactions of AP-1 and NFAT may determine distinct biological programs of gene expression (Macian *et al.*, 2000). More distantly separated GGA and TCA motifs have also been shown to support independent binding of NFAT and AP-1. In addition to the composite site at −77/−63 bp, there are GGA motifs at −111/−109 bp and at −90/−88 bp in the murine COX-2 promoter, but we have not found mutations of these more distant sites to affect COX-2 promoter activity (Pilbeam, unpublished data).

Runx2 (Cbfa1) is an essential transcription factor for osteoblast differentiation and can regulate the expression of many genes associated with osteoblastic differentiation. We identified a Runx2 consensus sequence (AACCACA) at −267/−261 bp in the murine COX-2 promoter and showed this site to mediate the BMP-2 induction of COX-2 (Chikazu *et al.*, 2005). The Runx2 site is the only *cis*-acting site lying between −371 and −150 bp, other than a regulatory sequence for TGFβ (Pilbeam *et al.*, 1997a), that we have found to mediate COX-2 promoter activity in MC3T3-E1 cells. Transcriptional activity of Runx2 is also necessary for maximal fluid shear stress induction of COX-2 promoter activity in MC3T3-E1 cells (Mehrotra *et al.*, 2006a). Multiple signaling pathways have been shown to regulate the activity of Runx2, and Runx2 can interact with many other transcription factors (Barakat and Lieu, 2003; Franceschi *et al.*, 2003; Gutierrez *et al.*, 2002; Porte *et al.*, 1999; Xiao *et al.*, 2000). Thus, transcriptional activity of Runx2 may be important for enhancing or integrating the actions of multiple other transacting factors on COX-2 induction.

Because RAW264.7 murine macrophages can be induced with receptor activator of NF-κB ligand (RANKL) to differentiate into osteoclasts, the regulation of COX-2 in these cells may reflect COX-2 regulation in osteoclast precursors. LPS induction of COX-2 in RAW264.7 cells is frequently studied as a model of inflammation. Mutation of the CRE or combined mutation of the C/EBP sites at −93/−85 bp and −138/−130 bp significantly reduced the LPS induction of COX-2 promoter activity, whereas mutation of the NF-κB binding sites had no effect on induction (Wadleigh *et al.*, 2000). A recent study identified a second CRE (CRE-2) upstream of the NF-κB site as important in the LPS induction of COX-2 promoter activity in these cells, along with the original CRE, the AP-1 site, and the C/EBP site (−138/−130 bp; Kang *et al.*, 2006). In that study, the E-box, which overlaps the CRE site (see Fig. 6), was found to be a transcriptional repressor. Several studies have suggested that the Ets family of transcription factors, including PU.1 (Joo *et al.*, 2004) and ESE-1 (Grall *et al.*, 2005), play a role in the regulation of COX-2 in RAW264.7 cells in response to LPS. RANKL has also been shown to induce COX-2 promoter activity in RAW264.7 cells (Han *et al.*, 2005). Although this induction was said to be abolished by deleting an NF-κB binding site, the NF-κB binding site was included in a large region from −518 to −362 bp that was deleted. Specific mutation of the NF-κB binding site will be necessary to confirm its involvement.

Transcriptional regulation of COX-2 by most factors appears to involve multiple signaling pathways and multiple cis-acting sites (Kang *et al.*, 2007; Tsatsanis *et al.*, 2006). Recent studies suggest that p300 may integrate these different pathways (Deng *et al.*, 2004; Kang *et al.*, 2006). p300 is a coactivator of transcription for multiple nuclear proteins and a histone acetyl transferase (HAT) that can generate a more accessible chromatin structure for transcription factors (Wolffe and Pruss, 1996). p300 is also capable of acetylating nonhistone proteins, such as Runx2 (Jeon *et al.*, 2006).

PGs AND BONE RESORPTION

Early work on the effects of PGs on bone resorption in organ culture showed that PGs of the E series are the most potent activators, with an effective concentration range of 1 n*M* to 10 μ*M* (Klein and Raisz, 1970; Raisz and Martin, 1983). $PGF_{2\alpha}$ is less effective than PGE_2, stimulating resorption at concentrations of 0.1 μ*M* and above, and the ability of $PGF_{2\alpha}$ to stimulate resorption is partly dependent upon its induction of PGE_2 (Raisz *et al.*, 1990). PGI_2 can also stimulate resorption, while PGD_2 is ineffective. Many factors that stimulate PG production also stimulate resorption in organ culture, and the resorption stimulated by such factors can be mediated in part by PG production (Akatsu *et al.*, 1991; Tashjian *et al.*, 1982, 1987). However, the dependence of resorption in organ culture on stimulated PG production is quite variable, and some potent stimulators of PGE_2, such as PTH, do not exhibit PG-dependent resorption in these systems (Igarashi *et al.*, 2002; Pilbeam *et al.*, 1989; Stern *et al.*, 1985).

PGE_1 and PGE_2, but not $PGF_{2\alpha}$, stimulate osteoclast formation in marrow cultures (Collins and Chambers, 1991). In contrast to organ culture resorption studies, studies in marrow culture consistently demonstrate a dependence of osteoclast formation on agonist-stimulated PG production. Multiple agonists can stimulate PG-dependent osteoclast formation in marrow cultures, including IL-1 (Akatsu *et al.*, 1991; Lader and Flanagan, 1998; Sato *et al.*, 1996); TNF-α (Lader and Flanagan, 1998); PTH (Inoue *et al.*, 1995; Sato *et al.*, 1997); $1,25(OH)_2D_3$ (Collins and Chambers, 1992); IL-11 (Girasole *et al.*, 1994; Morinaga *et al.*, 1998); IL-6 (Tai *et al.*, 1997); IL-17 (Kotake *et al.*, 1999); phorbol ester (Amano *et al.*, 1994); and FGF-2 (Hurley *et al.*, 1998). TGF-β can enhance osteoclastogenesis at low concentrations by a PG-dependent mechanism but inhibit osteoclastogenesis at higher concentrations (Shinar and Rodan, 1990).

Induction of COX-2 is likely to be responsible for the PG enhancement of stimulated osteoclastogenesis. In marrow cultures from COX-2 KO mice stimulated by $1,25(OH)_2D_3$ (Fig. 7) or PTH, PGE_2 production is markedly decreased and osteoclast formation is reduced by 60% to 70% or more compared to WT cultures (Okada *et al.*, 2000a). Marrow cultures from mice with disruption of only one COX-2 allele had levels of osteoclast formation and PGE_2 production intermediate to those in COX-2 WT and KO cultures. Treatment of WT cultures with a COX-2 inhibitor mimicked the results observed in cultures from COX-2 KO mice. There was no effect of disruption of both COX-1 alleles on stimulated PGE_2 production or osteoclastogenesis in these cultures. We found similar results for osteoclastogenesis stimulated by IL-1 (Pilbeam, unpublished data) and FGF-2 (see Fig. 7), suggesting that PGs produced by COX-2 are necessary for maximal *in vitro* osteoclastogenesis in response to multiple agents.

PGE_2 may stimulate osteoclastogenesis in several ways. PGE_2 can act on osteoclast supporting cells (osteoblasts or their precursors in the mesenchymal lineage) to increase expression of RANKL, which is essential for osteoclast differentiation (Li *et al.*, 2000; Suzawa *et al.*, 2000; Tsukii *et al.*, 1998). PGE_2 may also decrease expression of osteoprotegerin (OPG), the decoy receptor for RANKL, in osteoblasts (Suda *et al.*, 2004). PGE_2 may also act on cells of the hematopoietic lineage to increase differentiation of osteoclastic precursors. In cultured spleen cells, PGE_2 enhanced the effects of RANKL and M-CSF on osteoclast

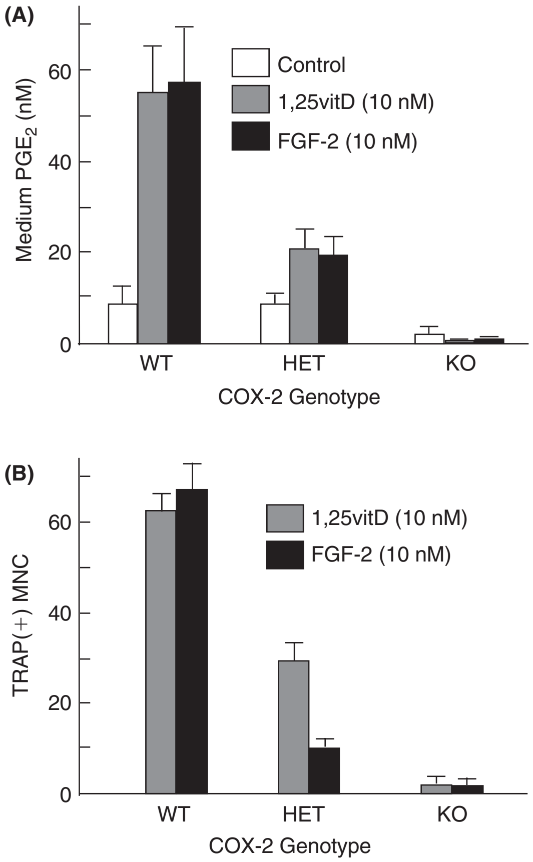

FIGURE 7 PGE_2 production and osteoclast formation in marrow cultures: comparison of COX-2 wild type (WT), heterozygous (HET), and knockout (KO) mice (Okada, Y., Tomita, M., Hurley, M., Raisz, L., and Pilbeam, C., unpublished data). Marrow was flushed from tibiae and femurs and cultured in α-MEM with 10% FCS. Cultures were treated for 7 days with vehicle (control), 1,25 $(OH)_2$ vitamin D_3 (1,25vitD), or FGF-2. (**A**) Medium PGE_2 accumulation measured by radioimmunoassay (Xu *et al.*, 2007b). (**B**) Osteoclasts per well. Cultures were stained for tartrate resistant acid phosphatase (TRAP) and TRAP positive cells with more than three nuclei were counted as osteoclasts. No osteoclasts were formed in control cultures. Bars are means ± SEM for three wells.

formation (Li *et al.*, 2000; Okada *et al.*, 2000a; Wani *et al.*, 1999). Osteoclast formation was reduced 50% in spleen cells from COX-2 KO mice compared to spleen cells from COX-2 WT mice (Okada *et al.*, 2000a). This reduction was associated with increased expression of granulocyte-macrophage colony-stimulating factor (GM-CSF), an inhibitor of osteoclast formation in these cultures, and could be reversed by addition to the cultures of blocking antibody to GM-CSF. GM-CSF may act by diverting progenitor cells into the macrophage rather than osteoclast pathway. PGE_2 also enhanced osteoclastogenesis in the presence of RANKL in RAW264.7 cells (Kaneko *et al.*, 2007; Kobayashi *et al.*, 2005a). This effect was attributed to cAMP-dependent PKA phosphorylation of a serine residue in TGFβ-activated kinase 1 (TAK1), a MAPK kinase that can enhance downstream signaling in response to RANKL and other cytokines (Kobayashi *et al.*, 2005a).

Studies using cells and tissues from EP receptor KO animals and selective EP receptor agonists have tried to define the specific receptors involved in the PGE_2 stimulation of resorption. Neonatal murine calvarial cultures express all PGE receptor subtypes, but PGE_2-stimulated resorption appears to be largely mediated via the EP4 receptor, with only a minor contribution from EP2 (Suzawa *et al.*, 2000). A marked decrease in resorptive response to PGE_2 was found in cultured calvariae from EP4 KO animals, and there was impaired response to PGE_2 even in calvariae from mice with only one EP4 allele disrupted (Miyaura *et al.*, 2000; Zhan *et al.*, 2005). In fetal rat long-bone cultures treated with agonists selective for EP2 and EP4, only the EP4 agonist was effective in stimulating resorption (Raisz and Woodiel, 2003). However, aged EP4 KO mice are reported to have significantly increased osteoclast numbers on trabecular surfaces and increased eroded endocortical surface compared to WT mice (Li *et al.*, 2005), and administration of an EP4 selective agonist to rats subjected to protocols causing bone loss, such as immobilization, suggested that the EP4 agonist increased bone formation but not resorption (Yoshida *et al.* 2002).

Both EP4 and EP2 receptors are involved in the PGE_2 effects on the osteoclastic support cells. A selective antagonist for EP4 partially inhibited osteoclastogenesis in murine marrow cultures, not only in response to PGE_2, but also in response to $1,25(OH)_2D_3$ and PTH (Tomita *et al.*, 2002). In bone marrow cultures and cocultures of osteoblasts and spleen cells, osteoclast formation was impaired when cells from either EP2 or EP4 receptor KO animals were used (Li *et al.*, 2000; Sakuma *et al.*, 2000). These studies confirmed earlier results using selective EP agonists and antagonists (Ono *et al.*, 1998). The EP2 receptor may also be involved in the effects of PGE_2 on hematopoietic precursors (Ono *et al.*, 2005). Spleen cells from EP2 KO animals showed an impaired osteoclastogenic response when treated with M-CSF, RANKL, and PGE_2 (Li *et al.*, 2000).

Some studies have found PGE_2 to have inhibitory effects on osteoclast formation from hematopoietic precursors. In murine spleen cell cultures, studied over an extensive time course, effects of PGE_2 were biphasic. PGE_2 had an initial inhibitory effect on osteoclast formation, possibly mediated by both EP2 and EP3 receptors, and a later stimulatory effect, mediated by the EP2 receptor, possibly acting on T-cells (Ono *et al.*, 2005). Although PGE_2 stimulated osteoclast formation in human marrow cultures (Lader and Flanagan, 1998), PGE_2 inhibited osteoclast formation in human CD14+ cells from peripheral blood mononuclear cells (PBMCs) in the presence of RANKL and M-CSF (Take *et al.*, 2005).

PGE_2 added to isolated osteoclasts actively resorbing bone *in vitro* transiently inhibits their activity (Fuller and Chambers, 1989). A similar transient inhibition of bone resorption and lysosomal enzyme release in mouse calvarial bone cultures by PGE_2 has also been observed (Lerner *et al.* 1987). Moreover, the transient decrease in serum calcium that occurs after systemic injection of IL-1 in mice was blocked by indomethacin and, thus, appears to be PG mediated (Boyce *et al.* 1989b). The inhibitory effect of PGE_2 on osteoclast activity or motility may involve both the EP4 and EP2 receptors (Mano *et al.*, 2000b; Okamoto *et al.*, 2004). EP2 and EP4 receptor expression is downregulated during differentiation in bone marrow macrophage cultures, and this may be one way osteoclasts escape the inhibitory effects of PGE_2 (Kobayashi *et al.*, 2005b). This inhibitory effect may be due to a direct activation of adenylate cyclase in osteoclasts, thereby mimicking the effect of calcitonin to inhibit osteoclast function. The physiologic relevance of this transient inhibition is unknown.

Little is known about the role of endogenous PGs in bone resorption *in vivo*. If PGs are stimulators of new osteoclast differentiation rather than osteoclast activity, a role for PGs in bone resorption might be most evident when the ability to generate new osteoclasts from osteoclastic precursors becomes rate limiting.

PGs AND BONE FORMATION

Studies in cell and organ culture indicate that PGs can have both stimulatory and inhibitory effects on bone formation. Anabolic effects can be seen in fetal rat calvarial organ cultures, in which PGE_2 stimulates both cell replication and differentiation (Woodiel *et al.*, 1996). At high concentrations, however, PGs can inhibit collagen synthesis in cell and organ culture (Fall *et al.*, 1994). This inhibitory effect appears to occur largely via transcriptional inhibition of collagen and to be mediated by the FP receptor rather than an EP receptor.

Systemic injection of PGE_2 in rats can increase both periosteal and endosteal bone formation in the rat and produce substantial increases in bone mass, similar to the effects of PTH (Jee and Ma, 1997; Lin *et al.*, 1994; Suponitzky and Weinreb, 1998). Systemic administration of PGE_2 or PGE_1 in humans (Faye-Petersen *et al.*, 1996; Ueda *et al.*, 1980) and dogs (Norrdin and Shih, 1988) has also been shown to increase cortical and cancellous bone mass. Although local injection of PGE_2 has been shown to have local anabolic effects in mice (Yoshida *et al.*, 2002), systemic injection of PGE_2 in mice, following the protocols established for rats, has generally been associated with a greater increase in resorption than formation, resulting in bone loss (Raisz, unpublished data). However, we recently found 3 mg/kg of PGE_2 injected twice a week for 4 weeks in mice increased bone formation rate and did not cause bone loss (Gao *et al.*, 2007b).

The most consistent anabolic effects of PGE_2 are seen in cultures designed to study osteoblastic differentiation. PGE_2 stimulates osteoblastic differentiation in marrow stromal cell and primary calvarial cell cultures (Flanagan and Chambers, 1992; Kaneki *et al.*, 1999; Nagata *et al.*, 1994; Scutt and Bertram, 1995; Xu *et al.*, 2007b). In addition, PGE_2 given to rats *in vivo* stimulates osteoblastic differentiation in *ex vivo* cultured bone marrow (Keila *et al.*, 1994; Weinreb *et al.*, 1997). In agreement with these studies, osteoblastic differentiation is decreased in marrow stromal cell cultures from mice deficient in COX-2 (Choudhary *et al.*, 2007; Okada *et al.*, 2000b; Zhang *et al.*, 2002b). An example of marrow stromal cells cultured from COX-2 WT and KO mice is shown in Figure 8. Medium PGE_2 production peaked between days 3 and 7 of culture in WT cultures and was generally undetectable in KO cultures despite constitutive expression of COX-1. Osteocalcin mRNA expression and Von Kossa staining for mineralization (see Fig. 8), as well as the area of staining for alkaline phosphatase and the area covered by all cells (Xu *et al.*, 2007b), were decreased in COX-2 KO cultures compared to WT cultures. Although decreased osteoblastic differentiation was also seen in primary calvarial cells from COX-2 KO mice, osteoblastic cell proliferation was increased relative to WT cultures (Xu *et al.*, 2007b).

Both EP2 and EP4 receptors have been implicated in the anabolic effects of PGE_2 *in vitro* (Alander and Raisz, 2006; Li *et al.*, 2007; Raisz and Woodiel, 2003; Woodiel *et al.*, 1996). *In vivo* studies of the skeletal phenotype of EP4 KO mice have been limited by the high rate of neonatal death (>95%) occurring within 72 hours of birth as a result of failure of the ductus arteriosus to close (Segi *et al.*, 1998). In an attempt to improve their survival, EP4 receptor deficient mice have been bred as mixtures of multiple genetic backgrounds. Aged EP4 receptor KO mice, studied in a highly mixed background, were shown to have reduced bone mass and impaired fracture healing compared to WT mice (Li *et al.*, 2005). Bone mechanical properties were reduced in both EP2 and EP4 KO mice (Akhter *et al.*, 2001, 2006).

PGE_2 can stimulate both the bone remodeling and angiogenesis needed for fracture healing. Early studies showed that exogenously applied PGE_2 stimulated callus formation in rabbits (Keller *et al.*, 1993). In addition, nonselective NSAIDs inhibited repair of spinal fusions (Dimar *et al.*, 1996) in rats and the fixation of implants in femora of rabbits (Jacobsson *et al.*, 1994). Following the development of selective COX-2 NSAIDs, multiple studies in animals showed that NSAIDs impaired fracture healing (Altman *et al.*, 1995; Bergenstock *et al.*, 2005; Brown *et al.*, 2004; Einhorn, 2003; Endo *et al.*, 2005; Murnaghan *et al.*, 2006; Simon *et al.*, 2002; Simon and O'Connor, 2007). The impairment of healing may be greater with selective COX-2 inhibitors, compared to nonselective NSAIDs, and is probably reversible after discontinuation of brief NSAID treatment (Gerstenfeld *et al.*, 2007). Fracture healing was impaired in COX-2 KO mice (Zhang *et al.*, 2002b), and a selective COX-2 inhibitor delayed

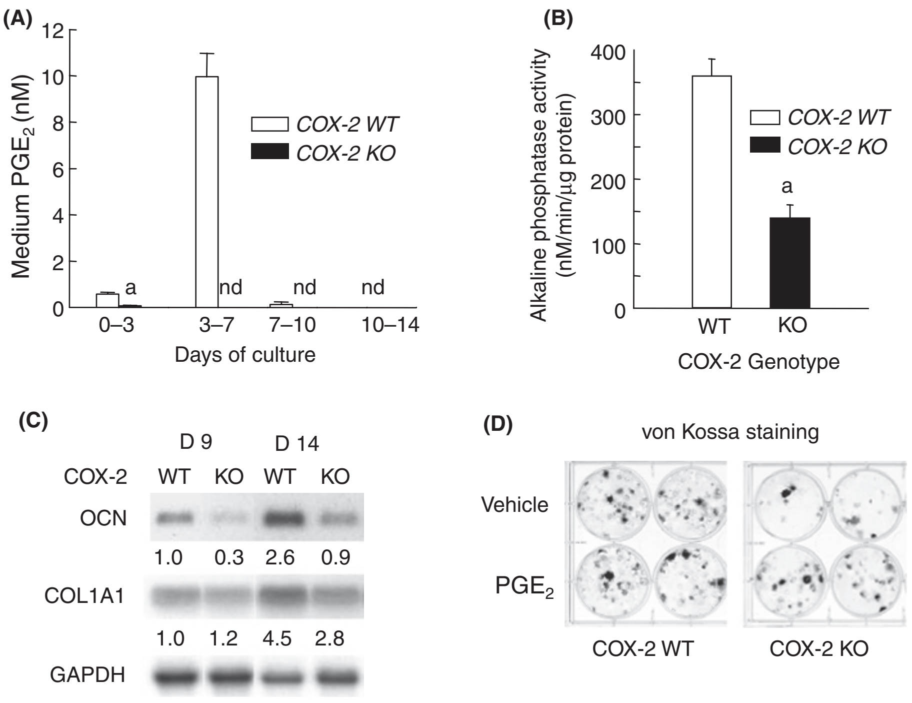

FIGURE 8 PGE$_2$ production and osteoblastic differentiation in marrow stromal cell (MSC) cultures from mice deficient in COX-2 (Okada, Y., Tomita, M., and Pilbeam, C., unpublished data). Marrow was flushed from tibiae and femurs, cultured in α-MEM with 10% FCS and 50 µg/mL phosphoascorbate. Ten millimolar β-glycerophosphate was added for the last 2 weeks of culture. (**A**) Medium PGE$_2$ accumulation measured by radioimmunoassay (Xu *et al.*, 2007b). nd, not detectable ($<0.1\,nM$). (**B**) Measurement of alkaline phosphatase (ALP) activity normalized to total protein in cells cultured for 14 days. Bars are the means ± SEM of three wells of cells. ^{a}Significant difference compared to WT, $P < 0.01$. (**C**) Northern analysis for type I collagen (COL1A1) and osteocalcin (OCN) mRNA. Ratios of mRNA levels to the housekeeping gene GAPDH are shown below the bands. (**D**) Von Kossa staining of mineralized nodules in cells cultured for 21 days with vehicle or PGE$_2$ ($1\,\mu M$). Means and SEM for stained areas were calculated from three wells. The mineralized areas in KO MSC were significantly less than in WT MSC ($P < 0.01$). There was also a significant effect of PGE$_2$ ($P < 0.01$) in the KO cultures.

allograft healing in a mouse model, whereas local delivery of PGE$_2$ enhanced bone formation at the cortical bone graft junction (O'Keefe *et al.*, 2006). On the positive side, perioperative treatment with aspirin or other NSAIDs may prevent the excessive bone formation response resulting in heterotropic ossification, a complication of hip arthroplasty that can adversely affect the outcome (Kienapfel *et al.*, 1999; Neal *et al.*, 2000; Nilsson and Persson, 1999).

Systemic PGE$_2$ is an unacceptable therapeutic option for enhancing fracture healing because of its side effects, such as diarrhea and hypotension. It is possible that selective EP receptor agonists could have the required anabolic effects without these side effects. Local injection of a nonprostanoid EP2 receptor agonist into the bone marrow or incorporated into a polymer matrix and then injected into the periosteum of rats increased bone formation, bone mass, bone strength, and fracture healing in rats (Li *et al.*, 2003). Bone healing was also shown to be improved by this EP2 agonist when applied locally to canine long-bone segmental and fracture model defects (Paralkar *et al.*, 2003). Systemic infusion or subcutaneous injection of an EP4 receptor agonist was able to restore bone mass in immobilized and ovariectomized rats (Yoshida *et al.*, 2002). These authors also showed that local infusion of PGE$_2$ onto the periosteal surfaces of rat femurs caused new bone formation in WT mice but not in EP4 receptor KO mice. Subcutaneous injection of a nonprostanoid EP4 receptor agonist restored bone mass and strength in aged ovariectomized rats (Ke *et al.*, 2006), and subcutaneous injection of an EP4 receptor agonist was shown to increase cortical bone healing in a rat drill hole model (Tanaka *et al.*, 2004). Thus, local application of an EP2 receptor agonist and local and systemic application of EP4 agonists have been shown to have anabolic effects *in vivo* and to enhance fracture healing. It is not yet clear if these selective agonists will have limiting side effects. Receptor pathways for the anabolic effects have not been fully elucidated, and the multiple signaling pathways identified for effects of PGs in tumor progression, discussed earlier, are likely to play a role in the anabolic effects in bone.

COX-2 KNOCKOUT MICE

Studies of mice in which either the gene for COX-1 or COX-2 is disrupted have shown that COX-1 and COX-2 have some overlapping functions, but that deficiency of COX-2 has more profound effects than deficiency of COX-1 (Dinchuk *et al.*, 1995; Langenbach *et al.*, 1995, 1999a; Morham *et al.*, 1995). Because few COX-2 KO mice could be generated when mice were backcrossed into the C57Bl/6 background (Scott Morham, personal communication), most early studies were done in a mixed C57Bl/6, 129 background. In this background, COX-1-deficient mice survive normally, whereas COX-2-deficient mice have increased mortality. One study reported that 35% of COX-2 KO mice died with a patent ductus arteriosus within 48 hours of birth (Loftin *et al.*, 2001). Other studies did not find a high incidence of neonatal death among COX-2 KO mice but found shortened life span secondary to renal dysplasia (Morham *et al.*, 1995). Renal development in COX-2 KO mice in the C57Bl/6, 129 background appeared normal until postnatal day 10, after which there was progressive renal architectural disruption and functional deterioration, with about 20% of COX-2 KO mice dying between 7 and 23 weeks of age from renal failure (Norwood *et al.*, 2000). Also, in this background, COX-2-deficient females are infertile, with multiple failures in female reproductive processes, including ovulation, fertilization, and implantation (Lim *et al.*, 1997).

We also found that COX-2 KO mice in the C57Bl/6, 129 background died 4 times faster after weaning than WT mice, with 40% dying between 2 and 10 months of age, whereas mice heterozygous for COX-2 gene disruption had normal mortality (Xu *et al.*, 2005b). Several 4-month-old KO mice with renal dysfunction had low serum calcium, high serum phosphate, and PTH levels greater than 2000 ng/dL, consistent with secondary hyperparathyroidism (HPTH). In COX-2 KO mice surviving to 10 months of age without evident renal dysfunction, PTH was still significantly increased compared to WT mice (114% in females, 150% in males). In addition, both male and female KO mice had increased $1,25(OH)_2D_3$ levels (180%), along with slightly elevated serum calcium, compared to WT mice, suggesting that older, apparently healthy, COX-2 KO mice might have primary HPTH. Skeletal analyses showed decreased lumbar BMD by DXA in 4-month-old KO females and decreased femur BMD in 10-month-old KO males, as well as decreased cortical width by μCT in 4-month-old KO males and 10-month-old KO females. Histomorphometric analyses of the distal femur showed increased mineral apposition rate and bone formation rate in 10-month-old KO males, whereas there was increased percent osteoclast surface in 10-month-old KO females. Interestingly, there was little difference in trabecular bone between KO and WT mice, by either μCT or histology, except for increased trabecular bone volume per total volume (BV/TV) in 10-month-old KO males. The overall picture for the 10-month-old KO male mice—increased PTH and $1,25(OH)_2D_3$ levels, decreased cortical but not trabecular bone, and increased bone formation rate—was consistent with effects expected for HPTH. We concluded that direct effects of COX-2 deficiency on bone might be obscured by effects of secondary HPTH in KO mice with renal dysfunction or effects of primary HPTH in healthy KO mice.

Another study of 4.5-month-old female COX-2 KO mice in a C57Bl/6, 129 background also reported reduced femoral BMD by DXA, reduced cortical thickness, but increased distal femoral BV/TV in KO compared to WT mice (Alam *et al.*, 2005). In this study, only 60% of KO mice survived to weaning and 28% died after weaning before they could be studied. In contrast, another study of 4-month-old mice in a 129 background found that only COX-2 KO males, and not KO females, had decreased bone volume fraction in the distal femur and decreased femoral BMD, both measured by μCT, compared to WT mice (Robertson *et al.*, 2006). The COX-2 KO mice in this study were said to have a lower survival rate than the KO mice in our study.

It is possible that the absence of type GIIA secretory PLA_2 in mice with C57Bl/6 or 129 genetic backgrounds might result in a more severe phenotype in the absence of COX-2 (Kennedy *et al.*, 1995). For example, COX-2 KO females in an outbred CD-1 background are fertile (Wang *et al.*, 2004b), and COX-2 KO mice in a C57Bl/6, DBA background have less severe renal pathology (Laulederkind *et al.*, 2002). A study of 3-month-old females in a C57Bl/6, DBA background showed KO mice to have thinner and more porous cortical bones than WT mice (Chen *et al.*, 2003). We studied a large group (30–40 per genotype) of 5-month-old male COX-2 KO and WT mice in an outbred CD-1 background and found that KO mice had no increased mortality after weaning compared to WT mice and no significant renal dysfunction (Xu *et al.*, 2005a). However, COX-2 KO mice still had increased serum PTH compared to WT mice (mean ± SEM of 66 ± 13 versus 27 ± 3 ng/dL, $p < 0.01$), but no difference in serum calcium, phosphorus, or $1,25(OH)_2D_3$. KO mice had small but significant decreases in femoral BMD (−5%) by DXA and in cortical area (−9%) by μCT, with increased cortical porosity (15%), compared to WT mice. In contrast to our earlier study, COX-2 KO mice had decreased distal femoral BV/TV (−20%) by histology compared to WT. A study of 3-month-old female mice in a mostly DBA background also reported no difference in survival or serum creatinine of COX-2 KO mice compared to WT mice but found increased PTH and serum calcium levels in KO mice compared to WT (Myers *et al.*, 2006). KO mice also had lower femoral BMD than WT mice.

Hence, apparently healthy COX-2 KO mice appear to have HPTH in several genetic backgrounds, although it is not clear whether the HPTH is primary or secondary. Most studies have also found COX-2 KO mice to have reduced BMD compared to WT mice, although there may be some protective effect of female gender in this regard. In addition, studies have consistently found bones from COX-2

KO mice to have inferior mechanical properties compared to WT mice (Alam *et al.*, 2005; Chen *et al.*, 2003; Myers *et al.*, 2006). *In vitro* studies would suggest that absence of COX-2 should reduce both bone resorption and formation. However, studies have not found decreased bone turnover in the COX-2 KO mice (Xu *et al.*, 2005b). Because PGE_2 and PTH can both stimulate osteoblast and osteoclast differentiation, the systemic effects of HPTH may compensate for the effects of COX-2 deficiency. Thus, it is not yet possible to make conclusive statements about the direct effects of COX-2 deficiency on bone *in vivo*.

Given that PTH induces COX-2 and that PGE_2 can have anabolic effects, we expected that COX-2 deficiency might impair the anabolic response to PTH. However, we found that the anabolic effect of intermittent PTH was greater in COX-2 KO mice than in WT mice (Xu *et al.*, 2007a). In addition, whereas PTH treatment of marrow stromal or primary osteoblast cultures *in vitro* is generally not anabolic, and often catabolic, PTH treatment of cultures from COX-2 KO mice is anabolic (Huang *et al.*, 2007). Because both PGE_2 and PTH stimulate cAMP signaling, it is possible that endogenous PGs might result in cross-desensitization of PTH-stimulated responses, or perhaps PGs can inhibit some anabolic responses to PTH.

The role of COX-1 in bone is still unclear. We have not seen effects of COX-1 deficiency on osteoblasts or osteoclasts in culture (Okada *et al.*, 2000a; Xu *et al.*, 2007b). One study reported a beneficial effect of COX-1 deficiency on bone parameters *in vivo* (Myers *et al.*, 2006). Several studies, which compared relative COX-1 mRNA expression in COX-2 WT and KO mice, have suggested that COX-1 may compensate for COX-2 deficiency in C57Bl/6, 129 mice (Alam *et al.*, 2005; Zhang *et al.*, 2002a) and that COX-1 compensation explains the rescue of female infertility in CD-1 mice (Wang *et al.*, 2004b). However, we have never found increased COX-1 mRNA expression in freshly isolated bone or cultured osteoblastic cells from COX-2 KO mice (C56Bl/6,129 or CD-1) under basal or stimulated conditions (Pilbeam, unpublished observations). It is possible that other aspects of the PG production pathway, such as phospholipases or terminal enzymes, may be upregulated in COX-2 KO mice. Clearly there must be compensation by COX-1 in some tissues because double COX-1, COX-2 deficient mice die from failure of ductus arteriosus closure shortly after birth (Loftin *et al.*, 2001).

PGs AND MECHANICAL LOADING OF BONE

Bone is a dynamic tissue that can adapt to mechanical loading with changes in structure to achieve a better balance between stresses and load-bearing capacity. Mechanical loading of bone is an important regulator of bone turnover. Loading of bone can lead to new bone formation, whereas unloading of bone can lead to bone loss. Multiple studies have shown that PGs mediate some of the anabolic effects of mechanical loading on bone (Li *et al.*, 2006a). New bone formation resulting from loading of an isolated avian ulna preparation *in vitro* or from loading of rat tail vertebrae *in vivo* was blocked by inhibiting PG production (Chow and Chambers, 1994; Pead and Lanyon, 1989). Several studies showed PG-dependent new bone formation in bone explants subjected to cyclic mechanical loading *in vitro* and suggested that the PG mediating these effects was PGI_2 (Cheng *et al.*, 1997; Lanyon, 1992; Rawlinson *et al.*, 1991). PGs produced in response to loading are thought to be largely the result of loading-induced COX-2 expression, and a selective COX-2 inhibitor (NS-398) prevented endosteal bone formation in a rat tibial bending model (Forwood, 1996). In humans, loading increased release of PGE_2 in the tibial metaphysis, measured by *in situ* microdialysis (Thorsen *et al.*, 1997), which was blocked by selective inhibition of COX-2 activity (Langberg *et al.*, 2003). However, selective COX-2 inhibitors can inhibit both COX isoforms at high doses, and immunohistochemical localization of COX-1 and COX-2 after tibial bending in rats suggested involvement of both isoforms in loading responses (Forwood *et al.*, 1998). In addition, another study reported no difference between COX-2 WT and KO mice in their periosteal anabolic responses to ulnar loading and suggested that COX-1 compensated for absent COX-2 (Alam *et al.*, 2005). It is possible that HPTH (either secondary or primary) might have compensated to some extent for COX-2 deficiency in this latter study. PGs may also be involved in other effects of loading. Orthodontic tooth movement stimulates PG production, and PGs enhance orthodontic movement of teeth, presumably as a result of PG-stimulated bone resorption (Giunta *et al.*, 1995; Kehoe *et al.*, 1996; Sandy *et al.*, 1993).

External forces applied to bone result in small deformations, or strains, in the bone matrix, which are converted into intracellular signals that frequently lead to new gene transcription (Pavalko *et al.*, 2003; Turner and Pavalko, 1998). Although the specifics of how mechanical loading is coupled to cellular responses in bone are still being debated, as well as which cells are involved, interstitial fluid flow in the lacunar-canalicular network in bone, where large stresses on cells can be generated by fluid flow in narrow channels, is likely to play an important role (Burger and Klein-Nulend, 1999; Han *et al.*, 2004; Hillsley and Frangos, 1994; Knothe *et al.*, 1998; Srinivasan and Gross, 2000; Turner *et al.*, 1994; Weinbaum *et al.*, 1994). *In vivo,* osteocytes may be the targets because of their location in the lacunar-canalicular network, but *in vitro* many cell types are mechanosensitive, and fluid shear stress (FSS) is often modeled *in vitro* by subjecting osteoblasts as well as osteocytes, plated on fixed surfaces, to cyclic laminar fluid flow.

When osteoblasts are subjected to FSS, there is an early release of PGE_2, probably due to FSS-stimulated release of AA that is converted to PGs by constitutively expressed

COX-1, followed by a more sustained production of PG (Klein-Nulend *et al.*, 1997; Reich and Frangos, 1993). FSS induces COX-2 mRNA expression and PG production in primary mouse osteoblastic cells with little effect on COX-1 mRNA levels, and the induction of COX-2 correlates with the sustained production of PG (Klein-Nulend *et al.*, 1997). In MC3T3-E1 cells, 5 minutes of FSS is sufficient to induce COX-2 mRNA (Saegusa *et al.*, 2004). Other studies on MC3T3-E1 cells have shown that the induction of COX-2 involves cytoskeletal-integrin interactions (Pavalko *et al.*, 1998) but does not require intact microfilaments or microtubules (Norvell *et al.*, 2004). FSS induced PGE_2 may also mediate intracellular communication via gap junctions through the prostaglandin EP2 receptor (Cherian *et al.*, 2003). Using MC3T3-E1 cells stably transfected with a COX-2 promoter-luciferase reporter construct and calvarial cells from mice transgenic for this construct, we showed pulsatile FSS as low as 0.1 dyn/cm^2 induced new COX-2 gene transcription via the ERK signaling pathway (Wadhwa *et al.*, 2002b). There are multiple signaling pathways upstream of ERK that may be involved in the effects of FSS on osteoblasts (Hughes-Fulford, 2004). We found the induction of COX-2 in osteoblasts by 4 hours of pulsatile FSS to be dependent on a PKA pathway (Wadhwa *et al.*, 2002a), whereas the early induction by 1 hour of FSS, followed by return to static culture, was dependent on a protein kinase D (PKD) pathway (Mehrotra *et al.*, 2004).

COX-2: INFLAMMATION AND CANCER

PG production is frequently found to be increased in inflammatory processes, such as rheumatoid arthritis. Because many cytokines involved in inflammation are potent inducers of COX-2 expression and PGE_2 production and because PGE_2 is a potent stimulator of bone resorption, it is often assumed that COX-2 derived PGE_2 contributes to the bone loss and cartilage destruction associated with inflammatory diseases. There are some studies to support this conclusion, showing that NSAIDs decrease alveolar bone loss in periodontitis (Howell *et al.*, 1991; Jeffcoat *et al.*, 1993). In addition, inflammatory responses are reduced in mice null for mPGES-1 (Kamei *et al.*, 2004) and in COX-1 and COX-2 KO mice (Langenbach *et al.*, 1999b). However, cytokines themselves are potent inducers of bone resorption, independent of PG production. In a study in which IL-1 was injected *in vivo* above the calvaria of mice for 3 days, IL-1 stimulated resorption was initially PG-independent (Boyce *et al.*, 1989a). However, after the IL-1 injections were stopped, increased resorption continued for 3 to 4 weeks, and this resorption was PG-dependent. Hence, PGs produced by proinflammatory cytokines may be important in prolonging resorption responses to cytokines.

Inflammatory bone loss may reflect not only increased bone resorption but also decreased bone formation. Cytokines can inhibit production of bone matrix proteins independently of cytokine-induced PG production (Rosenquist *et al.*, 1996). It is possible that cytokine-induced PG production might stimulate osteoblastic differentiation and have a compensatory effect for the catabolic actions of cytokines. In IL-1α transgenic mice, treatment *in vivo* with an inhibitor of COX-2 reduced joint inflammation but increased osteopenia by suppressing bone formation (Niki *et al.*, 2007). PGs themselves may also have anti-inflammatory properties (Gomez *et al.*, 2005; Hata and Breyer, 2004). Some studies have suggested that COX-2 metabolites are proinflammatory only during the early stages of an inflammatory response; and, at later times, COX-2 generates a different set of PGs, the cyclopentenone PGs derived from PGD_2, which have anti-inflammatory effects (Rajakariar *et al.*, 2006).

Inflammation has been associated with the progression of many cancers (Harris, 2007; Sarkar *et al.*, 2007). A role for PGs in tumorigenesis was initially suggested by epidemiological studies showing a reduction in the incidence of colorectal cancer in individuals taking NSAIDs and by studies showing constitutive upregulation of COX-2 in tumor cells. Hundreds of studies have now implicated COX-2 and PGE_2 in tumorigenesis in multiple tissues, including colon, breast, prostate, and lung, and COX-2 produced PGE_2 has been shown to enhance tumor cell growth and survival, promote angiogenesis, and facilitate metastasis (Cha and DuBois, 2007; Trifan and Hla, 2003; Wang and DuBois, 2006). One caveat is that many studies used NSAIDs at much higher concentrations than required to inhibit PGE_2 synthesis, and selective COX-2 inhibitors at high doses can have effects independent of PGE_2 inhibition to reduce cell growth and survival (Grosch *et al.*, 2006; Jang *et al.*, 2004). Studies have also suggested that genetic variants in the COX-2 gene or promoter, or chromosomal gain at the COX-2 locus associated with COX-2 overexpression, may correlate with the risk for various cancers or with survival of cancer patients (Ali *et al.*, 2005; Cox *et al.*, 2004; Gallicchio *et al.*, 2006; Gao *et al.*, 2007a; Knosel *et al.*, 2004; Shahedi *et al.*, 2006; Tan *et al.*, 2007; Zhang *et al.*, 2005). Use of selective COX-2 inhibitors to treat or prevent cancer is currently contraindicated because these inhibitors may increase the risk for cardiovascular problems, including myocardial infarction and stroke (Fitzgerald, 2004; Grosser *et al.*, 2006). Hence, there is interest in developing therapies against downstream targets, such as mPGES-1 (Mehrotra *et al.*, 2006b), to inhibit PGE_2 and not PGI_2, which may have beneficial cardiovascular effects, or against specific PGE_2 receptors involved in the tumor-promoting effects (Fulton *et al.*, 2006; Ma *et al.*, 2006).

The role of COX-2 in osteosarcomas has not yet been determined. Several studies have shown COX-2 to be expressed in osteosarcomas (Dickens *et al.*, 2003; Mullins *et al.*, 2004) and in cell lines derived from osteosarcomas (Wong *et al.*, 1997; Min *et al.*, 1998). Although one study reported that high COX-2 expression positively correlated with osteosarcoma grade (Masi *et al.*, 2007), another study reported that expression of COX-2 was characteristic of more benign osteoblastomas and not high-grade osteoblastic osteosarcomas (Hosono *et al.*, 2007). Our own research would argue that COX-2 overexpression may not enhance growth and survival of osteosarcoma cells. We observed that COX-2 mRNA expression was not detectable by Northern analysis in rat osteosarcoma ROS 17/2.8 cells (Pilbeam *et al.*, 1997b). We found expression and regulation of COX-2 to be quite variable among Saos-2, U2OS, and TE85 human osteosarcoma cell lines (Xu *et al.*, 2006b). Expression, measured by RT-PCR or Northern analysis, ranged from easily measurable constitutive or inducible expression to no detectable constitutive or inducible expression. Similar variability was seen in three "strains" of Saos-2 cells, all derived originally from the same tumor. (We used "strain" to denote cells carried for several years by different laboratories.) Although COX-1 was constitutively expressed in all three Saos-2 strains, PGE_2 production was not measurable ($<1\,pg/10^5$ cells) in the media of cells with little COX-2 mRNA expression. We overexpressed COX-2 in osteosarcoma cells, by both stable transfection and retroviral infection, and found that overexpression of COX-2 decreased cell growth and increased apoptosis, and these effects were not reversed by NSAIDs (Xu *et al.*, 2006b). Increased apoptosis was secondary to increased production of reactive oxygen species (ROS), probably from the COX-2 peroxidase site, and inhibition of ROS production by antioxidants blocked the COX-2 effect on apoptosis. In contrast, COX-2 overexpression decreased apoptosis and ROS in a colon cancer cell line, consistent with other studies in colon carcinoma cells, suggesting that the promotion of tumorigenesis by COX-2 overexpression is tissue/cell dependent. On the other hand, a recent study of U2OS cells reported that overexpression of COX-2 was associated with increased cell number, which could be decreased by treatment with high-dose NS-398 or celecoxib (Lee *et al.*, 2007). However, treatment of osteosarcoma cells with high-dose NS-398 has also been shown to have effects independent of COX-2 inhibition (Moalic *et al.*, 2001).

OMEGA-3 FATTY ACIDS AND BONE

Omega-3 (ω-3 or n-3) fatty acids have been proposed as dietary supplements to treat cardiovascular disease and inflammatory conditions (Calder, 2006; Fritsche, 2006; Mozaffarian and Rimm, 2006; Mozaffarian, 2007). Omega-3 and ω-6 essential fatty acids are derived from the dietary precursors, linoleic acid and α-linolenic acid, respectively. Linoleic acid and α-linolenic acid are converted via an alternating series of desaturations and elongations into AA and EPA, respectively (Vaddadi, 2004). EPA can be further converted into docosahexaenoic acid (DHA). In addition to being derived endogenously from α-linolenic acid, EPA and DHA can be obtained directly from the diet, primarily from fish oils. Both AA and DHA are abundant at the *sn*-2 site in structural phospholipids. AA and EPA, but not DHA, are substrates for COX.

The beneficial effects of ω-3 fatty acids are likely to be multifactorial (Calder, 2006; Wada *et al.*, 2007). Direct effects might be due to incorporation of ω-3 fatty acids into membranes and replacement of AA. This might change the physical properties of the membrane and reduce production of the 2-series of prostanoids by both the COX-1 and COX-2 pathways (Wada *et al.*, 2007). In rat bone organ cultures, PGE_3 and PGE_2 were equipotent in stimulating bone resorption, but there was less PGE_3 produced from EPA compared to PGE_2 produced from AA (Raisz *et al.*, 1989). Indirect effects of ω-3 fatty acids include alteration of the expression of inflammatory genes through effects on transcription factor activation (Calder, 2006; Wada *et al.*, 2007). In addition, EPA and DHA can give rise, via the lipoxygenase pathway, to a recently identified family of anti-inflammatory mediators called resolvins that may be important in the resolution phase of inflammation (Schwab *et al.*, 2007; Serhan, 2007).

Some animal studies suggest that ω-3 fatty acids may reduce bone loss due to ovariectomy (Sun *et al.*, 2003; Watkins *et al.*, 2006), inflammatory diseases (Bhattacharya *et al.*, 2005; Kesavalu *et al.*, 2006), and aging (Bhattacharya *et al.*, 2007). However, one study reported that high-dose EPA supplementation exacerbated the effects of ovariectomy on BMD (Poulsen and Kruger, 2006). Whether these animal studies are applicable to humans is unclear (Fritsche, 2007), and there are few human observational studies. One recent study, which correlated levels of serum fatty acid with BMD in young men, suggested that ω-3 fatty acids, especially DHA, are positively associated with bone mineral accrual and peak BMD (Hogstrom *et al.*, 2007). Another study, which relied on self-reported increased dietary intake, found that a higher ratio of ω-6 to ω-3 fatty acids was associated with lower BMD at the hip in both sexes (Weiss *et al.*, 2005). Frequent consumption of fish has also been associated with significantly greater BMD in Japanese postmenopausal women (Ishikawa *et al.*, 2000). Given their proposed effects on inflammation, perhaps the greatest effect of ω-3 fatty acids might be expected to be seen in inflammatory bone loss. However, a high-dose trial of fish-oil supplementation to patients with Crohn's disease did not show any effect on markers of bone turnover (Trebble *et al.*, 2005). More interventional trials are needed to determine if ω-3 fatty acid supplementation is beneficial for bone.

NSAIDs AND BONE LOSS IN HUMANS

Several studies have examined the effects of nonselective NSAIDs on BMD in humans. In a study of postmenopausal women 65 years of age or older, there was no significant difference found in N-telopeptide cross-link excretion, a marker of bone resorption, between self-reported users of NSAID or aspirin compared to nonusers (Lane *et al.*, 1997). In a similar cohort, the use of aspirin or NSAIDs was found to be associated with a small (1–3%), but significant, increase in BMD of the hip and spine, but there was no clinically significant protective effect on risk for fracture (Bauer *et al.*, 1996). In a study of older women from Rancho Bernardo, California, the regular use of propionic acid NSAIDs (ibuprofen, naproxen, ketoprofen), but not acetic acid NSAIDs (indomethacin, diclofenac, sulindac, tolmetin), was associated with higher BMD at multiple skeletal sites (Morton *et al.*, 1998). Those who concurrently used estrogen and propionic acid NSAIDs had the highest BMD at all sites. Another study stratified relative COX-1 and COX-2 selectivity of traditional NSAIDs and compared effects on men and women, mean age 74 years (Carbone *et al.*, 2003). Their data suggested that the combination of relatively COX-2 selective traditional NSAIDs and aspirin was associated with higher BMD at multiple skeletal sites in men and women. On the other hand, a cross-sectional study of men, average age 74 years, found no association between markers of bone turnover and NSAID use (Cauley *et al.*, 2005). A recent study examined effects of selective COX-2 inhibitors on BMD in men and women, age 65 and older (Richards *et al.*, 2006). In men, daily COX-2 inhibitor use was associated with a 2.4% to 5.3% *lower* hip and spine BMD compared to non-users. In contrast, in postmenopausal women not on estrogen replacement therapy, daily COX-2 inhibitor use was associated with a 0.9% to 5.7% *higher* BMD at hip and spine sites. The authors postulated that beneficial effects of mechanical loading might be reduced by COX-2 inhibition in men, whereas the proinflammatory state associated with estrogen withdrawal might be suppressed by COX-2 inhibition in postmenopausal women.

One factor that may complicate the interpretation of clinical studies with NSAIDs is the variability between individuals in their response to NSAIDs. A study of celecoxib and rofecoxib in healthy humans found considerable variability at an individual level in the degree of COX-2 inhibition and selectivity attained by both drugs (Fries *et al.*, 2006). Approximately one third of the variability was attributable to differences between individuals, suggesting the contribution of genetic sources of variance. Another potentially complicating factor is that some drugs thought to be devoid of significant PG inhibition, and therefore not taken into account in the studies, may also be COX inhibitors. An example is acetaminophen (paracetamol), which has recently been shown to have substantial COX-2 inhibition (Hinz *et al.*, 2007).

The highly selective inhibitors of COX-2, such as rofecoxib and celecoxib, were developed to minimize gastrointestinal complications of traditional NSAIDs, thought to be secondary to suppression of COX-1-derived PGs. Subsequently, clinical studies showed the COX-2 selective inhibitors to elevate the risk of myocardial infarction and stroke (Grosser *et al.*, 2006). Hence, it is unlikely that large clinical studies with highly selective COX-2 inhibitors will be undertaken to examine effects on bone in healthy humans, at least not until the cardiovascular risks are better understood. Given the potential importance of inhibiting the COX-2 pathway in treating or preventing cancer, it is likely that new drugs will be developed to target the involved tissues or to decrease specific downstream prostanoids.

LIPOXYGENASE AND BONE

An alternate pathway for AA metabolism is the lipoxygenase (LOX) pathway (Fig. 9). The three major forms of LOXs in mammalian species, 5-LOX, 12-LOX, and 15-LOX, initially oxidize AA to 5-, 12- and 15-hydroperoxyeicosatetraenoic acid (HPETE), respectively (Brash, 1999). Each HPETE can then be reduced by glutathione peroxidase to the corresponding 5-, 12-, and 15-hydroxyeicosatetraenoic acid (HETE). 5-LOX is a bifunctional enzyme that also metabolizes 5-HPETE to leukotriene (LT) A_4. The leukotrienes LTC_4, LTD_4, and LTE_4 are called peptidoleukotrienes (peptido-LT). Similar to PGs, LTs are generally considered to be proinflammatory agents. More recently identified eicosanoid products of the LOX pathway, lipoxins and resolvins, are proposed to be anti-inflammatory and to mediate processes required for resolution of acute inflammation (Serhan, 2007). Lipoxins are generated from AA via various LOX pathways or as the products of aspirin-acetylated COX-2 and may reduce bone loss associated with inflammation (Serhan *et al.*, 2003). Resolvins are derived via the LOX pathway from ω-3 PUFAs.

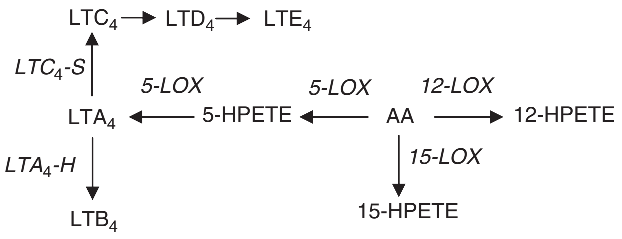

FIGURE 9 Lipoxygenase (LOX) pathways. 5-, 12-, and 15-LOX catalyze the insertion of molecular oxygen into arachidonic acid (AA) producing 5-, 12-, and 15-hydroperoxy-eicosatetraenoic acid (HPETE), respectively. Each HPETE can then be reduced by glutathione peroxidase to the corresponding 5-, 12-, and 15-hydroxyeicosatetraenoic acid (HETE). 5-LOX is a bifunctional enzyme that can also metabolize 5-HPETE to leukotriene A_4 (LTA_4). LTA_4 is converted to LTC_4 by LTC_4-synthase (LTA_4-S) and to LTB_4 by LTA_4 hydrolase (LTA_4-H). LTC_4 is unstable and converted rapidly to LTD_4 and LTE_4 by transpeptidases. Lipoxins can be generated by pathways initiated by both 5- or 15-LOX.

Although less studied than products of the COX pathway, products of the LOX pathway have been shown to play active roles in bone metabolism. A number of studies have shown LTs to directly stimulate resorption. In neonatal mouse organ cultures, LTs and HETEs had biphasic effects on bone resorption, with low doses stimulating and high doses inhibiting resorption (Meghji *et al.*, 1988). Effects of some LTs were partially inhibited by indomethacin, suggesting mediation by PGs. A cell line established from giant-cell tumors of bone produced an activity in the medium that stimulated bone resorption in neonatal mouse calvariae and pit formation by isolated rat osteoclasts on dentine (Gallwitz *et al.*, 1993). This activity was identified as 5-HETE and peptido-LT. Peptido-LTs were also shown to stimulate isolated avian osteoclasts to form resorption lacunae (Garcia *et al.*, 1996b). Isolated avian osteoclasts were shown to express both high and low affinity LTB$_4$ receptors, and LTB$_4$ increased both TRAP activity and pit formation by these osteoclasts (Flynn *et al.*, 1999). LTB$_4$ increased osteoclastic bone resorption and osteoclast numbers and eroded surfaces *in vivo* following local administration over the calvariae of normal mice and *in vitro* in organ cultures of neonatal mouse calvariae (Garcia *et al.*, 1996a). LTB$_4$ also increased the formation of resorption lacunae by isolated neonatal rat osteoclasts. Recently, LTB$_4$ was reported to induce osteoclastogenesis in cultured human PBMCs by a RANKL-independent mechanism (Jiang *et al.*, 2005).

Several studies have shown products of the LOX pathway to have negative effects on bone formation. LTB$_4$, LTD$_4$, and 5-HETE inhibited BMP-2 induced bone formation in mouse calvarial organ culture and blocked the formation of mineralized nodules in response to BMP-2 and dexamethasone in fetal rat calvarial cell culture (Traianedes *et al.*, 1998). 5-LOX KO mice were reported to have increased bone biomechanical properties and cortical thickness (Bonewald *et al.*, 1997). Recently, the mouse 15-LOX gene was identified as a negative regulator of peak BMD (Klein *et al.*, 2004). 15-LOX KO mice had higher femoral BMD and biomechanical properties than WT mice. Transient overexpression of 15-LOX in murine bone marrow stromal cell cultures reduced alkaline phosphatase activity and osteocalcin secretion. Pharmacological inhibitors of 15-LOX improved BMD and bone strength in two rodent models of osteoporosis. In contrast, overexpression of human 15-LOX in transgenic rabbits was shown to protect against bone loss associated with periodontal disease (Serhan *et al.*, 2003). This effect was attributed to the enhanced production of anti-inflammatory metabolite lipoxins in the rabbits.

The identification of 15-LOX as a susceptibility gene for peak BMD in mice may have relevance for human osteoporosis. An autosomal genome screen of 17 large pedigrees in humans detected linkage for hip, spine, and wrist BMD to the 17p13.1 chromosomal region, where 12- and 15-LOX genes are located (Deng *et al.*, 2002; Devoto *et al.*, 1998; Funk *et al.*, 2002). Another study reported association between a single polynucleotide polymorphism (SNP) located in the 5 -flanking region of the 15-LOX gene and total BMD in postmenopausal Japanese women (Urano *et al.*, 2005). Although the human 15-LOX gene shares significant sequence homology with the murine 15-LOX gene, the 12-LOX gene of humans is functionally more related to the 15-LOX gene of mice (Chen *et al.*, 1994). Considering this, two independent studies recently looked at the contributions of multiple SNPs in the 15- and 12-LOX genes of Caucasian men and women and found that polymorphisms in the 12-LOX gene, but not the 15-LOX gene, were associated with variations in hip and spine BMD (Ichikawa *et al.*, 2006; Mullin *et al.*, 2007).

It is possible that some effects of the LOX pathway may occur via PPARγ-dependent mechanisms. Products of the LOX pathway may be ligands for PPARγ (Bishop-Bailey and Wray, 2003). PPARγ haploinsufficient mice have increased BMD (Akune *et al.*, 2004), and an association between a SNP of the PPARγ gene and total body BMD was reported in postmenopausal women (Ogawa *et al.*, 1999).

SUMMARY

PGs are abundantly expressed in bone, and their production is highly regulated by multiple local and systemic factors that induce COX-2 expression. *In vitro,* PGE$_2$ generally stimulates the differentiation of both osteoblasts and osteoclasts, but inhibitory effects on bone formation and resorption are also seen. *In vivo,* the predominant effects of exogenous PGE$_2$ are to stimulate both bone formation and bone resorption, but the relative magnitude of these can vary so that in some models there is net bone loss whereas in others there is a gain in bone mass. Some of the complexity of PGE$_2$ actions may be explained by the multiplicity of receptors for PGE$_2$. Many studies have now implicated COX-2 and PGE$_2$ in the promotion of tumorigenesis, suggesting that PGE$_2$ functions as a general regulator of cell growth and apoptosis. Studies of the role of COX-2 and PGE$_2$ in cancer have highlighted new and complex signaling pathways for the EP2 and EP4 receptors that may also be important for PGE$_2$ effects in bone cells. The development of selective inhibitors for COX-2 and selective agonists and inhibitors for EP receptors, as well as transgenic mice deficient in PG synthetic enzymes and receptors, has led to a better understanding of the role of PGs in fracture healing and to the development of potential therapeutic agents to increase bone mass and accelerate fracture healing. Despite the availability of mice deficient in COX-2, the effects of endogenously produced PGs on bone remodeling under physiologic and pathologic conditions remain elusive. Reasons for this may be the redundancy of the PG pathways, as well as potential compensatory mechanisms involving other hormones, such as PTH. Although this complexity makes it more difficult to understand fully the role of PGs in bone, further studies that clarify the mechanisms of PG action could lead to a better understanding of the physiology and pathophysiology of bone and the discovery of new therapeutic applications of altering PGs in skeletal disorders.

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Chapter 63

Parathyroid Hormone-Related Peptide and Other Systemic Factors in Skeletal Manifestations of Malignancy

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INTRODUCTION

Hypercalcemia is one of the most frequent paraneoplastic syndromes and often occurs in advanced stages of the disease. The median survival rate often varies from 6 to 10 weeks following the onset of hypercalcemia (Body *et al.*, 2000); however, breast cancer patients experience a somewhat longer median survival of 3 to 4.5 months (De Wit and Cleton, 1994).

The association between elevated blood calcium and neoplastic disease was first noted in the 1920s (Zondek *et al.*, 1923), when it was postulated that release of calcium from bone by the direct osteolytic action of malignant cells was responsible for hypercalcemia associated with malignancy. However, hypercalcemia was often accompanied by hypophosphatemia, the hallmark of hyperparathyroidism (HPT), and also occurred in the absence of skeletal metastases. In 1941 it was hypothesized that a malignant tumor might release a systemically active factor resulting in hypercalcemia (Albright, 1941). This hypothesis was raised when a patient with a renal carcinoma, metastatic to bone, presented with hypercalcemia and hypophosphatemia in the absence of any obvious parathyroid dysfunction. The patient's biochemical abnormalities were temporarily normalized following tumor reduction but became apparent again as the tumor resumed its growth. It was reasoned that since hyperphosphatemia did not accompany hypercalcemia, despite evidence of osteolysis, perhaps the tumor was secreting a phosphaturic substance such as parathyroid hormone (PTH). Although no bioassayable hormone was detected in the tumor, the important observation had been made that hypercalcemia associated with malignancy (MAH) could have a humoral basis and that the factor might be related to PTH. Despite the striking similarities between the biochemical manifestations of HPT and MAH that precipitated Albright's hypothesis, it subsequently became apparent that there were also a number of important differences between these syndromes. Early attempts at the differential diagnosis of these hypercalcemic disorders identified not only subnormal concentrations of immunoreactive PTH (Powell *et al.*, 1973) and no detectable PTH mRNA in the tumors (Simpson *et al.*, 1983), but also a mild hypokalemic alkalosis in patients with malignancy-associated hypercalcemia (MAH). In contrast, elevated PTH and mild metabolic acidosis were associated with HPT (Lafferty, 1966). The search for a novel protein with PTH-like bioactivity intensified using several bioassays that had been developed for PTH (Goltzman *et al.*, 1981; Nissenson *et al.*, 1981; Rodan *et al.*, 1983). Using both *in vivo* and *in vitro* approaches, PTH-like bioactivity was identified in the blood and in tumor extracts from patients with MAH. These same bioassays were used in various combinations during the purification of a novel protein, named parathyroid-hormone-like peptide (PLP), from tumor tissue (Burtis *et al.*, 1987; Moseley *et al.*, 1987; Strewler *et al.*, 1987). When the gene was cloned and demonstrated to be structurally homologous to PTH, the protein was renamed parathyroid-hormone-related protein, or PTHrP (Mangin *et al.*, 1989; Suva *et al.*, 1987).

The term *humoral hypercalcemia of malignancy* (HHM) was later coined to distinguish the syndrome caused by a circulating factor, now known to be PTHrP, from hypercalcemia caused by the direct lytic action of tumor cells in bone. The demonstration that neutralizing antibodies to PTHrP could reduce or eliminate the hypercalcemia in animal models of HHM (Kukreja *et al.*, 1988; Henderson *et al.*, 1990) provided strong evidence that this was indeed the causal factor of this syndrome. HHM was associated primarily with renal cell carcinomas and squamous cell cancers originating in a variety of primary sites,

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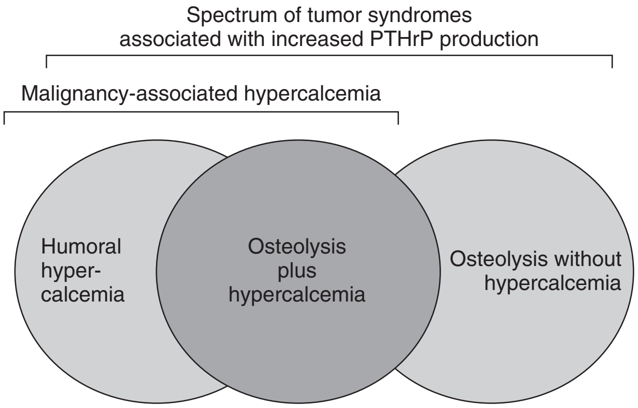
Spectrum of tumor syndromes associated with increased PTHrP production
Malignancy-associated hypercalcemia
Humoral hyper-calcemia
Osteolysis plus hypercalcemia
Osteolysis without hypercalcemia

FIGURE 1 Spectrum of tumor syndromes associated with increased PTHrP production. PTHrP may be produced and released by tumors that have not metastasized to bone. Circulating PTHrP can then produce hypercalcemia via an endocrine mechanism ("Humoral Hypercalcemia of Malignancy"). Other tumors that have metastasized to bone may also produce and release PTHrP. Hypercalcemia associated with these tumors may result from osteolysis but also from circulating PTHrP acting in an endocrine mode ("Osteolysis plus Hypercalcemia"). Some tumors that have metastasized to bone may produce PTHrP, but although osteolysis may occur, circulating concentrations are not increased and hypercalcemia does not occur ("Osteolysis without Hypercalcemia").

whereas hypercalcemia due to local osteolysis was commonly associated with metastatic breast disease and with hematologic malignancies. Nevertheless, it is now clear that the "direct" lytic action of tumor cells is also mediated in part by PTHrP, and that PTHrP released by these tumors may also act on a systemic basis (Fig. 1).

MOLECULAR AND CELLULAR BIOLOGY OF PTHrP

Characteristics of the PTHrP Gene

The genes encoding PTH and PTHrP have been localized to the short arms of human chromosomes 11 and 12, respectively, placing them among syntenic groups of functionally related genes and suggesting a common ancestral origin (Goltzman *et al*., 1989). Similarities in their structural organization and in the functional properties of their amino termini provide further support for the hypothesis that *PTH* and *PTHrP* are members of a single gene family. The human *PTHrP* gene is a complex unit that spans more than 15 kb of DNA. Its mRNA is transcribed from at least three promoters and undergoes differential splicing, giving rise to heterogenous mRNA species. The cDNA encodes a prototypical secretory protein with predicted mature isoforms of 139, 141, and 173 amino acids. The rat *PTHrP* gene is driven by a single promoter, and encodes a protein of 141 amino acids with marked sequence homology to human PTHrP up to residue 111, which suggests conserved functionality (Yasuda *et al*., 1989). Indeed species conservation of PTHrP is evident with high homology across a variety of mammalian species (Burtis *et al*., 1992).

PTHrP Overproduction in Malignancy

PTHrP is widely distributed in embryonic and adult tissues and has essential functions in development and growth. These include roles in the normal development of the cartilaginous growth plate (Amizuka *et al*., 1994), in bone anabolism (Miao *et al*., 2005), in mammary gland development including the development of mammary ducts and nipple formation (Foley *et al*., 2001), in calcium transport across the placenta (Kovacs *et al*., 1996), in smooth muscle relaxation, in vasodilatation (Schordan *et al*., 2004), and in tooth eruption (Philbrick *et al*., 1998). In view of its broad distribution pattern in many cell types, overproduction of PTHrP by malignant cells most likely results from deregulated expression of an endogenous protein during the process of malignant transformation, that is, "eutopic" rather than "ectopic" production.

A number of potential mechanisms have been invoked to explain overexpression of PTHrP in malignant cells, including gene amplification (Sidler *et al*., 1996) and alterations in the methylation status of critical regulatory regions of the gene during neoplastic transformation (Broadus and Stewart, 1994; Ganderton and Briggs, 1997). Furthermore, the complex organization of the human PTHrP gene suggests that changes in tissue-specific promoter usage or splice variants might also contribute to overproduction of PTHrP during the process of malignant transformation. One extensive clinical study examined a variety of tumors, including 13 breast malignancies, with exon-specific probes to identify transcripts arising from all three promoters and from the different 3′ splice variants (Southby *et al*., 1995). Although the authors failed to identify tissue-specific or tumor-specific transcripts, they did show that PTHrP was transcribed from multiple promoters in tumor samples, compared with a single promoter in normal tissue harvested from the same individual. The resulting overall increase in transcription could then lead to cumulative overexpression of the protein in neoplastic tissue. Others, however, have reported the presence of specific transcripts related to the stage of cancer progression. Thus Bouizar *et al*. (1999) investigated the differential expression of PTHrP isoforms in different stages of breast cancer progression as a result of differential use of a downstream TATA promoter. The amount of mRNA encoding the 1–139 isoform was much higher in the tumors of patients who later developed metastases than in those of patients who developed no metastases. The mRNA encoding this isoform was also more abundant in breast tumors from patients who developed bone metastases than in those of patients who developed metastases in soft tissues. In contrast the amounts of mRNA encoding the 1–141 isoform were similar in these different groups.

Regulation of PTHrP Production

Growth Factors and Cytokines

A variety of growth factors have been found to be potent stimulators of *PTHrP* gene transcription (Kremer *et al.*, 1991; Sebag *et al.*, 1994). Thus, basal levels of mRNA and unstimulated release of PTHrP into conditioned medium were both significantly higher in Ras-transformed keratinocytes, a model of epithelial carcinogenesis, compared with their nontransformed counterpart (Henderson *et al.*, 1991; Kremer *et al.*, 1991; Sebag *et al.*, 1992), and PTHrP gene expression could be stimulated by EGF. In contrast, insulin-like growth factor I (IGF-I) was a more effective stimulator of gene transcription in breast epithelial cells (Sebag *et al.*, 1994). Signaling by growth factors through receptor tyrosine kinases (RTKs) therefore appears to be an important mechanism for regulating PTHrP gene expression, and oncogenic transformation by the Ras component of the RTK signal transduction pathway in many malignant cells. Transfection of a constitutively active derivative of the hepatocyte growth factor receptor known as Tpr-Met into PTHrP-producing cells resulted in a substantial increase in the expression and release of PTHrP (Aklilu *et al.*, 1996), whereas introduction of a point mutation into Tpr-Met, which prevented its association with the Ras signaling pathway, led to a significant reduction in PTHrP expression and release. Similar reductions were observed in cells treated with agents that inhibit Ras function by preventing it from anchoring to the cell membrane and transducing a signal (Ras farnesylation inhibitors; Aklilu *et al.*, 1997, 2000). These studies demonstrated an important role for Ras in enhancing PTHrP production in transformed cells.

Other growth factors and cytokines have also been found to play a critical role in PTHrP overproduction, including, notably, transforming growth factor β (TGFβ). PTHrP mRNA is typically unstable with a short half-life, but cytokines such as TGFβ increase PTHrP mRNA stability (Sellers *et al.*, 2004).

Steroid Hormones

Several steroid hormones have been reported to influence the production of PTHrP. Thus estradiol has been reported to inhibit PTHrP gene promoter activity in breast cancer cell models *in vitro* and to diminish PTHrP production *in vivo* (Rabbani *et al.*, 2005). Similarly, androgens (dihydrotestosterone) have also been shown to inhibit PTHrP production in models of prostate cancer both *in vitro* and *in vivo* at least in part via a transcriptional mechanism (Pizzi *et al.*, 2003). Glucocorticoids have also been shown to inhibit PTHrP production (Sebag *et al.*, 1994; Tenta *et al.*, 2005; Glatz *et al.*, 1994).

The secosteroid 1,25 dihydroxyvitamin D (1,25$(OH)_2$D) has been shown to inhibit PTHrP gene transcription (Kremer *et al.*, 1991; Sebag *et al.*, 1992). A 1,25$(OH)_2$D responsive repressor sequence was identified between bases -1121 to -1075 upstream of the single promoter in the rat *PTHrP* gene (Kremer *et al.*, 1996a). Using nuclear extracts prepared from normal human keratinocytes in mobility shift analyses, it was determined that the PTHrP repressor site bound a vitamin D receptor (VDR)-retinoid X receptor (RXR) heterodimer. The VDR-RXR complex was also identified in extracts of nuclei from immortalized keratinocytes but not from the Ras transformed cells that had previously shown resistance to 1,25$(OH)_2D_3$-induced inhibition of PTHrP expression (Solomon *et al.*, 1998). Expression of wild-type RXRα in the transformed cells resulted in reconstitution of the VDR-RXR heterodimer. Subsequent work by the same group determined that disruption of the VDR-RXR complex in Ras transformed keratinocytes resulted from mitogen-activated protein kinase (MAPK)-stimulated phosphorylation of RXRα by the activated Ras/Raf/MAPK pathway (Solomon *et al.*, 1999). Analogues of vitamin D (EB1089) were also found to inhibit PTHrP production and prevents the development of malignancy-associated hypercalcemia in an animal model *in vivo* (Haq *et al.*, 1993).

Viral Proteins

Viral proteins, notably Tax, have been implicated in transcriptional stimulation of PTHrP production in malignant states. TAX is a 40-kDa nuclear phosphoprotein that transactivates its own promoter as well as those of a number of host genes. It interacts with a variety of transcription complexes that bind to DNA consensus elements in the *PTHrP* promoter including the cAMP response element, Ets-1, serum response element, and the AP-1 binding site (Dittmer *et al.*, 1993; Ejima *et al.*, 1993). PTHrP mRNA has been identified in samples harvested from asymptomatic HTLV-I carriers as well as from leukemic cells of ATL patients (Motokura *et al.*, 1989), and elevated circulating levels of PTHrP were detected in most hypercalcemic individuals in the acute phase of the disease (Ikeda *et al.*, 1994). In view of the documented role of human T-cell leukemia virus type I (HTLV-I) infection in the pathogenesis of adult T-cell leukemia-lymphoma (ATL) and the high incidence of PTHrP overexpression in these patients, it was proposed that the TAX might stimulate PTHrP gene transcription. The MT-2 cell line, in which TAX is overexpressed, was used to determine that PTHrP gene transcription was indeed enhanced and that maximal stimulation of *PTHrP* involved activation of PKA and PKC (Ikeda *et al.*, 1995).

Calcium

Various well-established cancer cell lines have been found to express both CaR mRNA and protein (Cattopadhyay, 2006.). These include cell lines derived from breast cancer (MCF-7, MDA-MB-231; Sanders *et al.*, 2000), and prostate cancer (PC-3, LnCaP; Sanders *et al.*, 2001).Both pharmacological and molecular evidence indicates that elevated Ca^{2+} upregulates PTHrP synthesis and release via

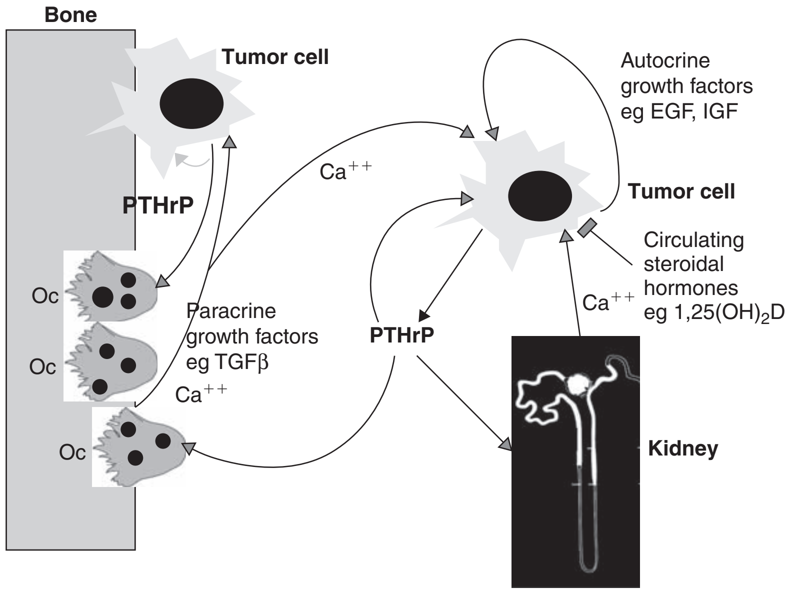
Bone
Tumor cell
PTHrP
Oc
Oc
Oc
Paracrine growth factors eg TGFβ
Ca++
Ca++
Autocrine growth factors eg EGF, IGF
Tumor cell
Circulating steroidal hormones eg 1,25(OH)2D
Ca++
PTHrP
Kidney

FIGURE 2 Regulation of PTHrP production by tumors that have and have not metastasized to bone. The production and release of PTHrP may be increased (→) by growth factors acting in an autocrine/paracrine mode such as EGF or IGF. PTHrP released from such tumors may enhance calcium (Ca^{++}) reabsorption by its action on the kidney nephron and by stimulating osteoclastic (Oc) osteolysis and calcium release from bone. The hypercalcemia itself then may augment PTHrP release from such tumors. Other circulating factors such as steroidal molecules may also modulate PTHrP production, generally by producing inhibitory effects (—|). Once tumors have metastasized to bone, they can also release PTHrP, which may cause osteoclastic osteolysis in a paracrine mode, resulting in the release of growth factors (such as TGFβ) and calcium from resorbed bone. Such growth factors, acting in a paracrine mode, and released local calcium may then further stimulate PTHrP release from metastases. PTHrP produced by tumors may also influence the course of progression of such tumors.

CaR activation in these cell lines. Stimulation of PTHrP release due to CaR activation involves *de novo* synthesis of PTHrP mRNA, in view of the fact that the pan-RNA polymerase inhibitor actinomycin D inhibits the effect of Ca^{2+} on the expression of PTHrP mRNA and protein release. Consequently elevated calcium induced by circulating PTHrP may have a stimulatory effect on further PTHrP release by the tumor. Furthermore the majority of calcium in the body is stored in bone, and bone-derived calcium is released during bone resorption resulting in an increase in extracellular calcium (Ca^{2+}) in the bony microenvironment to levels, in the vicinity of resorbing osteoclasts, that are manyfold higher (ranging from 8 to 40 m*M*) than the level of systemic Ca^{2+} (Silver *et al*., 1988). Evidence is accumulating that these large local changes in Ca^{2+} may be "sensed" by cancer cells metastatic to bone and could enhance further PTHrP release from tumor cells metastatic to bone.

Overall therefore, these studies indicate that increased PTHrP production can result from alterations in the interaction between stimulatory and inhibitory signaling pathways in malignant cells (Fig. 2).

Processing and Degradation of PTHrP

PTHrP appears to undergo endoproteolytic cleavage in the secretory pathway, resulting in the release of multiple fragments. The Fischer rat implanted with the Rice-H500 Leydig cell tumor is a well characterized model of HHM and has been used to investigate the regulation of PTHrP metabolism (Rabbani *et al*., 1993). Biosynthetic labeling of nascent PTHrP revealed rapid processing into three distinct amino-terminal species of 1–36, 1–86, and 1–141 amino acids, which were constitutively released into the extracellular environment. These observations were in agreement with similar studies that had been performed in malignant human cells (Soifer *et al*., 1992).

Like PTH, PTHrP is synthesized as a prohormone with an amino-terminal extension. The biological potency of pro-PTHrP is considerably less than that of PTHrP-(1-34) (Liu *et al*., 1995). A furin recognition sequence is found between the propeptide and the mature protein, and studies (Liu *et al*., 1995) have shown that pro-PTHrP was indeed a substrate for the prohormone convertase furin.

MECHANISMS OF ACTION OF PTHrP

Interaction of Amino-Terminal PTHrP with Cell Surface Receptors

Sequence homology between PTH and PTHrP is restricted to only eight of the first 13 residues; however, this domain is known to be required for activation of signal transduction cascades. Additional conformational similarities in the 14–34 region, a domain that appears critical for peptide binding to the receptor, permit amino-terminal fragments of the proteins to act as equivalent agonists for their common receptor, the type 1 PTH/PTHrP receptor (PTH1R). The PTH1R is a seven-transmembrane G-protein linked receptor that has the "signature" G protein-coupled receptor (GPCR) topology, a seven-membrane-spanning, "serpentine" domain, as well as a large extracellular ligand-binding domain and an intracellular COOH-terminal domain. The receptor couples to Gs and Gq leading to activation of the protein kinase A (PKA) and protein kinase C (PKC) pathways (Mannstadt *et al*., 1999) and, like other GPCRs, undergoes cyclical receptor activation, desensitization, and internalization (Weinman *et al*., 2006). After ligand binding and endocytosis, the PTH1R is either recycled to the cell membrane or targeted for degradation. Arrestins contribute to the desensitization of both Gs and Gq mediated PTH1R signaling. PTH1R activation and internalization can be selectively dissociated (Sneddon *et al*., 2004). PTH1R signaling can be modified by scaffolding proteins such as the Na^+/H^+ exchanger regulatory factor (NHERF) 1 and 2 through PDZ1 and PDZ2 domains (Mahon *et al*., 2002; Sneddon *et al*., 2003).

PTH1R signaling via the cAMP pathway, leading to PKA activation and phosphorylation of the cyclic AMP response element binding protein (CREB), has been extensively documented. CREB binds to the cyclic AMP response element (CRE) in the promoter region of many genes and transcriptionally modulates their expression.

PTH1R, as with PTHrP, is expressed in a wide variety of embryonic and adult tissues, including cartilage and bone, and therefore mediates the autocrine/paracrine actions of locally produced and secreted PTHrP.

Actions of Carboxy-Terminal PTHrP

In the past, several investigators have reported a variety of functions for fragments of PTHrP that share no homology with PTH. These include the pentapeptide PTHrP-(107–111), which was named osteostatin for its potential to inhibit osteoclastic bone resorption in culture (Fenton *et al*., 1991). Other studies using carboxy-terminal fragments of PTHrP have shown that such fragments inhibit production of the early osteoblast marker osteopontin (Seitz *et al*., 1995) in isolated osteoblasts and are almost as effective as PTHrP-(1–34) in stimulating functional osteoclast formation from progenitor cells (Kaji *et al*., 1995). These studies support *in vivo* observations demonstrating decreased osteoblastic and increased osteoclastic activity in association with elevated circulating levels of carboxy-terminal fragments of PTHrP in patients with HHM (Burtis *et al*., 1994). Several reports have reported the presence of cell surface binding proteins for carboxy-terminal fragments of PTHrP on skeletal cells (Orloff *et al*., 1995); however, to date, the molecular nature of these binding proteins remain unclear.

Intracellular Mechanism of PTHrP Action

Although most of the cellular actions of PTHrP have been attributed to the interaction of its amino terminus with the cell surface PTH1R, there are also reports that PTHrP may have a direct, intracellular mode of action. There is evidence in the literature for three potential mechanisms for the translocation of PTHrP, a prototypical secretory protein, into the cytoplasmic compartment of target cells. One involves the alternative initiation of PTHrP translation to exclude the "pre" or leader sequence, which is necessary for entry of the molecule into the endoplasmic reticulum and secretory pathway (Nguyen *et al*., 2001). A second involves retrograde transport of nascent PTHrP from the endoplasmic reticulum (Meerovitch *et al*., 1997, 1998) into the cytoplasm. Finally, a third involves internalization of secreted PTHrP via a PTH1R-independent cell-surface binding protein for PTHrP (Aarts *et al*., 1999).

The intracrine action of PTHrP appears to be mediated at least in part, through residues 87–107 in the mid-region of the protein. This region shares sequence homology with a lysine-rich bipartite nuclear localization sequence (NLS) in nucleolin (Schmidt-Zachmann and Nigg, 1993) and with an arginine-rich NLS in the retroviral regulatory protein TAT (Dang and Lee, 1989). The PTHrP NLS is both necessary and sufficient to direct the passage of PTHrP to the nuclear compartment of transfected cells and to localize endogenously expressed PTHrP to nucleoli in chondrocytes and osteoblasts *in vitro* and *in vivo* (Henderson *et al*., 1995). Endogenous PTHrP has been identified in the coarse fibrillar component of nucleoli by immunoelectron microscopy. This compartment is occupied by complexes of newly transcribed 45s ribosomal RNA and protein that are destined for assembly into ribosomes, and PTHrP has been shown to bind to GC-rich homopolymeric RNA and total cellular RNA with specificity and high affinity (Aarts *et al*., 1999), compatible with a role in ribosomal biogenesis. The biological impact of nucleolar PTHrP on cell function was demonstrated *in vitro* when chondrocytes expressing wild-type PTHrP were protected from apoptosis induced by serum deprivation, whereas cells expressing the protein without the NLS were not. Subsequently the nuclear localization of PTHrP was also found to be protective, in studies *in vitro*, for prostate cancer cell apoptosis (Dougherty *et al*., 1999) and breast cancer (Sepulveda *et al*., 2002).

Nuclear localization of PTHrP occurs in a cell cycle-dependent manner with higher expression in the G2 and M phases of the cycle (Okano *et al*., 1995; Lam *et al*., 1997). The cell cycle-dependent localization of PTHrP is regulated by the activity of the cyclin-dependent kinases (cdk) cdc2 and cdk2, which phosphorylate PTHrP at threonine^{85} within a consensus cdc2/cdk2 site (Lam *et al*., 2000). Phosphorylation increases as cells progress from G_1 to G_2 and M of the cell cycle and leads to decreased nuclear entry, perhaps by enhancing binding to a cytoplasmic retention factor. PTHrP appears to bind with high affinity to importin β1 and the GDP-bound protein, Ran (Lam *et al.*, 1999). PTHrP nuclear import seems dependent on microtubular integrity, implying a role for the cytoskeleton in transport to the nucleus (Lam *et al.*, 2002). After translocation across the nuclear envelope, GTP-bound Ran may release PTHrP into the nucleus, where it apparently can act in the nucleolus to bind RNA, thereby regulating mRNA processing or mRNA transport. Functionally, intranuclear PTHrP seems to delay apoptosis (Henderson *et al.*, 1995) and to increase cell proliferation.

Despite this evidence in favor of an intracrine action of PTHrP, ultimately, *in vivo* studies will be required to determine the role of nuclear PTHrP in physiology and in the pathophysiology of cancer.

PTHrP ACTIONS TO PRODUCE THE MANIFESTATIONS OF HHM

Actions in Kidney

An elevation in the circulating level of PTHrP in patients with HHM initially results in phosphate wasting and calcium retention by the kidney, in association with an increase in the nephrogenous component of excreted cAMP. This is analogous to the effects of excess circulating PTH in HPT and results from the interaction of PTHrP with the PTH1R. Thus in the proximal tubule, stimulation of adenylate cyclase causes internalization of the type II Na^+/Pi^- (inorganic phosphate) cotransporter leading to reduced apical Na^+/Pi^- cotransport, decreased phosphate reabsorption, and phosphaturia (Keusch *et al.*, 1998). About 20% of filtered calcium is reabsorbed in the cortical thick ascending limb of the loop of Henle (CTAL) and 15% in the distal convoluted tubule (DCT), and it is here that PTHrP also binds to the PTH1R and, again by a cyclic AMP-mediated mechanism, enhances calcium reabsorption. In the CTAL, at least, this appears to occur by increasing the activity of the Na/K/2Cl cotransporter that drives NaCl reabsorption and also stimulates paracellular calcium and magnesium reabsorption. PTHrP can also influence transcellular calcium transport in the DCT. This is a multistep process involving transfer of luminal Ca^{2+} into the renal tubule cell via the transient receptor potential channel (TRPV5), translocation of Ca^{2+} across the cell from apical to basolateral surface (a process involving proteins such as calbindin-D28K), and finally active extrusion of Ca^{2+} from the cell into the blood via a Na^+/Ca^{2+} exchanger, designated NCX1. PTHrP most likely stimulates Ca^{2+} reabsorption in the DCT primarily by augmenting NCX1 activity via a cyclic AMP-mediated mechanism. In the proximal tubule, PTH can, after binding to the PTH1R, also stimulate the 25-hydroxyvitamin D 1α hydroxylase [1a(OH)ase], leading to increased synthesis of $1,25(OH)_2D$. Consequently increased circulating levels of $1,25(OH)_2D$ are often observed in HPT. In contrast, circulating $1,25(OH)_2D$ levels are often low in patients with HHM (Stewart *et al.*, 1980). These apparent discrepancies were not resolved by early animal models of HHM in which circulating $1,25(OH)_2D_3$ levels were high (Sica *et al.*, 1983; Strewler *et al.*, 1986). Neither was the problem solved by *in vivo* and *in vitro* studies performed with synthetic amino-terminal fragments of PTHrP, which suggested that the peptides had identical bioactivity (Chorev and Rosenblatt, 1994; Fraher *et al.*, 1992; Rabbani *et al.*, 1988). More recently, continuous infusion of PTH(1–34) and of PTHrP(1–36) into healthy young adults for 2 to 4 days demonstrated that renal $1,25(OH)_2D$ synthesis was stimulated effectively by PTH but poorly by PTHrP (Horwitz *et al.*, 2005), supporting the apparent discrepancies between the circulating concentrations of $1,25(OH)_2D$ in HPT and HHM .

Actions in Bone

In bone, the PTH1R is localized on cells of the osteoblast phenotype that are of mesenchymal origin (Rouleau *et al.*, 1990). Nevertheless, both PTH and PTHrP enhance osteoclastic bone resorption. This effect of PTH on increasing osteoclast stimulation is indirect, with PTH binding to the PTH1R on preosteoblastic stromal cells (Rouleau *et al.*, 1990) and enhancing the production of the cytokine RANKL [receptor activator of nuclear factor (NF) kappaB ligand], a member of the tumor necrosis factor (TNF) family (Blair *et al.*, 2007). Simultaneously, levels of a soluble decoy receptor for RANKL, termed osteoprotegerin, are diminished facilitating the capacity for increased stromal cell-bound RANKL to interact with its cognate receptor, RANK, on cells of the osteoclast series. Under the influence of RANKL stimulation, multinucleated osteoclasts, derived from hematogenous precursors committed to the monocyte/macrophage lineage, then proliferate and differentiate as mononuclear precursors, eventually fusing to form multinucleated osteoclasts. These can then be activated to form bone-resorbing osteoclasts. RANKL can drive all of these proliferation/differentiation/fusion/ activation steps although other cytokines, notably monocyte-colony stimulating factor (M-CSF), may also participate in this process.

In comparing HPT and HHM, histomorphometric evaluation of bone biopsies also revealed that whereas catabolic activity was significantly increased in both disorders, discrepancies in bone formation were noted, with decreases in HHM but increases in those with parathyroid disease (Stewart *et al.*, 1982). Nevertheless, in animal models of HHM, bone turnover appeared to be coupled (Sica *et al.*, 1983; Yamamoto *et al.*, 1995). In recent infusion studies in humans however, both PTH and PTHrP appeared to produce inhibition of formation and stimulation of resorption (Horwitz *et al.*, 2005).

Actions in Tumor Cells

PTH1R and PTHrP are frequently coexpressed in tumors (Downey *et al.*, 1997), and PTHrP has been reported to stimulate cell proliferation in a variety of cancer cell models including MCF-7 breast cancer cells (Hoey *et al.*, 2003), SV-40 immortalized breast epithelial cells (Cataisson *et al.*, 2000), renal carcinoma cells (Massfelder *et al.*, 2004), and colon cancer cells (Shen and Falzon, 2005). The amino-terminal domain of PTHrP via PTH1R has also been reported to inhibit apoptosis (Hastings *et al.*, 2003) and to enhance cancer cell adhesion to bone matrix components (Shen and Falzon, 2003). PTHrP has also been shown, by tumors that secrete it, to promote growth and survival by the nuclear localizing domain via an intracrine mechanism (Sepulveda and Falzon, 2002). PTHrP could therefore offer a selective advantage to tumor cells that produce it by contributing to tumor cell survival and growth.

DETECTION OF PTHrP PRODUCED BY TUMORS

Circulating Levels of PTHrP

Shortly after the discovery and characterization of PTHrP, assays were developed to detect this factor in the circulation. Assays that have been employed to measure PTHrP include those recognizing epitopes in the amino-terminal region, the mid-region (Wu *et al.*, 1996), and the carboxyl region of PTHrP and those employing two-site assays to measure longer domains. Discrepancies in results among these assays may depend at least in part on the variable molecular nature of PTHrP forms that circulate, either due to variable tumor production or clearance, especially in the presence of altered hepatic and/or renal function in cancer patients. To date the production, circulating nature, and clearance of heterogeneous PTHrP forms has not been nearly as well characterized as has the production, circulating nature, and clearance of heterogeneous PTH forms. Such heterogeneous PTHrP moieties may be differently detected by individual assays and therefore yield different results. Nevertheless certain consistent themes have emerged from the use of PTHrP assays.

The first generalized finding is that circulating PTHrP levels are increased in virtually all patients with HHM (Stewart *et al.*, 1980). Second is that although renal cell carcinomas and squamous cell carcinomas have most frequently been associated with HHM, assays capable of directly measuring PTHrP demonstrated a much broader spectrum of tumors associated with excess PTHrP production than was previously considered, including breast, colon, and hematologic cancers (Henderson *et al.*, 1990). Furthermore, cases of prostate cancer (Kao *et al.*, 1990) and melanoma (Kageshita *et al.*, 1999), not typically believed to be part of the HHM spectrum, have been reported in association with high circulating PTHrP levels. Thus, circulating PTHrP levels have been reported as elevated in 50% to 90% of hypercalcemic cancer patients with solid tumors (Budayr *et al.*, 1989; Ratcliffe *et al.*, 1992; Ralston *et al.*, 1990; Burtis *et al.*, 1990; Kao *et al.*, 1990; Henderson *et al.*, 1990; Rankin *et al.*, 1997) and in 25% to 60% of such patients with hematologic malignancies (Kremer *et al.*, 1996b; Firkin *et al.*, 1996). Third, circulating PTHrP concentrations have frequently been reported to be elevated in hypercalcemic patients that have metastasized to the skeleton. Thus, hypercalcemia with breast cancer has as well been classically associated with osteolytic lesions, but assays have generally detected elevated PTHrP in breast cancer, whether or not skeletal metastases were present (Grill *et al.*, 1991). These studies therefore suggested that PTHrP as a systemic factor may contribute to the hypercalcemia of tumors that have spread to bone as well as to the hypercalcemia of those that have not.

To date, measurement of serum PTHrP has not however been a useful method for early tumor detection, possibly because of assay limitations related to measurement of PTHrP fragments but more likely because of the advanced stage of tumors that release PTHrP into the circulation (Kremer *et al.*, 1996b; Rankin *et al.*, 1997). Furthermore, the finding of hypercalcemia in association with suppressed PTH using highly sensitive and specific assays to measure intact PTH has often been taken as presumptive evidence of elevated circulating PTHrP as the cause of the hypercalcemia, in lieu of direct measurement of PTHrP.

In some studies sequential PTHrP measurement has correlated with response to therapy (Henderson *et al.*, 1990; Oda *et al.*, 1998), but to date, PTHrP has not been widely utilized to monitor disease status.

PTHrP may have value in prognostic evaluation. Thus, PTHrP assays have generally shown that there is a positive correlation between circulating PTHrP levels and more advanced stages of cancer (Rankin *et al.*, 1997; Kremer *et al.*, 1996b; Firkin *et al.*, 1996), and several studies have reported that elevation of serum PTHrP levels was associated with a worse prognosis (Truong *et al.*, 2003; Pecherstorfer *et al.*, 1994; Hiraki *et al.*, 2002). One study, which did not find that serum concentration of PTHrP was associated with a poor prognostic factor, did not correct for confounding factors (Lee *et al.*, 1997).

Overall, the clinical significance of PTHrP assays in diagnosis and prognosis requires more extensive evaluation.

PTHrP in Tumor Tissue

PTHrP has also been studied in tumor tissue and is frequently expressed in many tumors including breast cancer (Southby *et al.*, 1990; Kohno *et al.*, 1994), lung cancer (Brandt *et al.*, 1991), and renal cell cancer (Iwamura *et al.*, 1999). The prognostic value of PTHrP expression in breast cancer tissue is uncertain, because PTHrP immunostaining is reported to be either positively or negatively associated with prognosis. Thus, the presence of PTHrP in tissue samples obtained from breast cancer patients has been associated with a more favorable outcome by one group (Henderson *et al.*, 2001, 2006), but not by two others (Liapis *et al.*, 1993; Yoshida *et al.*, 2000). The discrepancies in these findings may perhaps be due to the use of different PTHrP antibodies for immunohistochemical analysis.

PTHrP expression in tumor samples appears to be correlated in some reports with poor prognosis in lung cancer (Hidaka *et al.*, 1998), renal carcinoma (Iwamura *et al.*, 1999), and colorectal tumors (Nishihara *et al.*, 1999).

RELATIONSHIP OF PTHrP-PRODUCING TUMORS WITH THE BONE MICROENVIRONMENT

Up to two-thirds of patients with MAH have bone metastases, and as indicated earlier, elevated circulating levels of PTHrP have been detected in association with hypercalcemic tumors that have spread to bone (Henderson *et al.*, 1990). Thus, certain malignancies—notably breast, lung, renal, and thyroid carcinomas, which are frequently associated with hypercalcemia—have as well a strong propensity to spread to bone. PTHrP is thus expressed not only by tissues of tumors associated with hypercalcemia that have not metastasized to bone but also by tumors which have colonized the skeleton (Powell *et al.*, 1991; Southby *et al.*, 1990; Vargas *et al.*, 1992). It seems unlikely that local osteolysis per se will produce sustained hypercalcemia in the absence of a reduction of the renal clearance of calcium caused either by kidney damage or by the calcium-reabsorptive action of a humoral agent such as PTHrP. Therefore PTHrP as an endocrine factor is an important contributor to the pathophysiology of the hypercalcemia, not only of those tumors that have not metastasized to bone but also to those which have already colonized bone.

Furthermore, increasing evidence suggests that it may also be a mediator of focal osteolysis induced by skeletal metastases while acting in a paracrine mode. Evidence for this has been presented in models of MDA-MB-231 breast cancer (El Abdaimi *et al.*, 2000; Guise *et al.*, 1996) and prostate cancer (Rabbani *et al.*, 1999). Tumor cells can thus release PTHrP which acts at both the systemic (primary tumor) and local (skeletal metastases) levels to stimulate osteoclastic bone resorption, at least in part, through the RANKL/RANK/OPG system.

Bone provides a fertile environment for growth of tumors that are metastatic to the skeleton with a well-developed blood supply (in hematopoietic marrow) as a source of nutrients and numerous growth factors that can provide a growth advantage. Thus, bone matrix is a unique storage site of immobilized growth factors such as transforming growth factor (TGF)-β, insulin-like growth factor (IGF) I and II, fibroblast growth factor (FGF)-1 and -2, and platelet-derived growth factors (PDGF). Tumor-derived PTHrP that produces osteolysis results in the release of such active growth factors from the matrix, which facilitates growth of the tumor cells. In turn, growth factors such as TGF-β (Kakonen *et al.*, 2002; Yin *et al.*, 1999), as well as calcium per se, following their local release during osteoclastic resorption, not only may enhance the growth of tumor cells but also may further stimulate PTHrP production (see Fig. 2). This enhancement of PTHrP production by tumors in the bone microenvironment may explain the observation that breast cancer tissue from skeletal metastases may be more frequently positive for PTHrP expression than from other sites (Powell *et al.*, 1991) and that patients with PTHrP-negative primary breast cancers may have PTHrP-positive metastatic lesions (Henderson *et al.*, 2006). Although there is no major evidence for an overall facilitatory effect of PTHrP in causing bone metastases, PTHrP may enhance skeletal invasion, once seeding to bone has occurred and micrometastases have been established. In addition to causing osteolysis and thereby facilitating tumor expansion, the enhanced release of PTHrP from such micrometastases may also augment direct effects of PTHrP on tumor growth, survival, and possibly adhesion to bone matrix (Shen and Falzon,, 2003). This process therefore can expand the bidirectional interaction between the tumor cells and bone cells.

OTHER SYSTEMIC MEDIATORS OF MAH

Cytokines

Cytokines such as interleukin IL-1, IL-6, and TNF have been identified as physiological regulators of skeletal metabolism. They are produced by cells in the bone microenvironment, where they regulate their own and each other's expression and activity, as well as that of their cell surface receptors and soluble binding proteins. The overall effect of this complex, interdependent activity is to stimulate bone resorption.

Circulating proinflammatory cytokines most probably contribute to a number of the systemic manifestations of malignancy such as anorexia, dehydration, and cachexia (Ogata, 2000). Mounting evidence also suggests that cytokines released into the systemic circulation by a variety of solid tumors, which often coexpress PTHrP, contribute to the bone destruction associated with MAH. In this respect it is interesting to note that circulating concentrations of IL-6 correlate in a positive manner with tumor burden in patients with squamous and renal cell carcinoma, which represent the two prototypical malignancies associated with hypercalcemia and elevated circulating levels of PTHrP (Costes *et al.*, 1997; Nagai *et al.*, 1998).

Many other factors, including vascular endothelial growth factor (VEGF) and cytokines such as interleukin-8 and -11, and soluble RANKL (Nagai *et al*., 2000) have been implicated in promoting hypercalcemia of malignancy (Horwitz and Stewart,, 2003). The relative contributions of systemically active PTHrP and cytokines to MAH have been examined in immune-compromised rodents carrying human tumors that overexpress both factors (Nagai *et al*., 1998). Nude rats transplanted with a human squamous carcinoma of the oral cavity (OCC), which was shown to overproduce both PTHrP and IL-6, rapidly developed severe hypercalcemia in association with high circulating levels of both PTHrP and IL-6. Hypercalcemic animals immunized with anti-IL-6 monoclonal antibody demonstrated a complete reversal in the biochemical abnormalities associated with elevated circulating IL-6, but only a small reduction in serum calcium. As measured by quantitative histomorphometry, there was a significant decrease in indices of bone resorption, as well as an increase in the mineral apposition rate in rats that received the neutralizing antibody. These results suggest that although IL-6 contributed to the skeletal abnormalities seen in OCC tumor-bearing rats, its contribution to the hypercalcemic syndrome was minor compared with that of PTHrP.

Using an alternate model, others demonstrated that mice carrying a human esophageal tumor (EC-GI), which coexpresses PTHrP and IL-1, develop a modest elevation in blood calcium (Sato *et al*., 1989). A comparable level of hypercalcemia was observed in normal mice receiving a continuous infusion of IL-1. However, a significant increase in blood calcium was noted in mice that received a minimal daily dose of PTHrP in addition to the IL-1 infusion, suggesting a synergistic effect of PTHrP and IL-1 on bone resorption in this model. This hypothesis was supported by experiments in which addition of a small amount of synthetic PTHrP to the culture medium greatly enhanced ^{45}Ca release from prelabeled mouse bones, in response to recombinant IL-1.

This work using animal models of human disease predicts that PTHrP is the principal mediator of MAH. The hypothesis is further supported by clinical studies in which elevated circulating levels of PTHrP correlate strongly with hypercalcemia in patients with tumors of widely diverse histological origin (Ogata, 2000). No such correlation has been demonstrated for hypercalcemia and elevated circulating levels of cytokines such as IL-6 or IL-1. However, it has been proposed that a systemic increase in PTHrP not only promotes hypercalcemia but also stimulates normal cells to produce factors such as TNF-α, IL-1, IL-5, IL-6, and IL-8 in patients presenting with end-stage malignancy. The observation that infusion of anti-PTHrP antibody into mice with high circulating levels of PTHrP and IL-6 resulted in a prolonged decrease in the concentration of both factors in the bloodstream lends some support to this hypothesis.

1,25-Dihydroxyvitamin D (1,25(OH)$_2$D)

The site of conversion of 25-hydroxyvitamin D [25(OH)D] to its active metabolite, 1,25(OH)$_2$D, by the 25(OH)D 1α-hydroxylase enzyme is known not to be restricted to the kidney. Although the kidney is still recognized as the primary site of circulating 1,25(OH)$_2$D production *in vivo,* several extrarenal sites of 1-α-hydroxylase activity have been identified. These include cells of the hematopoietic and immune systems, as well as cells in many other tissues that give may give rise to solid tumors associated with MAH. In contrast to the systemic role played by kidney-derived 1,25(OH)$_2$D$_3$ in calcium homeostasis, locally produced hormone is thought to regulate cell proliferation and differentiation in normal cells (Holick, 1999) and could modulate the development of neoplastic transformation within these cells.

Elevated serum concentrations of 1,25(OH)$_2$D$_3$ have been reported in some cases of non-Hodgkin's (Breslau *et al*., 1984) and Hodgkin's lymphoma (Jacobson *et al*., 1988), in contrast to the low circulating levels seen in the majority of hypercalcemic cancer patients. This increase in circulating 1,25(OH)$_2$D$_3$ was often seen in the presence of renal impairment, suggesting an extrarenal source of the hormone. Occasionally solid tumors, such as a human small cell lung cancer cell line, NCI H82, have been reported to synthesize a vitamin D metabolite with similar biochemical properties and bioactivity to authentic 1,25(OH)$_2$D$_3$ (Mawer *et al*., 1994). Whether or not the systemic increase in 1,25(OH)$_2$D$_3$ makes a significant contribution to hypercalcemia in these isolated cases remains a question of debate as the same malignancies often also release cytokines and PTHrP, which are known mediators of MAH.

PTH

Although PTH is normally only secreted by the parathyroid glands, authentic ectopic secretion of PTH (ectopic hyperparathyroidism) has been well documented in a variety of histological types of tumors including lung, ovary, thyroid, and thymus and has been associated with hypercalcemia (Nussbaum *et al*., 1990; Yoshimoto *et al*., 1989; Strewler *et al*., 1993; Rizzoli *et al*., 1994). Although this syndrome is well documented, it appears to represent an exceedingly rare event.

EXPERIMENTAL APPROACHES TO CONTROLLING OVERPRODUCTION OR OVERACTIVITY OF PTHrP IN MAH

Although effective antitumor treatment is still the best means to obtain long-term normalization of serum calcium in MAH, a marked reduction of tumor burden often is not attainable because hypercalcemia generally complicates advanced and refractory cancer. Consequently, volume repletion and bisphosphonates, which inhibit osteoclastic bone resorption, have become the standard of therapy for MAH (Body *et al*., 1998), and newer antiresorptive agents are being developed (Capparelli *et al*., 2000). Bisphosphonates normalize serum Ca levels in more than 90% of patients with MAH, but they appear to be less efficient when hypercalcemia recurs in association with high circulating levels of PTHrP, possibly due to the renal calcium-reabsorbing effects of PTHrP (Rizzoli *et al*., 1999; Onuma *et al*., 2005). Glucocorticoid treatment can be beneficial for steroid-responsive tumors such as lymphoma. The central role of PTHrP in MAH has, however, stimulated the exploration of preventive and therapeutic measures by targeting PTHrP.

Immune Approaches to Reducing PTHrP

Anti-PTHrP neutralizing antibodies have been shown to be effective to reduce skeletal metastasis, bone lesions, and hypercalcemia (Iguchi *et al*., 1996; Guise *et al*., 2002), and humanized anti-PTHrP antibody has been engineered for therapeutic purposes (Sato *et al*., 2003; Saito *et al*., 2005).

Because of the high expression of PTHrP in a variety of tumors, PTHrP could also be a common target molecule in specific immunotherapy for patients with many tumor types, particularly those with bone metastases. PTHrP-specific and cancer-reactive cytotoxic T lymphocytes have thus been generated from patients with different tumor types and may play a role in targeting tumor metastases (Arima *et al*., 2005).

Vitamin D-Mediated Repression of PTHrP

$1,25(OH)_2D_3$ is a potent inhibitor of PTHrP in a variety of cellular systems. This inhibitory activity is mediated through the classic VDR, which also mediates the effects of $1,25(OH)_2D_3$ on calcium homeostasis. For this reason, administration of $1,25(OH)_2D_3$ in cases of MAH is counterproductive because of its intrinsic hypercalcemic effects. A concentrated effort has, therefore, been focused on the development of low-calcemic analogues of $1,25(OH)_2D_3$ that will effectively suppress PTHrP production but will not stimulate calcium absorption by the gut, calcium reabsorption by the kidney, and, most importantly, bone resorption. EB1089 and MC903 are two $1,25(OH)_2D_3$ analogues with conservative side-chain modifications that have been used to inhibit PTHrP production *in vitro*, and with variable degrees of success to prevent the hypercalcemic syndrome in models of MAH (Haq *et al*., 1993; Yu *et al*., 1995; El Abdaimi *et al*., 1999, 2000; Nakagawa *et al*., 2005). These studies suggested that low calcemic vitamin D analogues or nonsteroidal activators of the VDR that selectively fail to augment intestinal calcium absorption may prove useful in the management of MAH and in inhibiting skeletal metastases associated with excess PTHrP production.

Inhibition of Signaling to Reduce PTHrP Production or Action

Growth factors acting via receptor tyrosine kinases (RTKs) have been shown to be potent stimuli of PTHrP gene transcription. One of the best characterized signal transduction pathways downstream of RTKs is the Ras/Raf/Mek pathway. Activation of this pathway by growth factors or activated Ras was shown to increase PTHrP expression and release from a variety of cells in culture. Conversely, inhibition of the pathway through pharmacologic intervention, e.g., by a small molecule that acts as a competitive inhibitor of Ras farnesylation (one of the metabolic conversions that is required for Ras to be anchored to the inner aspect of the cell membrane and transmit a signal downstream; Aklilu *et al*., 1997) or by using dominant negative forms of Ras and Raf (Aklilu *et al*., 2000) successfully reduced PTHrP production. This suggested that specific inhibitors of components of the Ras signaling pathway could be used for therapeutic intervention to prevent the hypercalcemic syndrome associated with PTHrP overproduction *in vivo*.

Other small molecule inhibitors of PTHrP have been proposed as therapeutics including two small nucleotide analogues that were reported to inhibit production of PTHrP by tumor cells and reduce bone lesions with higher survival rates in animal models (Gallwitz *et al*., 2002).

Because PKA is the major signaling pathway that mediates PTHrP-induced production of RANKL by osteoblasts, antagonists against PKA could be another promising target.

Antisense Inhibition and RNA Interference of PTHrP Production

An alternative approach for diminishing PTHrP overproduction in malignancy employed antisense technology to inhibit endogenous PTHrP expression and diminish hypercalcemia. Thus the use of antisense PTHrP (Rabbani *et al*., 1995) or the use of antisense furin (Liu *et al*., 1995) to reduce bioactive PTHrP in MAH models *in vivo* led to diminished hypercalcemia, decreased tumor cell proliferation, and prolonged survival of the host animals. Newer techniques using small interfering RNA (sɪRNA) may prove useful in the future in furthering these initial approaches.

Overall these studies suggest that knowledge of the regulation of PTHrP production, processing and action may identify targets that may be useful for the development of agents to reduce circulating concentrations of bioactive PTHrP *in vivo* and to diminish hypercalcemia. Furthermore such agents could prove beneficial in inhibiting the production of PTHrP by tumors that might diminish the establishment and growth of metastatic lesions and potentially of primary tumors.

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Chapter 64

Localized Osteolysis

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INTRODUCTION

As primary tumors grow, they frequently metastasize to distant organs. Patients who succumb to cancer usually die because of metastases. In many cancer patients, these metastases cause more serious effects than the primary tumor itself. Tumors such as lung, prostate, breast, kidney, and thyroid frequently metastasize to bone. Complications of skeletal metastasis include fractures, bone pain, hypercalcemia, and cachexia. Bone metastases are especially prevalent in patients with prostate and breast cancer, in which a large proportion of the patients develop skeletal metastases at late stages in the disease course. Most commonly, the tumor cells metastasize to highly vascularized areas of the skeleton, such as the vertebral column, ribs, and long bones (Kakonen & Mundy, 2003). These metastases can either be osteolytic (bone destructive) or osteoblastic (bone forming). Prostate tumors tend to produce mixed lesions while breast tumors are frequently osteolytic (Keller & Brown, 2004).

The skeletal complications of malignancy have received considerable attention in recent years, in part due to the widespread use of bisphosphonates for the treatment of localized osteolysis associated with multiple myeloma and solid tumors. It is interesting to note that there has been considerable evolution in research focus over the past 30 years in the three major manifestations of cancer on bone, namely hypercalcemia, osteoporosis and metastatic bone disease (including the osteolysis associated with myeloma). In the 1970's and 1980's, the major focus was on hypercalcemia which was often difficult to manage and whose cause was unknown. In the 1990's and first decade of the 21st Century, attention shifted to metastatic bone disease, and its importance as the cause of many clinical manifestations of advanced malignancy. In the next decade, it may be that major attention will also be focused on osteoporosis associated with cancer, which is becoming increasingly more prevalent, in part due to patients living longer with advanced disease, and in part related to therapies aimed at slowing tumor growth which also have effects on the skeleton.

In this chapter, we will review osteolytic bone disease and osteoporosis, with major attention on osteolytic and osteoblastic bone disease. Our plan is to focus primarily on myeloma, breast cancer, prostate cancer, and myeloma as true malignancies which exemplify the spectrum of bone changes that occupy advanced cancer.

FREQUENCY OF METASTATIC BONE DISEASE

Metastatic bone disease occurs commonly in solid tumors of the breast, prostate and lungs. These are the three commonest solid tumors that affect humankind, and they are usually associated with bone metastasis at the stage that the patient has advanced disease. In most patients with breast and prostate cancer, essentially all patients will have bone metastases when the disease is advanced, if methods are used that detect micrometastases. Patients with metastatic bone disease due to these solid tumors have a marked impairment of their quality of life due to skeletal-related events such as intractable bone pain, bone fragility, and

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susceptibility to pathologic fracture. These patients can be expected to have a major skeletal-related event (episodes of intractable bone pain, hypercalcemia, pathologic fracture) every three to four months when untreated with specific bone-active agents.

Eighty percent of patients with bone metastases have breast or prostate cancer.

BREAST CANCER AND BONE METASTASIS

Spectrum of Metastases in Breast Cancer

Breast cancer metastasizes to bone frequently, as noted above, and usually causes extensive osteolysis. However, there is also usually a weak osteoblastic response demonstrated in patients by a small increase in serum alkaline phosphatase. In about 20 percent of all patients with metastatic bone disease due to breast cancer, this osteoblastic response is markedly enhanced and the patient has an osteoblastic metastasis easily recognized radiologically.

The osteoblastic metastasis of breast cancer is better studied than that of prostate cancer because the preclinical models to study the process are better developed.

In this section, we will discuss the osteolytic and osteoblastic process in breast cancer, and the important cell-cell interactions that occur in the bone microenvironment.

Tumor-Bone Interaction: The "Vicious Cycle"

The presence of many growth factors present in the bone matrix provides a fertile environment for tumor cells to metastasize to bone. Once tumors establish in bone, they secrete factors that stimulate bone resorption. As bone is destroyed by the tumor cells, these growth factors are released from the bone matrix and stimulate tumor growth. As these factors stimulate tumor cell proliferation, more osteolytic factors are produced by the tumor leading to more bone resorption. This in turn causes the release of growth factors and the process continues as a vicious cycle of bone destruction and tumor growth.

Some of the major factors, but likely not the only ones, contributing to this cycle of bone destruction are tumor-produced parathyroid hormone-related peptide (PTHrP) and bone-derived transforming growth factor β (TGF-β). TGF-β released from the bone matrix during osteolysis has been shown to stimulate tumor PTHrP production

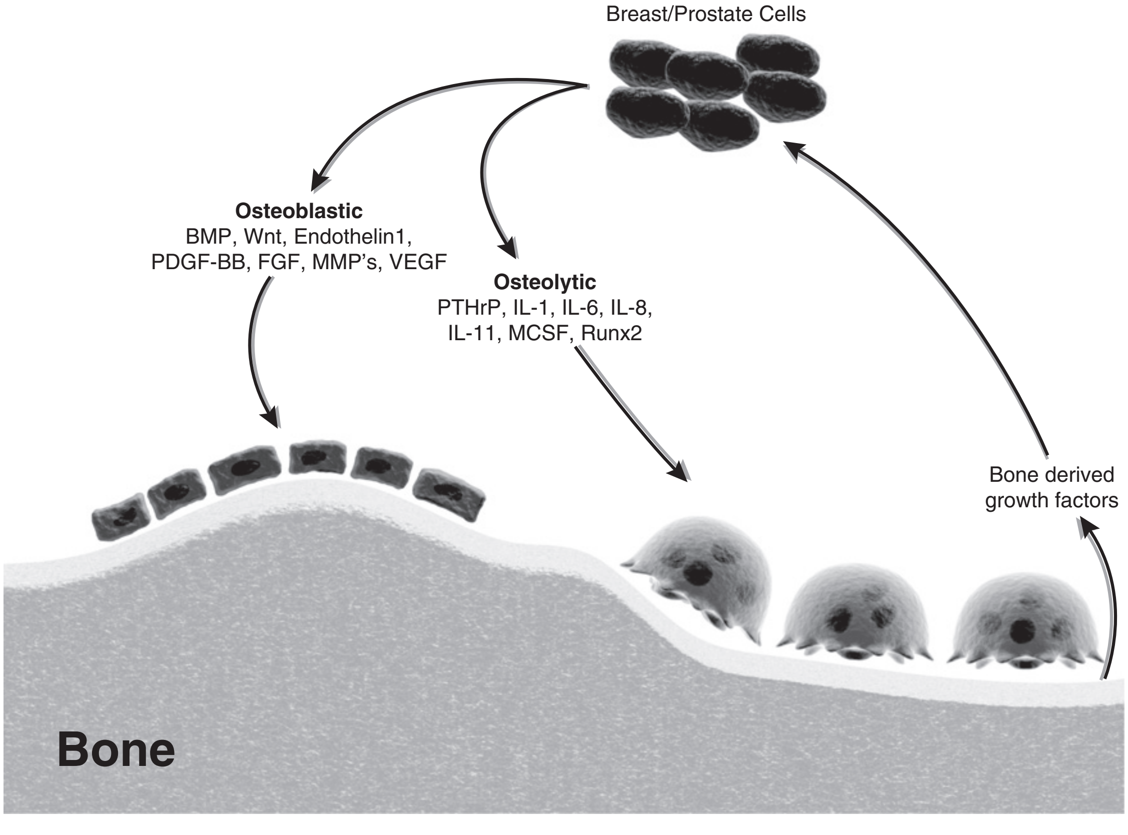

FIGURE 1 Factors implicated in the bone disease associated with breast and prostate cancer. When breast or prostate tumors metastasize to bone, factors secreted by the tumor cells disrupt normal bone remodeling. This alteration in bone remodeling can result in a spectrum of responses from osteolytic bone destruction to osteoblastic new bone formation depending on the factors produced by the tumor cells.

(Yin *et al.*, 1999). This relationship has been clearly shown using MDA-MB-231 cells transfected with a dominant-negative TGF-β receptor type II, which leaves the cells non-responsive to TGF-β (Yin *et al.*, 1999). In these cells the production of PTHrP was not enhanced in the bone microenvironment and there was a significant reduction in tumor-induced osteolysis (Yin *et al.*, 1999). The regulation of PTHrP by TGF-β was later demonstrated to be through both a smad-dependent and MAPK pathway (Kakonen *et al.*, 2002). In addition, recent experiments in several laboratories have indicated that inhibiting TGF-β inhibits osteolysis and tumor growth in bone (Bandyopadhyay *et al.*, 2006; Ehata *et al.*, 2007).

Many growth factors are stored in bone including bone morphogenetic proteins (BMPs), heparin-binding fibroblast growth factors, platelet derived growth factor, and Insulin like growth factor I and II (Hauschka, Mavrakos, Iafrati, Doleman, & Klagsbrun, 1986). These factors, like TGF-β, are released during bone resorption and likely contribute to tumor cell growth. While these and other factors likely play a role in the vicious cycle of bone destruction, the majority of studies have focused on the role of TGF-β.

Factors Responsible for Osteolysis

RANKL

Tumor cells that have established in the bone microenvironment secrete factors that stimulate osteoclastic bone resorption. One of the major factors responsible for this increased bone resorption is PTHrP, which can stimulate osteoblast expression of receptor activator of NF-κB ligand (RANKL) (Chikatsu *et al.*, 2000; Kitazawa & Kitazawa, 2002; Martin & Gillespie, 2001; Michigami, Ihara-Watanabe, Yamazaki, & Ozono, 2001; Thomas *et al.*, 1999). RANKL then binds to its receptor expressed on the osteoclasts, RANK. In addition, the presence of tumor cells frequently leads to a reduction in the expression of the decoy receptor for RANKL, osteoprotegerin (OPG) (Thomas *et al.*, 1999). Alteration of the expression of these proteins by the tumor cells results in an overall increase in RANKL/RANK binding and activation, often accompanied by a decrease in OPG, and a subsequent increase in osteoclast activation (Dougall & Chaisson, 2006; Wittrant *et al.*, 2004). Clinical trials using OPG or related molecules (RANK:Fc or RANKL antibodies) have successfully inhibited osteolysis caused by tumor cells that have metastasized to bone (Body *et al.*, 2003).

PTHrP

Parathyroid hormone-related peptide is expressed by tumor cells (Guise *et al.*, 2002; Powell *et al.*, 1991). Release of PTHrP by tumor cells stimulates RANKL-mediated osteoclastic bone resorption (Kitazawa & Kitazawa, 2002; Martin & Gillespie, 2001; Powell *et al.*, 1991; Thomas *et al.*, 1999). The production of PTHrP is regulated by TGF-β released from the resorbing bone (Kakonen *et al.*, 2002; Yin *et al.*, 1999) as described above. Factors which regulate PTHrP production are therefore important potential mediators of bone resorption. This was demonstrated by studies using PTHrP antibodies. Tumor-bearing mice which were treated with PTHrP displayed a reduction in tumor burden and osteolysis (Guise *et al.*, 1996). In addition, the chemotherapeutic agent, 6-thioguanine, which reduces PTHrP promoter activity, decreases tumor burden and osteolysis when used to treat tumor-bearing mice (Gallwitz, Guise, & Mundy, 2002).

Hedgehog Signaling

We have recently demonstrated that the hedgehog (Hh) signaling molecule Gli2 regulates PTHrP production and that overexpression of Gli2 leads to an increase in osteolysis (Sterling *et al.*, 2006). In addition, increasing evidence suggests that Hh signaling plays an important role in tumor invasion and metastasis (Mori, Okumura, Tsunoda, Sakai, & Shimada, 2006; Yanai *et al.*, 2007; Zhou & Hung, 2005). In tumor cell types such as prostate, in which Hh signaling is active at the primary site, inhibiting Hh signaling effectively inhibited tumor cell growth (Karhadkar *et al.*, 2004; Shaw & Bushman, 2007), indicating that this may be a potential method for inhibiting tumor cells that have metastasized to bone.

Interleukins

While tumor-produced PTHrP has been clearly shown to play a critical role in the activation of RANKL and subsequent osteolysis (Thomas *et al.*, 1999), tumor cells can secrete other factors that stimulate osteoclasts. One such family of cytokines is the Interleukins. IL-1, 6, 8, and 11 are each frequently produced by tumor cells and can lead to osteolysis (Bendre, Gaddy, Nicholas, & Suva, 2003; Bendre *et al.*, 2005; M. S. Bendre *et al.*, 2003). Of these, IL-8 expression has been best described to stimulate tumor-induced osteolysis. Studies have demonstrated a positive correlation in some human breast tumors between IL-8 expression and increased frequency of bone metastases in patients. Furthermore, they have demonstrated that IL-8 stimulates osteoclast differentiation and activity *in vitro* (M. S. Bendre *et al.*, 2003).

M-CSF

Another factor secreted by some tumor cells that can stimulate osteoclast formation is macrophage colony-stimulating factor (M-CSF). Breast cancer cells have been shown to secrete M-CSF, leading to an enhancement of RANKL expression (Mancino, Klimberg, Yamamoto, Manolagas, & Abe, 2001). Furthermore, expression of M-CSF in cancer patients has been correlated with poor prognosis. Analysis of patient samples has indicated that M-CSF levels are dramatically higher in men with prostate cancer that has metastasized to bone and in women with advanced metastatic breast cancer than to normal patients or patients without metastases (McDermott *et al.*, 2002). These data indicate that production of M-CSF may play a key role in the stimulation of osteolysis in some tumor types.

Other Factors

Other factors that are associated with osteolysis include Runx2, a critical factor for normal bone development, and the matrix metalloproteinases. Runx2 is expressed in mammary epithelial cells and inhibition of Runx2 expression by tumor cells decreases the ability of tumors to induce osteolysis (Javed *et al.*, 2005; Pratap *et al.*, 2006; Shore, 2005). This is likely to be partially due to the ability of Runx2 to regulate the expression of MMP-9 (Pratap *et al.*, 2005). The matrix metalloproteinases are a family of extracellular matrix degrading proteins. MMP-9 produced by the tumor cells, and not the micro-environment, leads to tumor growth and osteolysis of prostate tumors residing in bone (Bonfil *et al.*, 2006; Nabha *et al.*, 2006). Alternatively, MMP-7 in the micro-environment contributes to osteolysis through activation of RANKL (Lynch, Crawford, Matrisian, & McDonnell, 2004; Lynch *et al.*, 2005). Despite the promising pre-clinical trials, MMP inhibitors have not been successful in patients, probably related to non-specificity of the inhibitors (Coussens, Fingleton, & Matrisian, 2002; Wagenaar-Miller, Gorden, & Matrisian, 2004).

In addition to the tumor-produced factors described above, several other factors are also be produced by tumor cells that are known to stimulate osteoclast activity. Through screening conditioned media of cells known to stimulate osteoclastic bone resorption, IL-1, IL-6, M-CSF, GF-CSF, TGF-β1, TGF-β2, and PTHrP (Pederson, Winding, Foged, Spelsberg, & Oursler, 1999) were identified as factors secreted by the tumor cells which stimulate osteoclast activity. While some of these have been well-described and are discussed in detail above, little is known about the relative roles of others of these factors. In addition, this screening experiment indicated that no single factor stimulated the osteoclasts as well as the complete media (Pederson *et al.*, 1999). This and other experiments have suggested that production of multiple factors by the tumor cells may be required for the total stimulation of osteoclast activity seen at the tumor site (Kang *et al.*, 2003).

Factors Responsible for Osteoblastic Metastases in Breast Cancer

At autopsy, approximately 90% of patients with prostate cancer have skeletal metastases, with osteoblastic metastases accounting for the majority of the lesions (Vessella & Corey, 2006). While predominantly osteoblastic, prostate tumors have an osteolytic component that in many cases is required for the establishment of the osteoblastic component (Keller & Brown, 2004; Zhang *et al.*, 2001). This has been in part demonstrated by the effectiveness of inhibiting prostate tumor growth in bone by using drugs that inhibit bone resorption such as bisphosphonates and OPG (Vessella & Corey, 2006; Zhang *et al.*, 2001). Once established in bone prostate tumor cells secrete osteoblastic factors which stimulate bone formation (Hall, Kang, MacDougald, & Keller, 2006; Koeneman, Yeung, & Chung, 1999). Some of the major mediators may include BMPs, Wnt, and Endothelin-1 (Feeley, Krenek *et al.*, 2006; Hall *et al.*, 2006; Yin *et al.*, 2003).

BMP

Bone morphogenetic proteins are major regulators of bone formation, and prostate tumor cells frequently express several of the BMPs (Bentley *et al.*, 1992; Koeneman *et al.*, 1999). Of the BMP family BMP 2, 7, and 6 have been associated with osteoblastic metastases (Bentley *et al.*, 1992; Feeley, Krenek *et al.*, 2006; Hauschka *et al.*, 1986; Masuda, Fukabori, Nakano, Shimizu, & Yamanaka, 2004). Additionally, inhibiting BMP using the BMP inhibitor Noggin decreases the osteoblastic component of mixed lesions (Feeley, Liu *et al.*, 2006).

Wnt Signaling

The Wnt pathway is essential for normal bone formation, and may also be involved in tumor invasion and metastasis (Vessella & Corey, 2006; Zhou & Hung, 2005). Tumors that induce osteoblastic metastases have been demonstrated to secrete Wnt proteins. Furthermore, mixed prostate lesions initially produce the Wnt inhibitor DKK-1, which is decreased as the tumors transition into an osteoblastic phase (Clines *et al.*, 2007; Hall *et al.*, 2006).

Endothelin-1

Endothelin-1 is a factor secreted by osteoblastic tumor cells and has also been shown to regulate the expression of DKK-1, indicating an important relationship between these two pathways (Clines *et al.*, 2007; Koeneman *et al.*, 1999; Mohammad & Guise, 2003). Osteoblastic breast and prostate tumor cells express ET-1, which stimulates bone formation and osteoblast proliferation (Mohammad & Guise, 2003).

Other Factors

While these are the major pathways demonstrated to play important roles in osteoblastic metastases, other pathways that may be involved include factors that activate osteoblast activity including PDGF-BB (Mehrotra, Krane, Walters, & Pilbeam, 2004; Yi, Williams, Niewolna, Wang, & Yoneda, 2002), VEGF (Guise *et al.*, 2005; Shimamura *et al.*, 2005), FGFs (Matuo *et al.*, 1992; Valta *et al.*, 2006), and urokinase (Achbarou *et al.*, 1994; Festuccia *et al.*, 1997;

Rabbani, Harakidas, Davidson, Henkin, & Mazar, 1995; Rabbani, Rajwans, Achbarou, Murthy, & Goltzman, 1994). Other molecules that may be important are Prostate Specific Antigen (Goya *et al.*, 2006) and OPG (Corey *et al.*, 2005; Kiefer *et al.*, 2004; Zhang *et al.*, 2001), which inhibits bone resorption.

Tumor micro-environment and the pre-metastatic niche

As time has progressed and this field advanced, it has become clear that the vicious cycle concept is exceedingly more complex than originally thought. In addition to the tumor cell interactions with the bone matrix, other cells such as the immune cells, osteoblasts, osteoclasts, and precursor cells all play important roles in bone metastasis and developing the appropriate micro-environment for the growth of tumor cells in bone.

Recent work has focused on the movement of bone marrow-derived precursor cells to sites of tumor cell metastases which help establish a niche environment for tumor cell growth prior to the tumor cells arriving at the metastatic site. One group refers to these cells as bone marrow-derived cells, and has demonstrated that these cells are important for malignant transformation, migration, and tumor vascularization (Kaplan, Psaila, & Lyden, 2006, 2007; Kaplan, Rafii, & Lyden, 2006; Kaplan *et al.*, 2005). These studies have shown that these hematopoietic bone marrow progenitors arrive at sites of metastasis before the tumor cells and secrete factors that allow tumor cells to grow at these sites at a later time (Kaplan *et al.*, 2005). Furthermore, they have demonstrated that VEGFR1 expression by these cells is critical for later metastasis progression, and that factors secreted by tumor cells help determine where these cells and later the tumor cells grow (Kaplan *et al.*, 2005). Other groups have reported similar finding that immune precursor cells similarly colonize in metastatic target organs, and secrete factors that are important for establishing metastasis (Yang *et al.*, 2004).

In addition to these bone marrow precursor cells, immune cells such as the B and T cells can influence bone metastases. For example, tumor-produced factors such as PTHrP, IL-7, and IL-8 may recruit T-cells to sites of tumor in bone (Fournier, Chirgwin, & Guise, 2006). Once recruited, factors secreted by the T-cells may then stimulate bone resorption (Fournier *et al.*, 2006). Other immune cells such as the Natural Killer cells and B cells are also likely to play a role in the vicious cycle of bone metastasis.

PROSTATE CANCER AND BONE METASTASIS

Prostate cancer frequently metastasizes to bone and studies have shown that approximately 90% of the men who die from prostate cancer will have evidence of bone metastases (Bubendorf *et al.*, 2000; Keller & Brown, 2004). These observations suggest that metastatic prostate cancer cells (seed) have a high affinity for the soil of the bone although the pattern of blood flow from the prostate to the spinal lumbar region via the Batson's plexus may also favorably contribute to prostate to bone metastasis. However, vascular flow does not explain why in more advanced cases of prostate cancer, the bone remains almost exclusively the target for metastatic prostate cancer cells. A typical hallmark of prostate metastases in the bone is the robust osteoblastic response induced by the tumors that are often easily discernable by x-ray. However, based on clinical observations, prostate cancers are not solely blastic but often have a lytic compartment to the lesion suggesting that osteolysis also plays a functional role in prostate cancer progression in the bone (Roudier *et al.*, 2004; Vinholes, Coleman, & Eastell, 1996). As a consequence, the humoral factors involved in breast tumor induced osteolysis can also have a role in prostate tumor progression in the bone.

The molecular mechanisms that govern prostate osteotropism are key to understanding why prostate tumor cells home to bone and how the prostate tumor cells interact with the myriad of cells present in the bone in order to support metastatic progression in the bone. The development of several animal models that reflect the human clinical scenario of prostate tumor induced osteolytic and osteoblastic response have been crucial in determining the contribution of humoral factors involved in prostate cancer progression in the bone. Many models have used the human prostate cancer cell lines PC-3 and LnCAP and studies have found that the intra-cardiac or intratibial injection of these cells into various immunocompromised mice result in the growth of prostate tumors in the bone (Singh & Figg, 2005). However, these tumor cell lines typically induce osteolytic lesions which do not reflect the pathology of human prostate to bone metastases but do allow for the study of how prostate tumor cells can induce the recruitment and activation of osteoclasts at the tumor bone interface. Interestingly, several investigators have derived cell lines from these parental cell lines which upon hematogenous or direct inoculation into the bone can generate mixed lesions (Singh & Figg, 2005). A number of transgenic murine models of primary prostate cancer progression also exist. Until recently, bone metastasis in these models had not been reported. However, using the probasin promoter, transgenic mice expressing the SV40 antigen and the serine proteinase hepsin in the prostate have been shown to develop prostate cancer and demonstrate metastasis to the bone, lung and liver and therefore may allow for the study of prostate tumor homing to the bone (Klezovitch *et al.*, 2004).

Several rat prostate cancer cell lines have been described that can induce osteoblastic and osteolytic type metastases. The use of rat prostate cancer cell lines allows for the study of prostate tumor-bone interaction in syngeneic rats thereby ensuring that potential contributions of B and T cells to the process can be considered. Rat cell lines used include, the PA-III cell line which when deposited over the calvaria of Lobund-Wistar rats can induce osteolysis and a robust osteoblastic response (Pollard, 1996), and the MAT-Ly-Lu cell line which interestingly, when intravenously injected into Copenhagen rats induces systemic osteolytic lesions but when inoculated directly into the bone induces an osteoblastic response (Geldof & Rao, 1990). Other rat cell lines exist which mainly induce osteolytic lesions such as the Walker 256, 13762 and cSST2 cell lines (Blouin, Basle, & Chappard, 2005). Recently, Lynch *et al.*, have also have demonstrated that the transplantation of moderately differentiated primary prostate adenocarcinoma to the calvaria of F344 rats or immunocompromised mice induces an osteoblastic and osteolytic lesion that resembles the pathology of human prostate cancer progression in the bone.

Using these models, recent studies have begun to elucidate the humoral vocabulary that prostate cancer cells employ to communicate with bone stromal cells and vice versa. These factors include TGFβ, BMP, Wnts, ET-1, RANKL, vascular endothelial growth factor (VEGF), parathyroid hormone related peptide (PTHrP) and matrix metalloproteinases (MMPs) and the role of each in prostate cancer metastases is described herein:

TGF-β

The bone matrix represents that largest store of TGF-β in the body. During skeletal development, TGF-β is synthesized and incorporated into the bone matrix by osteoblasts. TGF-β is sequestered into the bone matrix in an inactive state via latency associated peptide (LAP) and latent TGF-β binding proteins (LTBPs) that are susceptible to cleavage by several MMPs (Saharinen, Hyytiainen, Taipale, & Keski-Oja, 1999). TGF-β effects are mediated by the TGF-β receptor (TβR) family which is comprised of three members, TβRI, -II and -III (Bierie & Moses, 2006). These receptors are expressed on both osteoblasts and osteoclasts (Janssens, Ten Dijke, Janssens, & Van Hul, 2005). While the role for TGF-β in regulating osteoblast function has been well described, TGF-β has also been shown to be important in mediating osteoclast differentiation and activation (Chambers, 2000; Fox, Haque, Lovibond, & Chambers, 2003; Quinn *et al.*, 2001). TGF-β is endogenously expressed at low levels in normal prostate tissue and in non-pathological scenarios, activation of the TGF-β signaling pathway promotes apoptosis (Bello-DeOcampo & Tindall, 2003). However, during prostate cancer progression, a number of studies have shown that TGF-β is often overexpressed in comparison to normal prostate (Zhu & Kyprianou, 2005). Furthermore, this increase in expression of the ligand has been correlated to decreased expression of the principle TβR, TβRII. This alteration in the TGF-β pathway is associated with more invasive hormone refractory prostate tumor phenotype (Guo, Jacobs, & Kyprianou, 1997). In the bone microenvironment, prostate tumor derived TGF-β can have several effects on the bone stromal cells including, inhibiting the proliferation and promoting the differentiation of bone marrow endothelial cells and in promoting the proliferation of osteoblast precursor cells and the expression of RANKL in the osteoblast cells in addition to the prostate cancer cells themselves (Barrett *et al.*, 2006; Zhang *et al.*, 2004). Therefore, the initial exposure of the bone stroma to tumor derived TGF-β can greatly alter normal bone matrix homeostasis and result in the proliferation and/or activation of several cell types such as the osteoblasts and osteoclasts. In addition, metastatic prostate tumor induced osteolysis can mediate TGF-β release from the bone matrix and further accelerate the ‘vicious cycle’.

BMPs

Over 30 members of the BMP family exist but to date functions with respect to modulating osteoblast function and bone formation have been best described for BMP-2 and BMP-7. BMPs mediate their action via signaling through the BMP receptors (BMPR-IA, BMPR-IB and BMPR2). Several of the BMPs can be inhibited by Noggin and Gremlin which bind directly to the BMP and prevent the interaction of the ligand with the cognate receptor (Chen, Zhao, & Mundy, 2004). Prostate tumors have been shown to express BMPs (BMP-2, BMP-4 and BMP-7) (Yang, Zhong, Frenkel, Reddi, & Roy-Burman, 2005). Prostate tumor cells have also been shown to express BMPR-IB and BMPR-II (Yang *et al.*, 2005; Ye *et al.*, 2007) and in a similar manner to the TGF-β pathway, as the prostate tumors become more invasive, there is a decrease in BMP receptor expression. Subsequent studies have shown that the BMPs may regulate the migration and invasion of prostate tumor cell line (Feeley, Liu *et al.*, 2006). The expression of BMPs and the BMP receptors in primary prostate cancer is intriguing and may play an important role in the seeding of the tumor cells to the soil of the bone, where the BMP signaling pathway is essential for bone function. In this regard, the overexpression of noggin in the prostate tumor cell line, PC-3 significantly reduced the size of the osteolytic lesions induced by the tumor but whether the inhibition of BMP in the surrounding bone cells or tumor cells is responsible for the decrease in prostate tumor progression remains to be determined (Feeley, Liu *et al.*, 2006).

TGF-β and BMPs influence osteomimicry by prostate tumor cells

Perhaps one of the most novel recent advancements in understanding the progression of prostate cancer in the bone involves the concept of ‘osteomimicry’ whereby metastatic prostate tumor cells acquire an osteoblast-like phenotype in response to bone derived humoral factors

(Chung, Baseman, Assikis, & Zhau, 2005). Heightened TGFβ and BMP signaling in the bone can induce the expression of the Runx transcription factor family in tumor cells that regulate several bone related genes such as RANKL, OPG, OPN and BSP (Banerjee *et al.*, 2001; Barnes *et al.*, 2003; Enomoto *et al.*, 2003; Inman & Shore, 2003). The induction of a genetic program controlling the expression of bone related genes in the prostate tumor cells in turn can impact the behavior of the surrounding bone cells and assist in driving the 'vicious cycle'.

Wnts

Wnts are critical mediators of bone formation and therefore, alterations in Wnt signaling by metastatic prostate tumor cells in the bone can have profound effects on osteoblast function (Hall & Keller, 2006). Wnts act by binding to Frizzled and the co-receptor and Lrp5/6. The interaction of Wnt with its receptor complex ultimately results in the stabilization and nuclear translocation of the adherens junction protein, β-catenin. β-catenin has been shown to be an important regulator of genes such as Runx (Gaur *et al.*, 2005). Primary prostate tumors and cell lines have been shown to express Wnts and therefore, metastatic prostate tumor cells in the bone environment can induce osteoblast differentiation, hence bone formation (Hall, Bafico, Dai, Aaronson, & Keller, 2005). Interestingly, inhibitors of Wnt signaling such as dick kopf-1 (DKK-1) prevent ligand interaction with the Frizzled/Lrp5/6 pathway are also expressed at high levels by prostate tumor cells in the bone (Hall & Keller, 2006). These studies suggest that the expression of DKK-1 early during prostate tumor progression in the bone prevents Wnt signaling but increases RANKL expression in osteoblasts resulting in enhanced osteoclast maturation and bone degradation. Subsequently as the prostate cancer cells grow in the bone levels of DKK-1 decrease allowing for Wnt mediated osteoblastogenesis and bone formation. Other inhibitors of the Wnt signaling also exist in the bone environment such as sclerostin, an osteocyte derived factor. Although a weak antagonist of BMP signaling sclerostin has recently has been shown to bind to Lrp5 and prevent Wnt interaction with Frizzled (Li *et al.*, 2005; Winkler *et al.*, 2003). Therefore, the role of sclerostin in attenuating Wnt signaling may play an important role prostate tumor induced osteoblastic and osteolytic changes in the bone.

Endothelin-1 (ET-1)

ET-1, originally identified as a potent regulator vasoconstriction (Yanagisawa *et al.*, 1988), is a powerful stimulator of osteoblast function mediates this activity by signaling through the ET receptor ET_A (Takuwa, Ohue, Takuwa, & Yamashita, 1989; Van Sant *et al.*, 2007). Interestingly, prostate and breast cancer cell lines that induce an osteoblastic response in the bone have been shown to express high levels of ET-1 (Nelson *et al.*, 1995; Yin *et al.*, 2003). Using the ZR-75-1 breast cancer cell line which induces mixed bone lesions, Yin *et al.*, found that blocking tumor derived ET-1 action using a receptor neutralizing antibody or a small molecule inhibitor of ET_A prevented tumor induced osteoblastic and osteolytic changes in the bone (Yin *et al.*, 2003).

Receptor activator of nuclear κ B ligand (RANKL)

RANKL is a type II transmembrane protein that is a member of the tumor necrosis factor (TNF) family of cytokines that is an essential mediator of osteoclast activation (Kong *et al.*, 1999). RANKL mediates its effects by binding to the receptor activator of nuclear kappa B (RANK). Furthermore, osteoprotegerin (OPG), a soluble decoy molecule can prevent the RANKL/RANK interaction by binding to RANKL (Simonet *et al.*, 1997). In breast tumor induced osteolysis, studies have shown that tumor derived PTHrP can stimulate RANKL expression in osteoblasts and that the presence of RANKL on the osteoblast cell surface drives osteoclast maturation (Mundy, 2002). Interestingly, normal human prostate and primary prostate tumors have been shown to endogenously express RANKL although the function of RANKL in the prostate remains to be determined (Brown *et al.*, 2001). However, the expression of RANKL by prostate to bone metastases could have major implications for prostate tumor induced osteolysis. These studies suggest that the human prostate cancer cell line LnCAP and the LnCAP derivative C4-2B which causes mixed lesions in the bone expressed endogenous RANKL and that the osteolysis induced by the C4-2B cell line was prevented by treating mice with C4-2B induced bone lesions with OPG (Zhang *et al.*, 2001). Surprisingly, the decrease in osteolysis was concomitant with a decrease in the osteoblastic response which suggests that the prostate tumor induced osteoblastic response is dependent on osteolysis. Furthermore, the direct interaction of the prostate tumor cells with osteoclasts at the tumor bone interface was demonstrated (Zhang *et al.*, 2001). These studies raise the interesting question that in the context of the tumor: bone microenvironment, metastatic prostate tumor cells may be able to act as surrogate osteoblasts and circumvent the necessity for osteoblast derived RANKL by interacting directly with the immature osteoclast cells in order to mediate localized osteolysis.

Vascular Endothelial Growth Factor (VEGF)

The VEGF family encompasses a number of ligands (VEGF-A, -B, -C, -D and –E as well as placental growth factor) with VEGF-A being the most studied family member. VEGF activity is mediated by the VEGF receptors VEGFR-1/Flt-1, VEGFR-2/KDR, VEGFR-3/Flt-4, and neuropilin-1 and 2 (Hicklin & Ellis, 2005). VEGF is a powerful stimulator angiogenesis and is an important requirement for tumor cell growth since in order for the tumor cell to grow beyond a certain size angiogenesis is required (Fidler, 2002). Several studies have shown that primary prostate cancers and prostate tumor cell lines express VEGF and the VEGF receptors and that in addition to contributing to angiogenesis, VEGF may also contribute to the migratory and metastatic phenotype of the cancer cells (Chen, De, Brainard, & Byzova, 2004; Muir, Chung, Carson, & Farach-Carson, 2006). Interestingly, the expression of VEGF by prostate cancer cells in the bone can also impact the behavior of the surrounding host cells. Rosenblum *et al.*, recently demonstrated using an inhibitor of the VEGF receptors that VEGF was important for osteoclast maturation both *in vitro* and *in vivo* (Mohamedali *et al.*, 2006). Keller *et al.*, have also demonstrated that in the bone environment, BMP-7 induced the expression of VEGF-A in the tumor cells and that VEGF-A subsequently contributed to osteoblast differentiation and mineralization (Dai *et al.*, 2004). Therefore, in addition to regulating angiogenesis, the presence of VEGF in the bone may also regulate prostate tumor cell migration, osteoclast and osteoblast function.

Parathyroid hormone-related peptide (PTHrP)

PTHrP is a potent mediator of metastatic breast tumor induced osteolysis (Mundy, 2002). Interestingly, PTHrP is expressed by primary prostate tumors where it is thought to have a role in cell survival and proliferation (Dougherty *et al.*, 1999; Guise, 2000; Ye, Falzon, Seitz, & Cooper, 2001). Studies have demonstrated PTHrP expression does not play a critical role in the homing of prostate tumor cells to the bone but in the bone environment, the contribution of tumor derived PTHrP to osteoclast activation cannot be ruled out (Blomme *et al.*, 1999).

Matrix Metalloproteinases

The MMPs are comprised of a family of at least 24 related proteinases that collectively are capable of degrading the entire extracellular matrix (ECM) and are often induced in stromal cells in response to the tumor (Lynch & Matrisian, 2002). While MMPs have been associated with primary prostate tumor development and metastasis, the role of MMP expression by prostate cancer tumor cells in the bone is less clear (Lokeshwar, 1999). In the bone, osteoclasts have been shown to be a rich source of MMPs which is not surprising given the role of these proteinases in ECM degradation (Delaisse *et al.*, 2003; Delaisse *et al.*, 2000). Osteoclast derived MMP-1, MMP-13 and cathepsin K are the principle mediators of fibrillar type I collagen degradation, the major constituent of the bone ECM. The resultant fragments of collagen generated by MMPs and cathepsin K have been identified as useful prognostic serum markers of prostate to bone metastasis (Fukumitsu *et al.*, 2002; Garnero *et al.*, 2003). The non-collagenous bone matrix proteins osteopontin (OPN) and bone sialoprotein (BSP) are also modulated by MMPs and the presence of OPN and BSP in the serum have been shown to be prognostic for patients with prostate to bone metastasis (Fedarko, Jain, Karadag, Van Eman, & Fisher, 2001; Ramankulov, Lein, Kristiansen, Loening, & Jung, 2007). MMP-3 and MMP-7 have been shown to mediate OPN cleavage and the resultant fragments can promote cell migration (Agnihotri *et al.*, 2001). OPN contains a functional RGD binding domain that interacts with the cell adhesion molecule alpha (v) beta 3 integrin. Alpha (v) beta3 is often overexpressed in metastatic prostate tumors and OPN has been shown to enhance the migration and invasion of several prostate cancer cell lines (Angelucci, Festuccia, D'Andrea, Teti, & Bologna, 2002). In a similar manner to OPN, BSP can form a complex with MMP-2 and alpha(v) beta 3 integrin and although BSP is not a direct substrate for MMP-2, the presence of this tri-molecular structure on the cell surface results in a more migratory and invasive cell phenotype (Karadag, Ogbureke, Fedarko, & Fisher, 2004). Therefore, MMP degradation and interaction with bone matrix proteins can potentially lead to a more migratory phenotype and given the prevalence of alpha (v) beta 3 expression by prostate cancer, collagen, OPN and BSP modulation by MMPs may influence prostate cancer homing to the bone (Cooper, Chay, & Pienta, 2002).

Surprisingly, mice deficient in MMPs, with the exception of MMP-13 and MMP-14, have subtle or no defects with respect to skeletal development (Holmbeck *et al.*, 1999; Inada *et al.*, 2004; Stickens *et al.*, 2004). This suggests that many of the MMPs expressed in the bone may have roles outside of direct bone matrix degradation. In this regard, it has become increasingly clear that the MMPs are potent mediators of cell-cell communication via the cleavage of growth factors and cytokines and therefore may play a role in facilitating prostate tumor bone interaction (Lynch & Matrisian, 2002).

MMP-2, MMP-3 and MMP-9 are important in mediating the release of TGF-β from either the LTBP molecule and/or the LAP molecule (Dallas, Miyazono, Skerry, Mundy, & Bonewald, 1995; Maeda, Dean, Gay, Schwartz, & Boyan, 2001; Maeda, Dean, Gomez, Schwartz, & Boyan, 2002; Yu & Stamenkovic, 2000). Therefore, prostate cancer induced osteoclast activation and MMP expression can control the release of TGF-β from the bone matrix and assist in driving the vicious cycle. Interestingly, TβRIII, also referred to as betaglycan, has been shown to enhance the binding of active TGF-β to TβRI but the solubilized form of TβRIII can act as a sink for free TGF-β and inhibit TGF-β signaling (Bierie & Moses, 2006). Recently, MMP-14 and MMP-16 have been found to mediate the shedding of TβRIII (Velasco-Loyden, Arribas, & Lopez-Casillas, 2004). Therefore, MMPs appear to key factors in regulating the bioavailabilty of TGF-β.

MMPs have been shown to mediate the shedding of the tumor necrosis factor (TNF) family members such as TNF and FasL (Haro *et al.*, 2000; Powell, Fingleton, Wilson, Boothby, & Matrisian, 1999) and roles for TNF in controlling bone matrix homeostasis have been defined (Gilbert, Rubin, & Nanes, 2005; Kitaura *et al.*, 2002). RANKL is also a member of the TNF family and has been shown to be cleaved by MMP-1, MMP-14 and the metalloproteinase TACE in several cell types (Lum *et al.*, 1999; Schlondorff, Lum, & Blobel, 2001). MMP-3 and MMP-7 were also found to cleave RANKL in the juxtamembrane stalk region which resulted in the release of a soluble bioactive form of RANKL (Lynch *et al.*, 2005). Recently, using a rat model of prostate tumor induced osteolytic and osteoblastic changes, MMP-7 was shown to contribute to the tumor-induced osteolysis via the cleavage of RANKL (Lynch *et al.*, 2005). These studies demonstrate the importance of MMPs in regulating the bioavailability of a soluble form of RANKL which may circumvent the necessity for premature osteoclasts to directly interact with RANKL presenting osteoblasts and as a consequence accelerate osteoclast recruitment and activation at the tumor bone interface.

MMP-1, -2 and -3 have previously been shown to process the major IGF-binding protein, IGF-BP3. IGF-BP3 directly binds to IGF-I and prevents the interaction of IGF-I with the IGF-I receptor (IGF-1R) (Denley, Cosgrove, Booker, Wallace, & Forbes, 2005; Fowlkes, Enghild, Suzuki, & Nagase, 1994; Thrailkill *et al.*, 1995). IGF-I is commonly found in the bone and as is an important regulator of osteoblast mediated bone formation and osteoclastogenesis (Canalis, Centrella, Burch, & McCarthy, 1989; Jiang *et al.*, 2006). IGF-I has also been shown to play an important role in the progression of primary prostate cancer (Gennigens, Menetrier-Caux, & Droz, 2006). Therefore, MMP processing of IGF-BP3 may enhance the levels of bioavailable IGF-I and subsequently assist in promoting prostate tumor progression in the bone microenvironment.

In addition to these described substrates, MMPs have been also shown to regulate the bio-availability of other humoral factors present in the bone such as stromal derived factor-1 (SDF-1)/ chemokine ligand 12 (CXCL12) and VEGF, factors that are important in hematopoietic stem cell mobilization from the bone marrow and in angiogenesis respectively (Bergers *et al.*, 2000; Heissig *et al.*, 2002; McQuibban *et al.*, 2001).

MYELOMA BONE DISEASE

Multiple myeloma is a hematological malignancy which is characterized by the development of a progressive and destructive osteolytic bone disease which differs from the osteolysis frequently seen with solid tumors. The bone destruction associated with multiple myeloma results in manifestations including lytic bone lesions, which primarily affect the axial skeleton, hypercalcaemia, severe bone pain, and pathological fractures which occur either spontaneously or following trivial injury. The majority of patients with multiple myeloma have discrete osteolytic lesions occurring in areas of myeloma cell infiltration. Myeloma cells are found in close association with sites of active bone resorption, and the interactions between myeloma cells, osteoclasts, stromal cells, myeloma-associated fibroblasts and osteoblasts are crucial both for the development of the osteolytic bone disease and for myeloma cell growth and survival in the bone marrow (Mundy, 1998; Mundy, Raisz, Cooper, Schechter, & Salmon, 1974). Although the precise molecular mechanisms responsible for the bone destruction in multiple myeloma are not completely understood, it is known that the bone destruction is primarily mediated by osteoclasts, and that this destruction is exacerbated by a reduction in osteoblastic bone formation. Patients with multiple myeloma have abnormal bone remodeling, where resorption and formation become uncoupled, with the end result being an increase in bone resorption and a decrease in bone formation. Histomorphometric studies have demonstrated that bone resorption is increased in patients with multiple myeloma, with an increase in both the number and activity of osteoclasts (Bataille *et al.*, 1991; Taube *et al.*, 1992; Valentin, Charhon, Meunier, Edouard, & Arlot, 1982). The increase in resorption is thought to be an early event in the development of myeloma bone disease (Bataille *et al.*, 1991). Although early stages of multiple myeloma have been associated with an increase in osteoblast recruitment, a very marked impairment of bone formation due to reduced osteoblast number and activity is a common feature in the later stages of the osteolytic bone disease (Bataille *et al.*, 1991; Bataille, Delmas, Chappard, & Sany, 1990; Evans, Galasko, & Ward, 1989). This has been confirmed in studies which demonstrate that markers of bone formation are decreased in patients with multiple myeloma (Abildgaard *et al.*, 1998; Woitge *et al.*, 2001).

Myeloma cells secrete a number of osteoclast activating factors which can promote osteoclast formation and activation, including lymphotoxin, TNF, IL-1, IL-3 and IL-6 (Garrett *et al.*, 1987; Kawano *et al.*, 1988; Lee *et al.*, 2004; Lichtenstein, Berenson, Norman, Chang, & Carlile, 1989). While *in vitro* studies have demonstrated a role for these factors in the development of myeloma bone disease, their role *in vivo* remains unclear. Cell-cell contact between myeloma cells and bone marrow stromal cells is critically important in the development of myeloma bone disease and plays a key role in the abnormal regulation of factors implicated in myeloma bone disease, including RANKL, OPG and Dkk1. Over recent years, our understanding of the osteoclastogenic and osteoblastic factors involved in the development of myeloma bone disease has improved. Factors which have been implicated include RANKL, OPG, MIP-1α, SDF-1, TGF-β, Dkk1 and sFRP-2. Many of these factors have been demonstrated to be dysregulated in patients with multiple myeloma, and all may play a role in either bone resorption or bone formation, either alone or in combination.

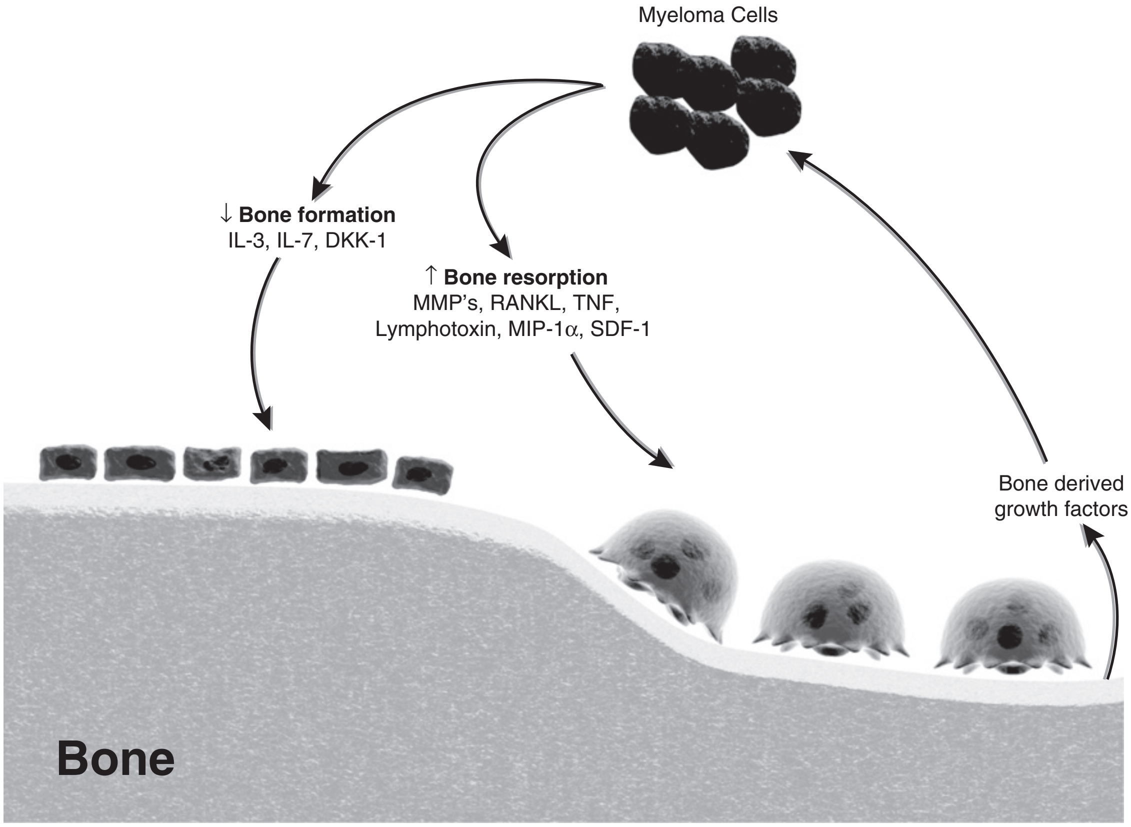

FIGURE 2 Factors implicated in myeloma bone disease. Myeloma bone disease is characterized by both an increase in bone resorption and a decrease in bone formation. These factors have been linked to the development of myeloma bone disease, by either increasing osteoclast activity or by inhibiting osteoblastic bone formation.

Pathophysiology of Osteoclast Stimulation

The mechanism of myeloma bone disease is an uncoupled bone remodeling with enhanced osteolytic resorption, which is mainly mediated by osteoclasts, and decreased bone formation. Both the number and activity of osteoclasts in myeloma patients are increased. Osteoclasts always occur adjacent to collections of myeloma cells, so the mechanism for the bone destruction in myeloma is locally mediated, presumably by cytokines released by the myeloma cells in the microenvironment of the osteoclasts (Mundy, Luben, Raisz, Oppenheim, & Buell, 1974; Mundy, Raisz *et al.*, 1974). Although it has been known for more than 30 years that osteoclasts are hyperstimulated by cytokines in myeloma, there are still unresolved questions as to the nature of the local mediators responsible for the increased osteoclast recruitment and activation.

The RANK/RANKL/OPG System

It is apparent that interactions between receptor activator of nuclear factor kappa B (RANK) expressed on the surface of the osteoclast lineage cells and RANK ligand (RANKL) expressed on stromal cells play a key role in the development and activation of osteoclasts in multiple myeloma. Bone marrow plasma cells derived from patients with multiple myeloma revealed high positive RANKL immunoreactivity as compared to healthy controls, and among patients with multiple myeloma RANKL immunoreactivity on plasma cells was positively correlated with the presence of osteolytic lesions (Heider *et al.*, 2004). RANKL induction by stromal cells was present in patients with multiple myeloma but not in patients with monoclonal gammopathy of undetermined significance (MGUS) (Pearse *et al.*, 2001; Roux *et al.*, 2002), indicating a specific threshold effect. There is some controversy as to whether myeloma cells directly express RANKL. A subset of myeloma cells have been described to express RANKL (Sezer, Heider, Jakob, Eucker, & Possinger, 2002). Several studies have unambiguously demonstrated that myeloma cells stimulate osteoclastogenesis by triggering an increase in RANKL expression by stromal cells through direct cell-to-cell contact (Giuliani, Bataille, Mancini, Lazzaretti, & Barille, 2001; Pearse *et al.*, 2001; Roux *et al.*, 2002; Sezer *et al.*, 2002), and this effect could be prevented by RANK-Fc, a specific inhibitor of RANKL (Pearse *et al.*, 2001). A decrease in osteoprotegerin (OPG), which is a decoy receptor for RANKL, may also be important. Myeloma patients have OPG serum levels that are lower than those of controls and inversely correlated with the number of lytic lesions. Serum levels of sRANKL in patients with MM were elevated and correlated with bone disease. The sRANKL/OPG ratio was also increased and correlated with markers of bone resorption (Terpos *et al.*, 2003). Human myeloma cells do not express or release OPG but myeloma cells may interact with bone marrow stromal cells and osteoblasts to reduce the concentration of OPG released from these cells, which is consistent with the hypothesis that decreased OPG may play a role in the pathogenesis of myeloma bone disease (Giuliani *et al.*, 2001; Pearse *et al.*, 2001).

MIP-1α

Macrophage inflammatory protein-1 alpha (MIP-1α) is a member of the C-C chemokine family originally purified from lipopolysaccharide-treated monocyte cell lines (Wolpe *et al.*, 1988). MIP-1α is normally expressed by stromal and hematopoietic stem cells (Kukita *et al.*, 1997). Using competitive PCR, mRNA for MIP-1α was shown to be elevated in bone marrow plasma of myeloma patients compared to normal controls and MIP-1α can directly stimulate osteoclast formation and differentiation in a dose-dependent way (Choi *et al.*, 2000; Han *et al.*, 2001). Blockade of bone destruction in an animal model using an antisense strategy against MIP-1α suggested that MIP-1α is responsible for the enhanced bone resorption in myeloma (Choi *et al.*, 2001). Oyajobi et al (Oyajobi *et al.*, 2003) demonstrated that athymic mice bearing intra-muscular Chinese hamster ovarian (CHO)/MIP-1α tumors developed lytic lesions earlier and larger than those in mice with CHO/empty vector (EV) tumors, suggesting MIP-1α is sufficient to induce MM-like destructive lesions in bone *in vivo*.

MIP-1α binds to CCR1 and CCR5 receptors on the surface of osteoclast precursors to exert its effects on osteoclast formation. Neutralizing antibodies to CCR1 or CCR5 as well as the CCR1-specific antagonist markedly inhibit osteoclast formation stimulated by MIP-1α (Oba *et al.*, 2005). In addition, injection of recombinant MIP-1α produced a strong increase in osteoclast formation in normal mice, but not in RANK-/- animals in a murine model of myeloma (Oyajobi *et al.*, 2003), suggesting MIP-1α effects on osteoclasts are mediated through the RANKL pathway.

Other factors

VEGF

Vascular endothelial growth factor (VEGF) has an important role in the induction of neovascularization in solid tumors. However it was not until 1994 that the microvessel density (MVD) of bone marrow in myeloma patients was shown to correlate with disease progression and poor prognosis (Vacca *et al.*, 1994). Myeloma cells may secrete VEGF. VEGF may substitute for M-CSF in the induction of *in vitro* osteoclast differentiation (Niida *et al.*, 1999). A recent study demonstrated that blockade of both VEGF and osteopontin almost completely abrogated angiogenesis and bone resorption enhanced by coculture of myeloma cells and osteoclasts (Tanaka *et al.*, 2007), suggesting VEGF probably supports osteoclastic bone resorption.

SDF-1 alpha/CXCR-4

Stromal derived factor-1 alpha (SDF-1α; CXCL12) is a CXC chemokine. Its ligand is CXCR4 which is expressed on hematopoietic stem cells and lymphocytes, as well as malignant cells and osteoclast precursors (Juarez & Bendall, 2004; Smith *et al.*, 2004; Yu, Huang, Collin-Osdoby, & Osdoby, 2003). SDF-1 is expressed by bone marrow stromal cells and endothelial cells. SDF-1α/CXCR-4 plays an important role in hematopoietic stem cell homing and tumor migration and proliferation. Recent data showed SDF-1α may not only mediate the migration and homing of myeloma cells (Alsayed *et al.*, 2007), but increase osteoclast motility and bone resorbing activity (Zannettino *et al.*, 2005). This was associated with an overexpression of osteoclast activation-related genes, including RANKL, RANKL, TRAP, MMP-9, carbonic anhydrase-11 and cathepsin K. Osteoclast activation mediated by myeloma cells was reduced by a CXCR-4 specific inhibitor (Zannettino *et al.*, 2005). These findings suggest that SDF-1α may also be an important factor in the pathogenesis of myeloma bone disease.

Cell-cell direct interaction

The direct attachment of myeloma cells to bone marrow stromal cells (BMSCs) induces NF-κB activation and leads to up-regulation of IL-6, VEGF and RANKL in BMSCs (Abe *et al.*, 2004; Michigami *et al.*, 2000; Oyajobi, Traianedes, Yoneda, & Mundy, 1998). Data from many studies provide compelling evidence implicating these cellular interactions, not only in the growth of myeloma in bone, but also in initiating the sequence of events that lead to promotion of osteoclastogenesis and bone destruction. An example is a link between VCAM-1/VLA-4 and osteoclast formation via induction of RANKL following direct cell-cell contact between BMSCs and myeloma cells. 5TGM1 myeloma cells exhibit tight adherence to the mouse marrow stromal cell line ST2 in vitro and contact between the two cell types increased RANKL mRNA expression compared with either cell type alone (Michigami *et al.*, 2000; Oyajobi *et al.*, 1998). The effect of these cell-cell interactions was mimicked by treating the myeloma cells with recombinant soluble VCAM-1 (Oyajobi *et al.*, 1998) and, conversely, the production of the soluble activity was blocked by neutralizing antibodies to either α4 or to VCAM-1 (Michigami *et al.*, 2000).

In addition, MIP-1α utilizes CCR1 and CCR5 not only to induce communication between myeloma cells and precursors of osteoclasts resulting in osteoclast formation, but also to increase adhesion of myeloma cells to BMSCs (Obaa, Leea, & Ehrlicha, 2005). MIP-1α induced enhancement of IL-6 production by BMSCs can be significantly inhibited by either anti-MIP-1α, anti-CCR1, anti-CCR5 antibodies or CCR1-specific antagonist, BX471, compared to the control culture treated with isotype specific IgG (Obaa *et al.*, 2005).

Myeloma cells also adhere to osteoclasts directly. Cell-to-cell interaction with peripheral blood mononuclear cell derived osteoclasts enhanced growth and survival of primary MM cells as well as MM cell lines more potently than stromal cells (Abe *et al.*, 2004). Osteoclasts also protected MM cells from apoptosis induced by serum depletion or doxorubicin (Abe *et al.*, 2004). The effects were only be partially inhibited by anti-IL-6 and anti-osteopontin (OPN) antibodies. However, prevention of cellular contact between myeloma cells and osteoclasts abolished the effect of osteoclasts on myeloma cell growth. In addition, a cell-to-cell interaction between human myeloma cells and osteoclasts potently enhanced vascular tubule formation (Tanaka *et al.*, 2007). Blockade of both VEGF and OPN actions almost completely abrogated such vascular tubule formation as well as migration and survival of human umbilical vascular endothelial cells (HUVECs) enhanced by conditioned medium from coculture of myeloma cells and osteoclasts (Tanaka *et al.*, 2007). These observations suggest the presence of a close link between myeloma cells, osteoclasts, and vascular endothelial cells to form a vicious cycle, that osteoclasts activated by myeloma cells can lead to bone destruction, enhance the aggressive behavior and survival of myeloma cells and promote angiogenesis via cell-to-cell interaction with myeloma cells.

Pathophysiology of Osteoblast Inhibition in Myeloma

A key component of myeloma bone disease is the reduction in bone formation, resulting in both a generalized loss of bone and an inability to repair the osteolytic lesions which occur as a result of increased bone resorption. While there is an extensive knowledge of the factors which mediate osteoclastic bone destruction, the cellular and molecular mechanisms which regulate bone formation in multiple myeloma are poorly understood. Several studies have demonstrated that myeloma cells can suppress osteoblast formation and differentiation, and a number of factors have been identified which are dysregulated in multiple myeloma and may play a role in the inhibition of osteoblast formation or function, including Dkk1, sFRP-2, IL-3, Runx2, and TGF-β.

Inhibition of Wnt signaling

Wnts are secreted glycoproteins that activate receptor-mediated signaling pathways which play critical roles in cell development and differentiation both in embryogenesis and in adults (Moon, Kohn, DeFerrari, & Kaykas, 2004). Canonical Wnt signaling is transduced by two transmembrane protein receptor families; frizzled proteins and lipoprotein receptor-related proteins 5 and 6 (LRP5/6). Activation of the canonical Wnt signaling pathway results in the translocation of β-catenin to the nucleus and activation of downstream target genes. This pathway plays a key role in the regulation of bone mass, as demonstrated by mutations in the gene for LRP5, resulting in osteoporosis-pseudoglioma syndrome in humans and osteopenia in mice (Gong *et al.*, 2001). Over-expression of β-catenin in osteoblasts has been demonstrated to induce osteoblast proliferation and a high bone mass phenotype. (Kato *et al.*, 2002), furthermore, increasing Wnt signaling by inhibition of GSK-3β has been demonstrated to increase bone mass *in vivo* (Clement-Lacroix *et al.*, 2005). The key role of Wnt signaling in stimulating bone formation has raised the possibility that this signaling pathway may be important in diseases associated with abnormal bone formation, including multiple myeloma. In support of this, increasing Wnt signaling in the bone microenvironment in multiple myeloma, by inhibition of GSK-β with LiCl, resulted in the inhibition of myeloma bone disease, using a murine model of myeloma (Edwards *et al.*, 2006).

There are 2 classes of extracellular antagonists of the Wnt signaling pathway, with distinct inhibitory mechanisms, acting either by binding directly to Wnt (secreted FRPs, WIF-1) or by binding to part of the Wnt receptor complex (Dkk family). There is recent evidence to suggest a role for each of these classes of inhibitors in the development of myeloma bone disease.

The soluble, extracellular antagonist of the Wnt signaling pathway, Dkk1, has been identified as a potential mediator of osteoblast dysfunction in myeloma bone disease (Tian *et al.*, 2003). Dkk1 is expressed by osteoblasts and bone marrow stromal cells, and has been demonstrated to inhibit bone formation in osteoblasts *in vitro* (Rawadi, Vayssiere, Dunn, Baron, & Roman-Roman, 2003). Over-expression of Dkk1 in bone marrow aspirates from patients with multiple myeloma was initially identified by cDNA microarray. Dkk1 concentrations were increased in bone marrow plasma and peripheral blood of patients with multiple myeloma, as compared to healthy individuals or to patients with MGUS (Tian *et al.*, 2003). In addition, osteoblast differentiation was blocked by bone marrow serum from patients with myeloma, and the inhibitory effect was found to be the result of Dkk1 in the serum. The development of myeloma bone disease is dependent upon interactions within the local bone marrow microenvironment. Myeloma cells have been demonstrated to interact with osteoblasts and up-regulate expression of Dkk1 by osteoblasts (Oyajobi *et al.*, 2004). Furthermore, interactions between myeloma cells and mesenchymal stem cells have been demonstrated to promote the growth of myeloma cells, resulting in an increase in Dkk1 and consequent inhibition of mesenchymal stem cell differentiation into osteoblasts (Gunn *et al.*, 2006). Preclinical studies have demonstrated the potential for targeting Dkk1 in myeloma bone disease. The treatment of myeloma-bearing SCID-rab mice with anti-Dkk1 antibody reduced osteolytic bone lesions, increased osteoblast number and significantly reduced tumor burden (Yaccoby *et al.*, 2007). Thus, Dkk1 may represent a novel therapeutic target for the treatment of myeloma bone disease.

Secreted Frizzled related protein-2 (sFRP-2) is a soluble antagonist of Wnt signaling which has been shown to be expressed by human myeloma cells(Oshima *et al.*, 2005). Furthermore, both exogenous sFRP-2 and myeloma-cell derived sFRP-2 have been shown to play a key role in bone formation *in vitro*, raising the possibility that sFRP-2 may play a role in the development of myeloma bone disease.

Transforming Growth Factor β

Transforming growth factor β is a ubiquitous, multifunctional growth factor that is released from the bone matrix during osteoclastic bone resorption and acts to inhibit osteoblast differentiation. In addition to inhibiting myeloma growth, inhibition of TGF-β has been demonstrated to block the ability of myeloma cells to inhibit osteoblast differentiation *in vitro*. These results suggest a role for TGF-β in mediating osteoblast dysfunction in myeloma, and the potential for targeting TGF-β in the treatment of myeloma bone disease (Takeuchi *et al.*, 2006).

Runx2

Runt-related transcription factor 2 (Runx2) is a transcription factor which acts to promote the formation and differentiation of osteoblasts from mesenchymal stem cells. The critical role for Runx2 in osteoblastogenesis is demonstrated by the complete lack of osteoblasts and bone formation in mice deficient in Runx2. Myeloma cells were found to inhibit Runx2 activity and reduce osteoblast differentiation in osteoprogenitor cells; an effect that was mediated by both cell-cell contact and interleukin 7(Giuliani *et al.*, 2005). In support of a role for Runx2 in the pathophysiology of myeloma bone disease, a significant reduction in the proportion of Runx2 positive osteoblasts was observed in patients with myeloma with evidence of osteolytic bone lesions, compared to those patients with no evidence of bone disease (Giuliani *et al.*, 2005).

IL-3

In addition to a role in osteoclastogenesis, recent evidence suggests that IL-3 may also play a role in regulation of bone formation in myeloma bone disease. (Ehrlich *et al.*, 2005; Lee *et al.*, 2004). While IL-3 had no effect on osteoblast cell lines, IL-3 inhibited basal and BMP-2 stimulated osteoblast formation from primary stromal cells *in vitro*. The inhibitory effects of IL-3 on osteoblast differentiation were found to be an indirect effect as a result of increasing the number of CD45 + hematopoietic cells in stromal cultures. Furthermore, bone marrow plasma from patients with myeloma was found to inhibit osteoblast differentiation, and this effect could be blocked with a neutralizing antibody to IL-3. These data identify IL-3 as a potential mediator of myeloma bone disease, with a dual effect to directly increase osteoclastic bone resorption, and indirectly inhibit osteoblast differentiation.

HGF

Hepatocyte growth factor (HGF) is produced by myeloma cells and is increased in the serum of patients with multiple myeloma (Seidel *et al.*, 1998). Levels of HGF are correlated with a poor prognosis and HGF acts as a growth and survival factor for myeloma cells (Andersen *et al.*, 2005). More recently, serum concentrations of HGF were negatively correlated with levels of bone specific alkaline phosphatase in patients with multiple myeloma, suggesting a role for HGF in myeloma bone disease(Standal *et al.*, 2007). HGF was found to inhibit BMP-induced osteoblastogenesis, including expression of the transcription factors Runx2 and Osterix, potentially by inhibiting nuclear translocation of receptor-activated SMADs (Standal *et al.*, 2007).

Proteasome Inhibitors

Proteasome inhibition is a new therapeutic strategy in the treatment of multiple myeloma, which is based on the exquisite sensitivity of myeloma cells to the activity of the proteasome. Bortezomib (Velcade™; PS-341) is a potent inhibitor of myeloma cell growth and survival *in vitro* and results from several completed or on-going clinical trials indicate striking therapeutic efficacy and acceptable toxicity profile of intravenous Bortezomib in myeloma patients when administered alone or in combination with dexamethasone and/or chemotherapeutic agents, after relapse or at first presentation (Jagannath, Barlogie *et al.*, 2005; Jagannath, Durie *et al.*, 2005; Richardson *et al.*, 2005a, 2005b; Richardson *et al.*, 2003; Yang, Vescio, Schenkein, & Berenson, 2003). Proteasome inhibitors are more effective in myeloma than in other cancers, which may reflect a dependence of myeloma cells on proteasome function and degradation of NF-κB. The tremendous impact of proteasome inhibition in the treatment of multiple myeloma is all the more exciting because we have recently shown that structurally-unrelated inhibitors of the ubiquitin-proteasome pathway (including Bortezomib) have beneficial anabolic effects on the skeleton *in vivo* (Garrett *et al.*, 2003; Oyajobi *et al.*, 2004). In addition to direct effects on osteoblasts, proteasome inhibitors have also recently been demonstrated to inhibit osteoclast formation and bone resorption (Zavrski *et al.*, 2005). In support of these observations, Bortezomib has been demonstrated to reduce tumor burden and increase BMD in myeloma-bearing mice using the SCID-rab model of myeloma, an effect associated with an increase in osteoblasts and a reduction in osteoclasts (Pennisi *et al.*, 2006). Clinical studies have demonstrated significant increases in markers of bone formation, including alkaline phosphatase and osteocalcin in those patients who responded to Bortezomib treatment (Giuliani *et al.*, 2006; Shimazaki *et al.*, 2005; Zangari *et al.*, 2005; Zangari, Yaccoby, Cavallo, Esseltine, & Tricot, 2006). In addition to changes in markers of bone formation, Bortezomib treatment has also been shown to result in a reduction in serum Dkk1 and RANKL (Terpos *et al.*, 2006). These studies also suggest that the effect of Bortezomib on bone formation is specific to proteasome inhibition, and not a result of reducing tumor burden. Thus, Bortezomib may be effective both for the treatment of myeloma and of the associated bone disease.

Management of Patients with Metastatic Bone Disease

Management of patients with advanced cancer with cytotoxic agents or hormonal therapy will not be considered here. Rather, we will focus on the specific approaches aimed at treating the bone disease. This in essence means the use of agents which target the bone microenvironment rather than the tumor directly.

The most frequently used modalities are radiation therapy and bisphosphonates, drugs which specifically inhibit osteoclastic bone resorption. Radiation therapy is widely used for the treatment of severe bone pain when the disease is localized, and is often effective. The mechanism by which radiation therapy causes a palliative effect on bone pain remains unknown.

Bisphosphonates have been widely used for the past 30 years for patients with metastatic bone disease. They are now FDA-approved for all types of bone metastases, and have proven to be effective in reducing skeletal-related events by approximately 50% (Hortobagyi *et al.*, 1996; Lipton *et al.*, 2002; Theriault *et al.*, 1999). This is good but not optimal therapy for patients with metastatic bone disease. Bisphosphonates are associated with some complications, including propensity to precipitate renal failure, and osteonecrosis of the jaw (AAOMS, 2006; ADACSA, 2006; Marx & Stern, 2002; Ruggiero, Fantasia, & Carlson, 2006; Ruggiero, Mehrotra, Rosenberg, & Engroff, 2004; Surgeons, 2006) Impaired renal function has been seen most frequently in patients treated with zoledronic acid, but is unlikely to be an issue if the drug is given in appropriate doses to patients with normal renal function over the recommended periods of infusion time. Osteonecrosis of the jaw has been seen much more frequently in recent years, particularly in patients treated with intravenous bisphosphonates for cancer. This may be related to the cumulative dose and the fact that the bisphosphonates used are powerful and given intravenously. Patients with osteonecrosis of the jaw are frequently given relatively large doses and commonly have had either a recent dental procedure or have active dental disease. The pathophysiology of this condition is still not known, but may be related to bacterial infection in the oral cavity, and the high rate of bone turnover that is usually seen in the jaw.

Bisphosphonates are drugs which effectively reduce bone resorption, but do not restore bone that has been lost. Therefore, some patients with severe bone fragility caused by metastatic cancer will continue to have skeletal-related events despite optimal bisphosphonate therapy, and an underlying malignant disease which is in remission. This remains an ongoing problem for the treatment of patients with these advanced malignancies, particularly because many of these patients are living for prolonged periods of time.

Currently, the mostly widely used bisphosphonates for this indication are zoledronic acid, pamidronate and clodronate. There are differences in mode of administration (zoledronic acid 15–30 minute infusion, pamidronate 2–4 hours infusion, clondronate oral), and potency. There are less convincing differences in efficacy based on current evidence, although it is possible zoledronic acid is slightly more efficacious. Zoledronic acid has been associated with impaired renal function more frequently than the other agents, but it is also probably used more commonly for this indication.

Experimental approaches are now being developed for the treatment of patients with metastatic bone disease. These include approaches based on inhibition of TGF-β signaling in the stromal cells in the bone microenvironment, which may promote osteoblast differentiation and tends to inhibit PTHrP. Other approaches are focused on inhibiting PTHrP action or effects, and include anti-sera to PTHrP (Guise et al 1996) and small molecule inhibitors of PTHrP transcription (Gallwitz *et al.*, 2002).

OSTEOPOROSIS IN PATIENTS WITH CANCER

Osteoporosis is common in patients with advanced malignancy. Just how common is not known with any accuracy, but it is clear that it is not only frequent, but becoming more prevalent. Much of the emphasis in recent years has been on patients who have been treated with various therapies for the underlying malignancy, and osteoporosis is a side effect of a number of effective anti-cancer treatments (see below). This could be anticipated because attempts to kill tumor cells with cytotoxic agents are also likely to kill those normal cells with relatively high rates of cell turnover and proliferation, such as precursors of osteoblasts.

This has always been an issue in patients with malignancy treated with chemotherapy or corticosteroids, but has been emphasized much more in recent years in the hormone-dependent tumors, namely in carcinomas of the breast and prostate, which are frequently treated with therapies that influence hormones and control bone-cell activity. In the case of breast cancer, the widespread use of aromatase inhibitors and SERMs has led to generalized bone loss in patients with breast cancer, and in prostate cancer, the use of androgen deprivation therapy either by LHRH or by castration, or by the use of estrogen-related compounds has also caused widespread bone loss.

However, what is not emphasized so much is that osteoporosis is probably also common in patients with malignancy who are treatment-naïve and untreated with any of these drugs. This was first documented by Kanis and colleagues (1999) (Kanis *et al.*, 1999), who found a marked increase in the rate of vertebral fractures in untreated breast cancer patients. Kanis *et al.*, 1999 examined the prevalence and incidence of vertebral fractures in women with breast cancer at the time of diagnosis, during the next three years in women without skeletal metastases, and in women with advanced disease without skeletal metastases. They found that at the time of diagnosis, the prevalence of vertebral fractures was the same as in the control population but the annual incidence rate was five-fold greater per year over the first three years after diagnosis, and in women with advanced disease without skeletal metastases, was twenty-fold that of appropriate controls.

This has long been appreciated in myeloma. The pathophysiology of osteoporosis in this situation is unknown, but this type of low turnover osteoporosis is similar to that seen in men, in patients of advanced age, or patients treated with corticosteroid therapy. Treatment of this type of osteoporosis may be best accomplished with an anabolic agent.

Cancer-treatment-induced osteoporosis

It has been noted by the Institute of Medicine, National Research Council and NCI Office of Cancer Survivorship that systemic bone loss is an unfortunate feature of the majority of non-surgical cancer-treatments (Hewitt, 2006). The use of chemotherapy, radiation and hormonal therapy has undoubtedly improved the long-term prognosis for cancer sufferers, but the skeletal complications of such approaches often result in decreased bone mass and increased fracture risk for the patient. One mechanism for this effect in premenopausal women involves the decreased levels of circulating estrogen through primary ovarian failure as a result of chemotherapy and the onset of premature menopause (Hirbe, Morgan, Uluckan, & Weilbaecher, 2006; Shapiro, Manola, & Leboff, 2001). Aromatase inhibitors also have the potential to cause decreased BMD by disrupting normal estrogen levels (Ganz *et al.*, 2004; Mackey & Joy, 2005) and the resulting hypogonadism following androgen deprivation therapy is a primary cause of acquired osteoporosis (Hewitt, 2006; Krupski *et al.*, 2004). Interestingly, this effect is thought to be due to decreases in estrogen as well as testosterone levels (Smith, 2006).

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Chapter 65

Genetic Regulation of Parathyroid Gland Development

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Congenital anomalies of parathyroid gland development, which lead to hypoparathyroidism, are common and occur in more than 1 in 4000 births. The four parathyroid glands, in man, develop from transient bilateral outpocketings of the pharyngeal endoderm, which are referred to as the pharyngeal pouches. There are four pairs of pharyngeal pouches, and the superior parathyroid glands develop from the fourth pharyngeal pouches, which are the most caudal, whereas the inferior parathyroid glands together with the thymus develop from the third pharyngeal pouches. The pharyngeal pouches together with the branchial arches and grooves form the branchial apparatus, which gives rise to a number of structures that include the face, jaws, oral cavity, neck, pharynx, larynx, ear, aortic arch, tonsils, thyroid, parathyroids, thymus, ultimobranchial body that results in calcitonin-producing cells, carotid glomus, and the epibranchial placodes that contribute to the facial, glossopharyngeal, and vagus nerves. Thus, developmental abnormalities of the branchial apparatus and pharyngeal pouches may lead to hypoparathyroidism that may be part of a complex congenital defect, as for example in the DiGeorge syndrome or a solitary endocrinopathy which is called *isolated* or *idiopathic* hypoparathyroidism. In addition, hypoparathyroidism may occur in association with other developmental anomalies involving dysmorphic features, sensorineural deafness, lymphedema, nephropathy, and cortical thickening of tubular bones. The molecular genetic basis for such forms of hypoparathyroidism has been investigated, and this has helped to elucidate further the mechanisms involved in the genetic regulation of parathyroid gland development. This chapter will focus on those forms of hypoparathyroidism that often present in early life, as these are likely to be associated with parathyroid gland agenesis or hypoplasia, or a congenital deficiency of parathyroid hormone (PTH), or an early destruction of the parathyroids (Table I). The hypoparathyroidism in these forms is characterized by hypocalcemia and hyperphosphatemia due to a deficiency in PTH secretion (Thakker, 1993; Bilezikian and Thakker, 1998).

COMPLEX SYNDROMES ASSOCIATED WITH HYPOPARATHYROIDISM

Hypoparathyroidism may occur as part of a complex syndrome that may be associated either with a congenital development anomaly or with an autoimmune syndrome. The congenital developmental anomalies associated with hypoparathyroidism include the DiGeorge, HDR (hypoparathyroidism, deafness, and renal anomalies), Kenney-Caffey, and Barakat syndromes, and also syndromes associated with either lymphedema or dysmorphic features and growth failure (see Table I).

DiGeorge Syndrome

Clinical Features and Genetic Abnormalities

Patients with DiGeorge syndrome (DGS) typically suffer from hypoparathyroidism, immunodeficiency, congenital heart defects, and deformities of the ear, nose, and mouth. The disorder arises from a congenital failure in the development of the derivatives of the third and fourth pharyngeal pouches with resulting absence or hypoplasia of the parathyroids and thymus. Most cases of DGS are sporadic, but an autosomal dominant inheritance of DGS has been observed, and an association between the syndrome and an unbalanced translocation and deletions involving 22q11.2 have also been reported (Scambler *et al.,* 1991); this is referred to as DGS type 1 (DGS1). In some patients, deletions of another locus on chromosome 10p have been observed in association with DGS (Monaco *et al.,* 1991), and this is referred to as DGS type 2 (DGS2). Mapping studies of the DGS1 deleted region on chromosome 22q11.2 defined a 250-kb to 3000-kb critical region (Gong *et al.,* 1996; Scambler et al., 2000), that contained approximately 30 genes. Studies of DGS1 patients reported deletions of several of the genes (e.g., rnex40, nex2.2–nex 3, UDFIL, and TBX1) from the critical region (Augusseau *et al.,* 1986; Budarf *et al.,* 1995; Yamagishi *et al.,* 1999;

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TABLE I Inherited Forms of Hypoparathyroidism and Their Chromosomal Locations

Disease	Inheritance	Gene Product	Chromosomal Location
Hypoparathyroidism associated with			
complex congenital syndromes	Autosomal dominant		22q11
DiGeorge type 1 (DGS1)	Autosomal dominant	Unknown	10p13–p14
DiGeorge type 2 (DGS2)	Autosomal dominant	GATA3	10p15
HDR			
Hypoparathyroidism associated with KSS,	Maternal	Mitochondrial	
MELAS, and MTPDS		genome	21q22.3
APECED	Autosomal dominant	AIRE1	?
Kenney–Caffey	Autosomal dominant[a]	Unknown	?
Barakat	Autosomal recessive[a]	Unknown	?
Lymphedema	Autosomal recessive	Unknown	?
Nephropathy, nerve deafness	Autosomal dominant[a]	Unknown	?
Nerve deafness without renal dysplasia	Autosomal dominant	Unknown	1q42–q43
Dysmorphology, growth failure	Autosomal recessive	Unknown	11p15
Isolated hypoparathyroidism	Autosomal dominant	PTH[b]	11p15
	Autosomal recessive	PTH[b]	Xq27.1
	X-Linked recessive	SOX3	

APECED, *autoimmune polyendocrinopathy candidiasis-ectodermal dystrophy*; HDR, *hypoparathyroidism, deafness and renal anomalies*; KSS, *Kearns-Sayre syndrome*; MELAS, *mitochondrial encephalopathy, strokelike episodes, and lactic acidosis*; MTPDS, *mitochondrial trifunctional protein deficiency syndrome.*

[a] *Most likely inheritance shown.*

[b] *Mutations of* PTH *gene identified only in some families.*

[?] *Location not known.*

Scambler *et al.,* 1991), and studies of transgenic mice deleted for such genes (e.g., Udf1l, Hira, and Tbx1) revealed developmental abnormalities of the pharyngeal arches (Lindsay *et al.,* 1999; Magnaghi *et al.,* 1998; Jerome et al., 2001). However, point mutations in DGS1 patients have only been detected in the TBX1 gene (Yagi *et al.,* 2003), and TBX1 is now considered to be the gene causing DGS1 (Baldini *et al.,* 2003). TBX1 is a DNA binding transcriptional factor, of the T-Box family, that is known to have an important role in vertebrate and invertebrate organogenesis and pattern formation. The TBX1 gene is deleted in ∼ 96% of all DGS1 patients. Moreover, DNA sequence analysis of unrelated DGS1 patients who did not have deletions of chromosome 22q11.2 has revealed the occurrence of three heterozygous point mutations (Yagi *et al.,* 2003). One of these mutations resulted in a frame shift with a premature truncation, whereas the other two were missense mutations (Phe148Tyr and Gly310Ser). All of these patients had the complete pharyngeal phenotype but did not have mental retardation or learning difficulties. Interestingly, transgenic mice with deletion of Tbx1 have a phenotype that is similar to that of DGS1 patients (Jerome *et al.,* 2001). Thus, Tbx1-null mutant-mice (−/−) had all the developmental anomalies of DGS1 (i.e., thymic and parathyroid hypoplasia; abnormal facial structures and cleft palate; skeletal defects; and cardiac outflow tract abnormalities), whereas Tbx1 haploinsufficiency in mutant mice (+/−) was associated only with defects of the fourth branchial pouch (i.e., cardiac outflow tract abnormalities). The basis of the phenotypic differences between DGS1 patients, who are heterozygous, and the transgenic +/− mice remain to be elucidated. It is plausible that Tbx1 dosage, together with the downstream genes that are regulated by Tbx1, could provide an explanation, but the roles of these putative genes in DGS1 remains to be elucidated.

Some patients may have a late-onset DGS1, and these develop symptomatic hypocalcemia in childhood or during adolescence with only subtle phenotypic abnormalities (Scire *et al.,* 1994; Sykes *et al.,* 1997). These late-onset DGS1 patients have similar microdeletions in the 22q11 region. It is of interest to note that the age of diagnosis in the families of the three DGS1 patients with inactivating Tbx1 mutations ranged from 7 to 46 years, which is in keeping with late-onset DGS1 (Yagi *et al.,* 2003).

Mouse Models Developing Features of DiGeorge Syndrome Reveal Roles of Hox and Pax Genes in Parathyroid and Thymus Development

In the mouse, the parathyroids develop with the thymus from common primordia that arise from the third pharyngeal pouch endoderm (Gordon *et al.,* 2001). The molecular mechanisms that regulate parathyroid/thymus organogenesis from the pharyngeal pouch endoderm have been investigated using knockout models, and the roles of transcription regulators e.g., Hoxa3, Pax1, Pax9, Eyal, Six1 and Six4 defined (Manley and Capecchi, 1998; Xu *et al.,* 2002; Zou *et al.,* 2006). The roles of Hoxa3 and Pax1 genes will be briefly reviewed because they form a genetic pathway with GCM2 (see later discussion) that regulates parathyroid organogenesis.

Homeobox (HOX) Genes

The HOX genes are a group of genes that specify the body plans of invertebrates, e.g., *Drosophila* and, in all likelihood, vertebrates (Deschamps, 2007). In *Drosophila* these homeobox genes specify the identity of cells within each parasegment. The function of the evolutionarily conserved genes in man and mouse is not known, but because the order of these genes on the chromosomes of *Drosophila*, man, and mouse is the same, and because this gene order reflects the order of the anterior boundaries of gene expression along the anteroposterior body axis of the early embryos of all three species, it would appear that these homeobox genes are equally important in mammalian development (Deschamps, 2007). The HOX genes all contain a 180-bp motif, which encodes a 60 amino acid DNA biding domain called the homeodomain. In man and mouse this set of 30 genes are known collectively as the HOX genes, and they are distributed in the genome in four separate clusters (HOXa, b, c and d), which may have arisen during chordate evolution as the result of two duplications of chromosomal segments (Kappen *et al.,* 1989; Manley and Capecchi, 1998). The homeobox genes of *Drosophila* encode transcription factors that share the DNA-binding motif, and these genes act as master switches directing the course of morphogenetic development of each segment. Because the human and mouse genes share similar homeobox sequences, the HOX proteins are thought to function also as transcriptional factors participating in the specification of regional information in the early mammalian embryo (Manley and Capecchi, 1998; Deschamps, 2007).

In order to determine the genetic function of some of the HOX genes, mouse knockout models have been generated. Disruption of HOXa3 resulted in an abnormal phenotype that had similarities to the DiGeorge syndrome (Chisaka and Capecchi, 1991; Manley and Capecchi, 1995, 1998). Null mutant mice (−/−) died in the neonatal period and were found to be lacking the thymus and parathyroids because of a failure to initiate the formation of the parathyroid/thymus primordial. These HOXa3 −/− mice also had defects of the heart and arteries, craniofacial abnormalities, and a reduction in the mass of the thyroid and submaxillary tissues. The HOXa3 +/− mice were phenotypically normal. Thus, although the HOXa3 −/− mice clearly indicated an important role for the HOXa3 gene in parathyroid and thymus organogenesis, there were nevertheless important differences between this mouse and the DiGeorge syndrome, which is an autosomal dominant trait, whereas the mouse syndrome is autosomal recessive.

Paired Box (PAX) Genes

The paired box (PAX) genes encode transcriptional factors that are expressed in a spatially and temporally restricted manner during embryonic development (Robson *et al.,* 2006). The PAX genes contain a highly conserved 128-amino-acid DNA-binding domain called the paired box. Man and mouse have nine PAX genes, and mice deficient (−/−) in either PAX1 or PAX9 have congenital parathyroid and thymic defects (Peters *et al.,* 1998; Su *et al.,* 2001). Studies of double knockout mice indicated that there is a genetic pathway that involves Hoxa3, PAX1, and glial cells missing 2 (Gcm2, see later discussion), in parathyroid development (Su *et al.,* 2001). Thus, gcm2 expression is absent in HOXa3 −/− mouse embryos that are 10.5 days post coitum (dpc), reduced in PAX1 −/− mouse embryos that are 11.5 dpc, and absent in Hoxa3 (+/−)/PAX1 (−/−) mice at 11.5 dpc (Su *et al.,* 2001). These studies have revealed that Hoxa3 is required for initiation of parathyroid organogenesis and for initial Gcm2 expression and that both HOXa3 and PAX1 are required for Gcm2 expression. Failure of Gcm2 expression results in a lack of parathyroids, and this is due to programmed cell death (apoptosis) of the parathyroid primordia at 12 dpc in mouse embryos (Su *et al.,* 2001; Liu *et al.,* 2007). Thus, there is an important transcription cascade involving HOXa3, PAX1 and Gcm2, in the embryonic development of parathyroids.

Hypoparathyroidism, Deafness, and Renal Anomalies Syndrome

Clinical Features and Role of GATA3 Mutations

The combined inheritance of hypoparathyroidism, deafness, and renal dysplasia as an autosomal dominant trait was reported in one family in 1992 (Bilous *et al.,* 1992). Patients had asymptomatic hypocalcemia with undetectable or inappropriately normal serum concentrations of PTH, and normal brisk increases in plasma cAMP in response to the infusion of PTH. The patients also had bilateral, symmetrical, sensorineural deafness involving all frequencies. The renal abnormalities consisted mainly of bilateral cysts that compressed the glomeruli and tubules and lead to renal impairment in some patients. Cytogenetic abnormalities were not detected, and abnormalities of the PTH gene were excluded (Bilous *et al.,* 1992). However, cytogenetic abnormalities involving chromosome 10p14–10pter were identified in two unrelated patients with features that were consistent with HDR. These two patients suffered from hypoparathyroidism, deafness, and growth and mental retardation; one patient also had a solitary dysplastic kidney with vesicoureteric reflux and a uterus bicornis unicollis (Fryns *et al.,* 1981), and the other patient, who had a complex reciprocal, insertional translocation of chromosomes 10p and 8q, had cartilaginous exostoses (Van Esch *et al.,* 1999). Neither of these patients had immunodeficiency or heart defects, which are key features of DGS2 (see earlier discussion), and further studies defined two nonoverlapping regions; thus, the DGS2 region was located on 10p13–p14 and HDR on 10p14–10pter. Deletion mapping studies in two other HDR patients further defined a critical 200-kb region that contained the GATA3 gene (Van Esch *et al.,* 2000).

GATA3 belongs to a family of dual zinc-finger transcription factors that are involved in vertebrate embryonic development. DNA sequence analysis in other HDR patients identified mutations that resulted in a haploinsufficiency and loss of GATA3 function (Van Esch *et al.,* 2000; Nesbit *et al.,* 2004a; Ali *et al.,* 2007). To date, 39 different heterozygous germline GATA3 abnormalities, which consist of 33 mutations and 6 whole gene deletions, have been reported (van Esch 2000; Muroya *et al.,* 2001; Nesbit *et al.,* 2004a; Zahirieh *et al.,* 2005; Mino *et al.,* 2005; Adachi *et al.,* 2006; Chiu *et al.,* 2006; Ali *et al.,* 2007). The GATA3 mutations are scattered throughout the coding region, and approximately 18% are nonsense mutations, 42% are frame-shift deletions or insertions, 15% are missense mutations, 8% are splice site mutations, 3% are in-frame deletions, and 15% are whole-gene deletions. Thus, the majority (>75%) of these HDR-associated mutations are predicted to result in truncated forms of the GATA3 protein. Each proband and family will generally have its own unique mutation, and there appears to be no correlation with the underlying genetic defect and the phenotypic variation, e.g., the presence or absence of renal dysplasia. More than 90% of patients with two or three of the major clinical features of the HDR syndrome, i.e., hypoparathyroidism, deafness, or renal abnormalities, have a GATA3 mutation (Ali *et al.,* 2007). The remaining 10% of HDR patients, who do not have a GATA3 mutation of the coding region, may harbor mutations in the regulatory sequences flanking the GATA3 gene, or else they may represent heterogeneity. The phenotypes of HDR patients with GATA3 mutations appear to be similar to those without GATA3 mutations (Ali *et al.,* 2007).

The HDR-associated GATA3 mutations are predicted to disrupt either one or both of the zinc-finger domains (Nesbit *et al.,* 2004a). The C-terminal finger (ZnF2) of GATA proteins binds DNA, whereas the N-terminal finger (ZnF1) stabilizes this DNA binding and interacts with other zinc-finger proteins, such as friends of GATA (FOGs; Tsang *et al.,* 1997; Tevosian *et al.,* 1999; Svensson *et al.,* 1999). The functional consequences of these HDR-associated GATA3 mutations have been assessed by electrophoretic mobility-shift assays to detect alterations in DNA binding, and by yeast two-hybrid assays to detect alterations in protein-protein interactions (Nesbit *et al.,* 2004a; Ali *et al.,* 2007). These studies have revealed that HDR-associated mutations involving the GATA3 ZnF2 or the adjacent basic amino acids result in a loss of DNA binding to the consensus motif, GATA, that would be associated with the promoter of the gene being regulated by GATA3 (van Esch *et al.,* 2000; Nesbit *et al.,* 2004a; Ali *et al.,* 2007). However, those HDR-associated mutations involving ZnF1 lead to either a loss of interaction with FOG2 ZnFs or altered DNA-binding affinity (Nesbit *et al.,* 2004a; Zahirieh *et al.,* 2005; Ali *et al.,* 2007). These findings are consistent with the proposed three-dimensional model of GATA3 ZnF1, which has separate DNA and protein-binding surfaces (Nesbit *et al.,* 2004a; Ali *et al.,* 2007). Thus, the HDR-associated GATA3 mutations can be subdivided into two broad classes that depend on whether they disrupt ZnF1 or ZnF2, and their subsequent effects on interactions with FOG2 and altered DNA binding, respectively.

Phenotype of GATA3 Knockout Mouse Model

The HDR phenotype is consistent with the expression pattern of GATA3 during human and mouse embryogenesis in the developing kidney, otic vesicle, and parathyroids (Labastie *et al.,* 1994, 1995; George *et al.,* 1994; Debacker *et al.,* 1999). However, GATA3 is also expressed in the developing central nervous system (CNS) and the hematopoietic organs in humans and mice, and this suggests that GATA3 may have a more complex role (George *et al.,* 1994; Debacker *et al.,* 1999; Pandolfi *et al.,* 1995). Indeed, studies of mice that are deleted for a Gata3 allele (+/−), or both Gata3 alleles (−/−) have revealed important roles for Gata3 in the development of the brain, spinal cord, peripheral auditory system, T-cells, fetal liver hematopoiesis and urogenital system (Pandolfi *et al.,* 1995).

Heterozygous (+/−) Gata3 Knockout Mice

Gata3 +/− mice are viable, appear to be normal with a normal life span, and are fertile (Pandolfi *et al.,* 1995). The lymphoid organs, peripheral blood, and bone marrow of the +/− mice have no abnormalities when compared to the +/+ mice. The +/− mice were not assessed for hypoparathyroidism, but renal abnormalities were absent. In contrast, analysis of auditory brainstem response (ABR) thresholds in alert Gata3 +/− mice, aged from 1 to 19 months of age, revealed a hearing loss when compared to their wild-type (+/+) littermates (van der Wees *et al.,* 2004). The hearing loss in these Gata3 +/− mice was associated with cochlear abnormalities that consisted of a significant progressive morphological degeneration which started with the outer hair cells at the apex and eventually involved all the inner hair cells, pillar cells, and nerve fibers (van der Wees *et al.,* 2004; van Looij *et al.,* 2005). These studies have shown that hearing loss in Gata3 haploinsufficiency commences in the early postnatal period and is progressive through adulthood, and that it is peripheral in origin and is predominantly due to malfunctioning of the outer hair cells of the cochlea (van der Wees *et al.,* 2004; van Looij *et al.,* 2005).

Homozygous (−/−) Gata3 Knockout Mice

Gata 3 −/− mice are embryonically lethal, and these null embryos die between 11 and 12 dpc (Pandolfi *et al.,* 1995). Examination of these Gata3 −/− embryos revealed a variety of abnormalities that included massive internal bleeding, resulting in anemia, marked growth retardation, severe deformities of the brain and spinal cord, a hypopigmented retina, gross aberrations in fetal liver hematopoiesis, a total block of T-cell differentiation, and a retarded or missing lower jaw area (Pandolfi *et al.,* 1995; Lim *et al.,* 2000). Abnormalities of parathyroid development were not reported. These Gata3 −/− mice had an anatomically normal sympathetic nervous system, yet the sympathetic ganglia lacked tyrosine hydroxylase and dopamine beta-hydroxylase, which are key enzymes that convert tyrosine to L-DOPA and dopamine to noradrenaline, respectively, in the catecholamine synthesis pathway. Thus, the Gata −/− mice lacked noradrenaline in the sympathetic neurons, and this was contributing to the early embryonic lethality (Lim *et al.,* 2000). Feeding of catecholamine intermediates to the pregnant dams helped to partially rescue the Gata −/− embryos to 12.5 to 16.5 dpc. These older, pharmacologically rescued Gata −/− embryos showed abnormalities that could not be detected in the untreated mice (Lim *et al.,* 2000). These late embryonic defects included thymic hypoplasia, a thin-walled ventricular septum, a poorly developed mandible, other developmental defects in structures derived from the cephalic neural crest cells, renal hypoplasia, a failure to form the metanephros, and an aberrant elongation of the nephric duct along the anteroposterior axis of the embryo (Lim *et al.,* 2000; Grote *et al.,* 2005). The defect of the nephric duct, which consisted of an abnormal morphogenesis and guidance in the developing kidney, was characterized by the loss of Ret expression that is an essential component of the glial-derived-nerve-factor (GDNF) signaling pathway involved in ureteric bud formation and nephric duct guidance (Grote *et al.,* 2005). Parathyroid development was not studied in the catecholamine rescued Gata3 −/− mice. Thus, Gata3 has a role in the differentiation of multiple cell lineages during embryogenesis as well as being a key regulator of nephric duct morphogenesis and guidance of the nephric duct in its caudal extension in the pro/mesonephric kidney (Lim *et al.,* 2000; Grote *et al.,* 2005).

Role of GATA3 in Developmental Pathogenesis

The mechanisms whereby GATA3 haploinsufficiency leads to the phenotypic features of the HDR syndrome remain to be elucidated. Important clues have been obtained from *in vitro* studies and from *in vivo* studies of the mouse knockout. The *in vitro* studies have shown that the HDR-associated GATA3 mutations do result in impaired binding to the target DNA motif, and this in turn will alter the transcription role of GATA3. However, the genes that are under the transcription regulation of GATA3 in the different organs remain largely unknown with the exceptions of the T-cell antigen receptor subunits, interleukin 5 (IL5) and Ret (Marine et al., 1991; Zheng *et al.,* 1997; Zhang *et al.,*1997; Grote *et al.,* 2005). Despite the essential role of GATA3 in determining T-cell lineage (Zhang *et al.,* 1997), it is important to note GATA3 haploinsufficiency in the HDR patients is not associated with immunodeficiency (van Esch *et al.,* 2000; Lichtner *et al.,* 2000), indicating either that reduced amounts of GATA3 are sufficient for T-cell development, or that GATA1 and GATA2 may be able to compensate for the loss of GATA3. The regulation of Ret expression by GATA3 is of importance in the development of the kidney and nephric duct (Grote *et al.,* 2005). Moreover, Gata3 and Ret form part of an important transcriptional cascade, whereby Pax proteins (see earlier discussion)—Pax2 and Pax8—participate upstream to activate Gata3 (Grote *et al.,* 2005). However, it is important to note that the altered renal morphogenesis is observed only in the Gata3 −/− mice and not in the Gata3 +/− mice, which unlike the HDR patients who are also heterozygous for the GATA3 mutation, have normal kidneys (Pandolfi *et al.,* 1995). Thus, there are important phenotypic differences between Gata3 +/− mice and HDR patients, who are heterozygous for GATA3 mutations, and the mechanisms underlying these interspecies differences and the tissue-specific differences that conference susceptibility to GATA3 haploinsufficiency remain to be elucidated. In contrast to these renal differences between HDR patients and Gata +/− mice, there are marked similarities in the hearing loss (Bilous *et al.,* 1992; Lichtner *et al.,* 2000; van der Wees *et al.,* 2004; van Looij *et al.,* 2005). It has not been possible to perform detailed functional and histological studies on the auditory system of HDR patients, but such studies in the Gata3 +/− mice have revealed that the hearing loss is due to degeneration and loss of the outer hair cells which progressively involves the other types of hair cells and nerve fibers in the cochlea (van der Wees *et al.,* 2004, van Looij *et al.,* 2005). The genes under the transcriptional regulator of GATA3 in the cochlea remain to be identified. The morphological changes and genetic pathways involved in the parathyroids of HDR patients and Gata3 knockout also remain unknown. Thus, although the HDR phenotype is consistent with the expression pattern of GATA3 during human and mouse embryogenesis in the developing kidney (ureteric bud, collecting duct system, and mesangial cells), otic vesicle, and parathyroids, much remains unknown regarding the underlying transcription pathways and the basis of the interspecies differences between the Gata3 +/− mice and the HDR patients.

Kenney-Caffey and Sanjad-Sakati Syndromes

Hypoparathyroidism has been reported to occur in over 50% of patients with the Kenny-Caffey syndrome, which is associated with short stature, osteosclerosis and cortical thickening of the long bones, delayed closure of the anterior fontanel, basal ganglia calcification, nanophthalmos, and hyperopia (Fanconi *et al.,* 1986; Bergada *et al.,* 1988; Franceschini *et al.,* 1992). Parathyroid tissue could not be found in a detailed postmortem examination of one patient (Boynton *et al.,* 1979), and this suggests that hypoparathyroidism may be due to an embryological defect of parathyroid development. In the Sanjad-Sakati syndrome, hypoparathyroidism is associated with severe growth failure and dysmorphic features, and this has been reported in 12 patients from Saudi Arabia (Sanjad *et al.,* 1991). Consanguinity was noted in 11 of the 12 patients' families, the majority of which originated from the western province of Saudi Arabia. This syndrome, which is inherited as an autosomal recessive disorder, has also been identified in families of Bedouin origin, and homozygosity and linkage disequilibrium studies mapped this gene to chromosome 1q42–q43 (Parvari *et al.,* 1998). Molecular genetic investigations have identified that mutations of the tubulin-specific chaperone (TBCE) are associated with the Kenny-Caffey and Sanjad-Sakati syndromes (Parvari *et al.,* 2002). TBCE encodes one of several chaperone proteins required for the proper folding of α-tubulin subunits and the formation of α-β tubulin heterodimers (Parvari *et al.,* 2002).

Additional Familial Syndromes

Single familial syndromes in which hypoparathyroidism is a component have been reported (see Table I). The inheritance of the disorder in some instances has been established, and molecular genetic analysis of the PTH gene has revealed no abnormalities. Thus, an association of hypoparathyroidism, renal insufficiency, and developmental delay has been reported in one Asian family in whom autosomal recessive inheritance of the disorder was established (Parkinson *et al.,* 1993). An analysis of the PTH gene in this family revealed no abnormalities (Parkinson *et al.,* 1993). The occurrence of hypoparathyroidism, nerve deafness, and a steroid-resistant nephrosis leading to renal failure, which has been referred to as the *Barakat syndrome* (Barakat *et al.,* 1977), has been reported in four brothers from one family, and an association of hypoparathyroidism with congenital lymphedema, nephropathy, mitral valve prolapse, and brachytelephalangy has been observed in two brothers from another family (Dahlberg *et al.,* 1983). Molecular genetic studies have not been reported from these two families.

Mitochondrial Disorders Associated with Hypoparathyroidism

Hypoparathyroidism has been reported to occur in three disorders associated with mitochondrial dysfunction: the Kearns-Sayre syndrome (KSS), the MELAS syndrome, and a mitochondrial trifunctional protein deficiency syndrome (MTPDS). KSS is characterized by progressive external ophthalmoplegia and pigmentary retinopathy before the age of 20 years and is often associated with heart block or cardiomyopathy. The MELAS syndrome consists of a childhood onset of mitochondrial encephalopathy, lactic acidosis, and strokelike episodes. In addition, varying degrees of proximal myopathy can be seen in both conditions. Both the KSS and MELAS syndromes have been reported to occur with insulin-dependent diabetes mellitus and hypoparathyroidism (Moraes *et al.*, 1989; Zupanc *et al.*, 1991). A point mutation in the mitochondrial gene tRNA leucine (UUR) has been reported in one patient with the MELAS syndrome who also suffered from hypoparathyroidism and diabetes mellitus (Morten *et al.*, 1993). Large deletions, consisting of 6741 and 6903 bp and involving more than 38% of the mitochondrial genome, have been reported in other patients who suffered from KSS, hypoparathyroidism, and sensorineural deafness (Zupanc *et al.*, 1991; Isotani *et al.*, 1996). Rearrangements (Wilichowski *et al.*, 1997) and duplication (Abramowicz *et al.*, 1996) of mitochondrial DNA have also been reported in KSS. Mitochondrial trifunctional protein deficiency is a disorder of fatty-acid oxidation that is associated with peripheral neuropathy, pigmentary retinopathy, and acute fatty liver degeneration in pregnant women who carry an affected fetus. Hypoparathyroidism has been observed in one patient with trifunctional protein deficiency (Dionisi-Via *et al.*, 1996). The role of these mitochondrial mutations in the etiology of hypoparathyroidism remains to be further elucidated.

Pluriglandular Autoimmune Hypoparathyroidism

Hypoparathyroidism may occur in association with candidiasis and autoimmune Addison's disease, and the disorder has been referred to as either the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) syndrome or the autoimmune polyglandular syndrome (APS1) type 1 (Ahonen *et al.,* 1990). This disorder has a high incidence in Finland, and a genetic analysis of Finnish families indicated autosomal recessive inheritance of the disorder (Ahonen *et al.,* 1985). In addition, the disorder has been reported to have a high incidence among Iranian Jews, although the occurrence of candidiasis was less common in this population (Zlotogora *et al.,* 1992). Linkage studies of Finnish families mapped the APECED gene to chromosome 21q22.3 (Aaltonen *et al.,* 1994). Further positional cloning approaches led to the isolation of a novel gene from chromosome 21q22.3. This gene, referred to as AIRE1 (autoimmune regulator type 1), encodes a 545-amino-acid protein that contains motifs suggestive of a transcriptional factor and includes two zinc-finger motifs, a proline-rich region, and three LXXLL motifs (Nagamine *et al.,* 1997; Finnish-German APECED Consortium 1997). Four AIRE1 mutations are commonly found in APECED families, and these are Arg257Stop in Finnish, German, Swiss, British and Northern Italian families; Arg139Stop in Sardinian families; Tyr85Cys in Iranian Jewish families; and a 13-bp deletion in exon 8 in British, Dutch, German, and Finnish families (Nagamine *et al.,* 1997; Finnish-German APECED

Consortium 1997; Pearce *et al.*, 1998; Scott *et al.*, 1998; Rosatelli *et al.*, 1998; Bjorses *et al.*, 2000). AIRE1 has been shown to regulate the elimination of organ-specific T cells in the thymus, and thus APECED is likely to be caused by a failure of this specialized mechanism for deleting forbidden T cell and establishing immunologic tolerance (Liston *et al.*, 2003).

ISOLATED HYPOPARATHYROIDISM

Isolated hypoparathyroidism may be inherited either as an autosomal-dominant (Barr *et al.*, 1971; Ahn *et al.*, 1986), autosomal-recessive (Bronsky *et al.*, 1968; Parkinson and Thakker, 1992), or X-linked recessive (Peden, 1960; Whyte and Weldon, 1981; Thakker *et al.*, 1990) disorder. Some autosomal forms of hypoparathyroidism have been shown to be due to abnormalities of the PTH and Gcm2 genes.

PTH Gene Abnormalities

PTH Gene Structure and Function

The PTH gene is located on chromosome 11p15 and consists of three exons, which are separated by two introns (Naylor *et al.*, 1983). Exon 1 of the PTH gene is 85 bp in length and is untranslated (Fig. 1), whereas exons 2 and 3 encode the 115-amino-acid prepro-PTH peptide. Exon 2 is 90 bp in length and encodes the initiation (ATG) codon, the prehormone sequence, and part of the prohormone sequence. Exon 3 is 612 bp in size and encodes the remainder of the prohormone sequence, the mature PTH peptide, and the 3′ untranslated region (Vasicek *et al.*, 1983). The 5′ regulatory sequence of the human PTH gene contains a vitamin D response element 125 bp upstream of the transcription start site, which downregulates PTH mRNA transcription in response to vitamin D receptor binding (Okazaki *et al.*, 1988; Demay *et al.*, 1992). PTH gene transcription (as well as PTH peptide secretion) is also dependent on the extracellular concentrations of calcium and phosphate (Naveh-Many *et al.*, 1995; Slatopolsky *et al.*, 1996; Almaden *et al.*, 1996), although the presence of specific upstream "calcium or phosphate response element(s)" has not yet been demonstrated (Russell *et al.*, 1983; Naveh-Many *et al.*, 1989). The mature PTH peptide is secreted from the parathyroid chief cell as an 84-amino-acid peptide, and this is regulated through a G protein-coupled calcium-sensing receptor (CaSR) which is also expressed in renal tubules. However, when the PTH mRNA is first translated it is as a prepro-PTH peptide. The "pre" sequence consists of a 25-amino-acid signal peptide (leader sequence) that is responsible for directing the nascent peptide into the endoplasmic reticulum to be packaged for secretion from the cell (Kemper *et al.*, 1974). The "pro" sequence is six amino acids in length and, although its function is less well defined than that of the "pre" sequence, it is also essential for correct PTH processing and secretion (Kemper *et al.*, 1974). After the 84-amino-acid mature PTH peptide is secreted from the parathyroid cell, it is cleared from the circulation with a short half-life of about 2 minutes, via nonsaturable hepatic uptake and renal excretion. PTH shares a receptor with PTH-related peptide (PTHrP, also known as PTHrH, PTH-related hormone; Jüppner *et al.*, 1991), and this receptor is a member

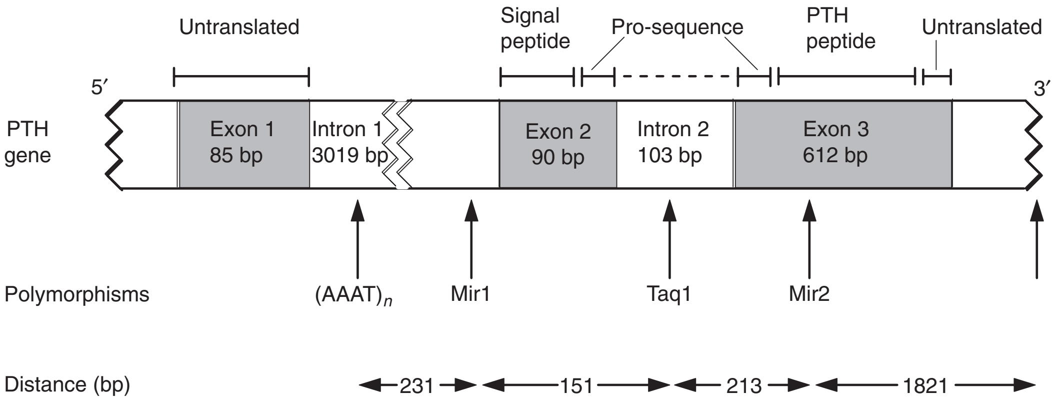

FIGURE 1 Schematic representation of the PTH gene. The PTH gene consists of three exons and two introns; the peptide is encoded by exons 2 and 3. The PTH peptide is synthesized as a precursor that contains a pre- and a prosequence. The mature PTH peptide, which contains 84 amino acids, and larger carboxy-terminal PTH fragments are secreted from the parathyroid cell. The polymorphic sites associated with the PTH gene are indicated. Two restriction fragment length polymorphisms (RFLPs) are associated with the PTH gene: The *Taq*I polymorphic site is within intron 2, and the P*st*I polymorphic site is 1.7 kbp downstream in the 3′ direction of the gene (Schmidtke *et al.*, 1984). Two other polymorphisms (Miric and Levine, 1992) of the PTH gene designated Mir1 and Mir2 are located in intron 1 and exon 3, respectively, and the tetranucleotide (AAAT)$_n$ polymorphism is in intron 1 (Parkinson *et al.*, 1993). The distance between the tetranucleotide (AAAT)$_n$ polymorphism and the Mir1 polymorphism is 231 base pairs (bp), that between the Mir1 polymorphism and the *Taq*1 RFLP is 152 bp, that between the *Taq*I RFLP site and the Mir2 polymorphism is 212 bp, and that between the Mir2 polymorphism and the P*st*I RFLP is 1821 bp. Linkage disequilibrium between the (AAAT)$_n$, T*aq*I, and P*st*I polymorphic sites has been established (Parkinson *et al.*, 1993). Adapted from Parkinson and Thakker (1992).

of a subgroup of G protein-coupled receptors. The PTH/PTHrP receptor gene is located on chromosome 3p21–p24 (Gelbert *et al.*, 1994) and is highly expressed in kidney and bone, where PTH is its predominant agonist (Abou-Samra *et al.*, 1992). However, the most abundant expression of the PTH/PTHrP receptor occurs in chondrocytes of the metaphyseal growth plate where it mediates predominantly the autocrine/paracrine actions of PTHrP (Segré, 1996; Potts and Jüppner, 1997). Five polymorphisms of the PTH gene have been reported and two of these are associated with restriction fragment length polymorphisms (RFLPs; Schmidtke *et al.*, 1984), another two are the result of single base changes that are not associated with RFLPs (Miric and Levine, 1992), and one is due to a variation in the length of a microsatellite repetitive sequence in intron 1 (Parkinson *et al.*, 1993). These polymorphisms are inherited in a Mendelian manner and are thus useful as genetic markers in family studies. Mutations involving the PTH gene affect the regulation of calcium homeostasis and are associated with hypoparathyroidism (see Table I).

Autosomal Dominant Hypoparathyroidism

DNA sequence analysis of the PTH gene (see Fig. 1) from one patient with autosomal dominant isolated hypoparathyroidism has revealed a single base substitution (T→C) in codon 18 of exon 2 (Arnold *et al.*, 1990), which resulted in the substitution of arginine (CGT) for the normal cysteine (TGT) in the signal peptide. The presence of this charged amino acid in the midst of the hydrophobic core of the signal peptide impeded the processing of the mutant prepro-PTH, as demonstrated by *in vitro* studies. These revealed that the mutation impaired the interaction with the nascent protein and the translocation machinery, and that cleavage of the mutant signal sequence by solubilized signal peptidase was ineffective (Arnold *et al.*, 1990; Karaplis *et al.*, 1995). Ineffective cleavage of the prepro PTH sequence results in a molecule that does not proceed successfully through the subsequent intracellular steps required for ultimate delivery of PTH to secretary granules. The parathyroid cell, therefore, cannot respond to hypocalcemia with the secretion of native, biologically active PTH.

Autosomal Recessive Hypoparathyroidism

Autosomal recessive hypoparathyroidism has usually arisen in families with consanguineous marriages (Parkinson and Thakker, 1992; Parkinson *et al.*, 1993). Abnormalities in the PTH gene have been sought (Parkinson *et al.*, 1993) and mutations identified in two unrelated families (Parkinson and Thakker, 1992; Sunthornepvarakul *et al.*, 1999). In one such family a donor splice site at the exon 2-intron 2 boundary has been identified (Parkinson and Thakker, 1992). This mutation involved a single base transition (**g**→**c**) at position 1 of intron 2, and the effects of this alteration in the invariant **gt** dinucleotide of the 5′ donor splice site consensus on mRNA processing were assessed by an analysis of the non-tissue-specific transcription of the normal and mutant PTH genes. This non-tissue-specific expression of genes has been estimated to be at the rate of one molecule of correctly spliced mRNA per 1000 cells (von Heijne, 1983; Chelly *et al.*, 1989). Although the physiological relevance of this low level of non-tissue-specific or illegitimate transcription is not known, it is of clinical importance. Easily accessible peripheral blood lymphocytes can be used to detect abnormalities in mRNA processing, thereby avoiding the requirement for tissue that may only be obtainable by biopsy. Use of these methods revealed that the donor splice site mutation resulted in exon skipping, in which exon 2 of the PTH gene was lost and exon 1 was spliced to exon 3. The lack of exon 2 would lead to a loss of the initiation codon (ATG) and the signal peptide sequence, which are required, respectively, for the commencement of PTH mRNA translation and for the translocation of the PTH peptide. Thus, the patients' parathyroid cells would not contain any translated PTH products. In the other family a single base substitution (**T**→**C**) involving codon 23 of exon 2 was detected. This resulted in the substitution of proline (CCG) for the normal serine (TCG) in the signal peptide (Sunthornepvarakul *et al.*, 1999). This mutation alters the −3 position of the prepro-PTH protein cleavage site (von Heijne, 1983). Indeed, amino acid residues at the −3 and −1 positions of the signal peptidase recognition site have to conform to certain criteria for correct processing through the rough endoplasmic reticulum (RER), and one of these is an absence of proline in the region −3 and +1 of the site (von Heijne, 1983). Thus, the presence of a proline, which is a strong helix-breaking residue, at the −3 position is likely to disrupt cleavage of the mutant prepro-PTH that would be subsequently degraded in the RER, and PTH would not be available (Sunthornepvarakul *et al.*, 1999).

GCMB Gene Abnormalities

GCMB (glial cells missing B), which is the human homologue of the *Drosophila* gene Gcm and of the mouse gcm2 gene, is expressed exclusively in the parathyroid glands, suggesting that it may be a specific regulator of parathyroid gland development (Günther *et al.*, 2000; Kim *et al.*, 1998). In order to investigate this, mice deleted for Gcm2 were generated by the method of homologous recombination using embryonic stem cells. Mice that were heterozygous (+/−) for the deletion were normal, whereas mice that were homozygous (−/−) for the deletion lacked parathyroid glands and developed the hypocalcemia and hyperphosphatemia observed in hypoparathyroidism (Günther *et al.*, 2000). However, despite their lack of parathyroid glands, Gcm2-deficient (−/−) mice did not have undetectable serum PTH levels, but instead had PTH levels identical to those of normal (+/+ , wild-type) mice. This endogenous level of PTH in the Gcm2-deficient (−/−) mice was too low to correct the hypocalcemia, but exogenous continuous PTH infusion could correct the hypocalcemia (Günther *et al.*, 2000). Interestingly, there were no compensatory increases in PTHrP or 1,25(OH)$_2$ vitamin D$_3$. These findings indicate that Gcm2 mice have a normal response (and not resistance) to PTH and that the PTH in the serum of Gcm2-deficient mice was active. The auxiliary source of PTH was determined by combined expression and ablation studies (Günther *et al.*, 2000). These revealed a cluster of PTH-expressing cells under the thymic capsule in both the Gcm2-deficient (−/−) and wild-type (+/+) mice. These thymic PTH-producing cells also expressed the calcium-sensing receptor (CaSR), and long-term treatment of the Gcm2-deficient mice with 1,25(OH)$_2$ vitamin D$_3$ restored the serum calcium concentrations to normal and reduced the serum PTH levels, thereby indicating that the thymic production of PTH can be downregulated (Günther *et al.*, 2000). However, it appears that this thymic production of PTH cannot be upregulated, because serum PTH levels are not high despite the hypocalcemia in the Gcm2-deficient mice. This absence of upregulation would be consistent with the very small size of the thymic PTH-producing cell cluster, when compared to the size of normal parathyroid glands. The development of the thymic PTH-producing cells likely involves Gcm1, which is the other mouse homologue of *Drosophila* Gcm (Kim *et al.*, 1998). Gcm1 expression, which could not be detected in parathyroid glands, colocalized with PTH expression in the thymus (Günther *et al.*, 2000). The specific role of Gcm2 in the development of the parathyroids from the third pharyngeal pouch has been further investigated by studying the expression of the Hoxa3-Pax1/9-Eya1 transcription factor and Sonic hedgehog–Bone morphogenetic protein 4 (Shh-Bmp4) signaling networks (Liu *et al.*, 2007). These studies have revealed that Gcm2 (−/−) embryos that are 12 dpc have a parathyroid-specific domain, but that this parathyroid domain undergoes coordinated programmed cell death (apoptosis) by 12.5 dpc in the Gcm2-null mouse embryos (Liu *et al.*, 2007). Moreover, the expression of the transcription factors Hoxa3 (see earlier discussion), Pax 1, Pax 9, Eya1, and Tbx1 and of Shh and Bmp4 was normal in the third pharyngeal pouch of these Gcm2-null mouse embryos. These findings indicate that the Hoxa3-Pax1/9-Eya transcription factor cascade, the transcription factor Tbx1, and the Shh-Bmp4 signaling network all act upstream of Gcm2 (Liu *et al.*, 2007). Moreover, these studies have revealed that Gcm2 has a role in promoting differentiation and survival of parathyroid cells in the developing embryo (Liu *et al.*, 2007).

Gcm genes have similar roles in human parathyroid development, as homozygous GCMB mutations have been identified in patients with autosomal recessive hypoparathyroidism (Ding *et al.*, 2001), whereas in another family a homozygous missense mutation (Arg47Leu) of the DNA binding domain was identified (Baumber *et al.*, 2005). Functional analysis, using electrophoretic mobility shift assays (EMSAs), of this Arg47Leu GCMB mutation revealed that is resulted in a loss of DNA binding to the GCM DNA binding site (Baumber *et al.*, 2005). More recently, heterozygous GCMB mutations, which consist of single nucleotide deletions (c1389deT and c1399delC) that introduce frame shifts and premature truncations, have been identified in two unrelated families with autosomal dominant hypoparathyroidism (Mannstadt *et al.*, 2007). Both of these mutations were shown, by using a GCMB-associated luciferase reporter, to inhibit the action of the wild-type transcription factor, thereby indicating that these GCMB mutants have dominant-negative properties (Mannstadt *et al.*, 2007).

X-LINKED RECESSIVE HYPOPARATHYROIDISM

Hypoparathyroidism with an X-linked recessive transmission pattern has been reported in two multigenerational kindreds (Peden, 1960; Whyte and Weldon, 1981). Only males were affected, and they suffered from infantile epilepsy and hypocalcemia. The hypoparathyroidism is due to a defect in parathyroid gland development (Whyte *et al.*, 1986). Linkage studies utilizing X-linked RFLPs in these two families assigned the mutant gene to chromosome Xq26–q27 (Thakker *et al.*, 1990). An approach utilizing mitochondrial DNA analysis established a common ancestry in these two X-linked hypoparathyroid kindreds (Mumm *et al.*, 1997). A common ancestry for these two kindreds from eastern Missouri had been suspected, but it could not be established despite five generations of extensive genealogical records (Whyte and Weldon, 1981). The mitochondrial genes are transmitted through the maternal line exclusively. If relatedness among the two kindreds involved the maternal lines, analysis of mitochondrial genetic markers would reveal common features. The DNA sequence of the mitochondrial (mt) D-loop was compared among individuals in both kindreds. The mt DNA sequence was identical among affected males and their maternal lineage for individuals in both kindreds, but differed at three to six positions when compared with the mitochondrial DNA of the fathers. These results demonstrated that the two kindreds with X-linked recessive hypoparathyroidism are indeed related and that an identical gene defect is likely to be responsible for the disease. Additional studies refined the location of this gene to be between the locus for diffuse B cell lymphoma (DBL) and DXS984; a 906kb region in Xq27.1 (Trump *et al.*, 1998, Nesbit *et al.*, 2004b). Furthermore, DNA sequence analyses of the coding regions of the three genes—adenosine triphosphatase 11C (ATP11C), U7snRNA homologue, and Sry-box3 (SOX3)—that are contained within this 906-kb interval did not reveal any abnormalities (Nesbit *et al.*, 2004b). These findings together with the reported absence of hypocalcemia in a boy with hemophilia B and mental retardation who had a chromosomal deletion that encompassed the entire 906-kb interval containing ATP11C, U7snRNA, and SOX3 genes (Stevanovic *et al.,* 1993) suggested that other genomic abnormalities such as molecular duplications for translocations, which could cause altered gene function may underlie the etiology of X-linked recessive hyperparathyroidism (Bowl *et al.,* 2005). Indeed, this proved to be the case, and a complex interstitial deletion-insertion involving chromosomes 2p25.3 and Xq27.1, near SOX3, was shown to cosegregate with X-linked recessive hypoparathyroidism (Bowl *et al.,* 2005). This deletion-insertion was located approximately 67 kb downstream of SOX3, and hence it was likely to exert a position effect on SOX3 expression. Moreover, SOX3 was shown to be expressed in the developing parathyroids of mouse embryos, and this indicates a likely role for SOX3 in the embryonic development of the parathyroid glands (Bowl *et al.,* 2005).

SOX3 belongs to a family of genes encoding high-mobility group (HMG) box transcription factors and is related to SRY, the sex-determining gene on the Y chromosome. The mouse homologue is expressed in the prestreak embryo and subsequently in the developing central nervous system (CNS), which includes the region of the ventral diencephalon that induces development of the anterior pituitary and gives rise to the hypothalamus, olfactory placodes, and parathyroids (Solomon *et al.,* 2004; Rizzoti *et al.,* 2004; Collignon *et al.,* 1996; Bowl *et al.,* 2005). Patients with X-linked hypopituitarism have been reported to have duplications involving a 686-kb to 13-Mb region, that contains SOX3 (Solomon *et al.,* 2002, -2004; Woods *et al.,* 2005), and overexpression of SOX3 has been reported to inhibit Wnt signaling, which has an important role in pituitary development (Zorn *et al.,* 1999). Furthermore, increased levels of SOX3 have been shown to cause developmental hypoplasia of tissues, such as the lens and otic placodes in fish embryos (Koster *et al.,* 2000). Reduced levels of SOX3 expression also result in hypopituitarism. Thus, SOX3-null mice have abnormal pituitary development associated with hypopituitarism, craniofacial abnormalities, and midline CNS defects (Rizzoti *et al.,* 2004). These phenotypic features are similar to those observed in patients with X-linked hypopituitarism, and with X-linked mental retardation and growth hormone deficiency, who have in-frame duplications of 21 bp or 33 bp encoding for seven or 11 alanines, respectively, in a polyalanine tract of the SOX3 gene (Woods *et al.,* 2005; Laumonnier *et al.,* 2002). The polyalanine tract expansion resulted in a reduction of SOX3 transcriptional activity that was associated with an impaired nuclear localization of the mutant protein (Woods *et al.,* 2005). These findings demonstrate that pituitary development is sensitive to SOX3 dosage, and that both loss- and gain-of-function mutations can result in X-linked hypopituitarism (Solomon *et al.,* 2002, 2004; Laumonnier *et al.,* 2002; Rizzoti *et al.,* 2004).

Patients with X-linked hypopituitarism have not been reported to suffer from hypoparathyroidism (Solomon *et al.,* 2002, 2004; Woods *et al.,* 2005; Laumonnier *et al.,* 2002), and conversely, the patients affected with X-linked recessive hypoparathyroidism do not suffer from hypopituitarism (Peden, 1960; Whyte and Weldon, 1981). These clinical differences may be due to the differences in the temporal expression patterns of SOX3 in the pituitary and parathyroids, or to interactions with different tissue-specific enhancers or repressors. Alternatively, they may be due to differences in the locations of the associated SOX3 genomic abnormalities, and it is important to note that X-linked hypopituitarism is associated either with duplications of the entire SOX3 coding region or with an intragenic expansion of a polyalanine tract (Solomon *et al.,* 2002, 2004; Woods *et al.,* 2005; Laumonnier *et al.,* 2007), whereas X-linked recessive hypoparathyroidism is associated with a deletion that is approximately 67 kb downstream from the SOX3 coding region. This situation may be analogous to that reported to occur in disorders associated with abnormalities of the sonic hedgehog (SHH) gene (Kleinjan and van Heyningen, 2005). SHH is a secreted protein that provides key inductive signals for the patterning of ventral neural tube, the anterior posterior limb axis, and the ventral somites. SHH gene abnormalities lead to holoprosencephaly type 3 (HPE3) and preaxial polydactyly (PPD) in man. HPE3 is caused by deletions or point mutations that involve the coding region and result in haploinsufficiency of SHH, whereas PPD is caused by breakpoints or point mutations within a limb regulatory element that is 1 Mb upstream of SHH (Kleinjan and van Heyningen, 2005). These findings illustrate that phenotypes caused by mutations in regulatory elements can be very different from those caused by mutations of coding regions, and that point mutations in regulatory elements at a distance as far as 1 Mb from the gene promoter can have a detrimental affect on embryonic development (Kleinjan and van Heyningen, 2005). Thus, it seems likely that the differences between X-linked recessive hypoparathyroidism and hypopituitarism occur because the hypoparathyroidism deletion-insertion, which is about 67 kb downstream of SOX3, involves disruption of regulatory elements, whereas the abnormalities resulting in hypopituitarism involve alterations of the SOX3 coding region. It is also important to note that the disruption in the regulatory region could also lead to upregulation or misregulation of the gene, thereby resulting in phenotypes due to gain-of-function (i.e., hypermorphic or neomorphic).

The location of the deletion-insertion about 67 kb downstream of SOX3 in X-linked recessive hypoparathyroid patients is likely to result in altered SOX3 expression, because SOX3 expression has been reported to be sensitive to position effects caused by X-chromosome abnormalities (Kleinjan and van Heyningen, 2005). Indeed, reporter-construct studies of the mouse Sox3 gene have demonstrated the presence of both 5′ and 3′ regulatory elements (Brunelli *et al.,* 2003), and thus it is possible that the deletion-insertion in the X-linked recessive hypoparathyroid patients may have a position effect on SOX3 expression and parathyroid development from the pharyngeal pouches. Indeed, such position effects on SOX genes, which may be exerted over large distances, have been reported. For example, the very closely related Sox2 gene has been shown to have regulatory regions spread over a long distance, both 5′ and 3′ to the coding region (Uchikawa *et al.,* 2003), and disruption of sequences at some distance 3′ has been reported to lead to loss of expression in the developing inner ear and absence of sensory cells, whereas expression in other sites is unaffected (Kiernan *et al.,* 2005). Similarly, for the SRY gene, which probably originated from SOX3 (Stevanovic *et al.,* 1993), both 5′ and 3′ deletions result in abnormalities of sexual development, and translocation break points over 1 Mb upstream of the SOX9 gene have been reported to result in camptomelic dysplasia due to removal of elements that regulate SOX9 expression (Kleinjan and van Heyningen, 2005). The molecular deletion-insertion identified in X-linked recessive hypoparathyroidism may similarly cause position effects on SOX3 expression, and this points to a potential role for the SOX3 gene in the embryological development of the parathyroid glands from the pharyngeal pouches.

CONCLUSIONS

Application of the methods of molecular genetics to the study of the hypoparathyroid disorders has resulted in considerable advances that have identified some genes and their encoded proteins that are involved in the embryological development of the parathyroids, mediating its actions in different target issues. In addition, the identification of mutations has helped to provide molecular explanations and insights into a variety of familial and sporadic forms of hypopara thyroidism. Moreover, investigation of mouse models has helped to increase our understanding of the molecular regulation of pharyngeal pouch development. However, many challenges remain in this complex field, and in particular to resolving the issues of phenotypic variance and interspecies differences.

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Chapter 66

Genetic Disorders Caused by PTH/PTHrP Receptor Mutations

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INTRODUCTION

The PTH/PTHrP receptor (also referred to as type I PTH/PTHrP receptor or PTH1R) mediates the actions of two ligands, parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHrP) and stimulates at least two distinct second messenger pathways, cAMP/PKA and IP_3/Ca^{2+}/PKC (Jüppner *et al.,* 1991; Abou-Samra *et al.,* 1992; Schipani *et al.,* 1993). This receptor is most abundantly expressed in kidney, bone, and growth plates, and at lower levels in a large variety of other tissues (Tian *et al.,* 1993; Urena *et al.,* 1993). The PTH-dependent endocrine actions that are mediated through the PTH/PTHrP receptor, i.e., the regulation of mineral ion homeostasis, had been explored through studies in intact and parathyroidectomized animals. The role of the PTH/PTHrP receptor in mediating the PTHrP-dependent autocrine/paracrine regulation of chondrocyte growth and differentiation became apparent only through the analysis of genetically manipulated mice. For example, animals that are “null” for PTHrP or the PTH/PTHrP receptor die *in utero* or shortly thereafter and show a profound acceleration of growth plate mineralization. In contrast, mice overexpressing PTHrP under the control of a growth plate-specific promoter are viable, but showed a severe delay in chondrocyte maturation, which leads to impaired bone growth and elongation (Karaplis *et al.,* 1994; Amizuka *et al.,* 1994; Weir *et al.,* 1996; Lanske *et al.,* 1996; Vortkamp *et al.,* 1996). These studies in mice thus provided important clues regarding the phenotypic abnormalities that were to be expected in humans with PTH/PTHrP receptor mutations and consequently led to the identification of different activating and inactivating PTH/PTHrP receptor mutations in several genetic disorders. Because the biological consequences of these PTH/PTHrP receptor mutations require a detailed understanding of the PTH- and PTHrP-dependent actions that are mediated through this G protein-coupled receptor, we will first review the physiological roles of these two peptide hormones.

PARATHYROID HORMONE (PTH)

Besides $1,25(OH)_2$ vitamin D_3 ($1,25(OH)_2D_3$), PTH is the most important endocrine regulator of extracellular calcium homeostasis in mammals (Kronenberg *et al.,* 1993, Jüppner *et al.,* 2005). PTH is predominantly expressed in the parathyroid glands. However, lower protein and mRNA levels were identified in the hypothalamus and the thymus of *glial cells missing 2* (*gcm2*)-ablated mice (Günther *et al.,* 2000). These findings confirmed earlier studies in rats (Nutley *et al.,* 1995), but it remains unlikely that PTH derived from tissues other than the parathyroid glands is involved in the regulation of mineral ion homeostasis. Its synthesis and secretion by the parathyroid glands are dependent predominantly on the extracellular concentration of calcium (Brown, 1983; Silver and Kronenberg, 1996), which is monitored by a calcium-sensing receptor (Brown *et al.,* 1993, 1999), and to a lesser extend by $1,25(OH)_2D_3$ and phosphate (Silver and Kronenberg, 1996; Almaden *et al.,* 1996; Slatopolsky *et al.,* 1996; Moallem *et al.,* 1998).

PTH acts primarily on kidney and bone, where it binds to cells expressing the PTH/PTHrP receptor and thereby initiates a series of processes that serve to maintain blood calcium and phosphate concentrations within narrow limits (Fig. 1). In kidney, the mRNA encoding the PTH/PTHrP receptor is expressed primarily in the convoluted and

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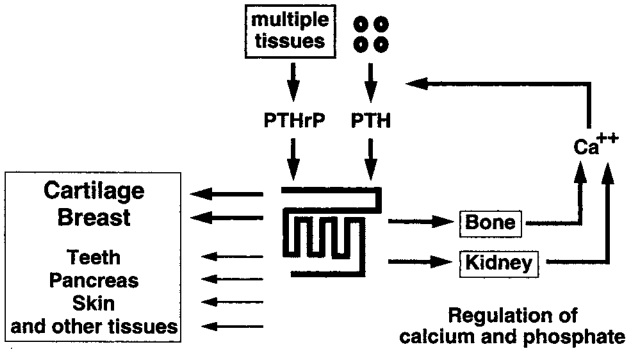

FIGURE 1 The PTH/PTHrP receptor is abundantly expressed in kidney and bone, where it mediates the PTH-dependent regulation of calcium and phosphate homeostasis. It is also expressed in numerous other tissues, particularly in the growth plate chondrocytes, where it mediates the regulation of cellular proliferation and differentiation during development.

straight proximal tubules, the cortical portion of the thick ascending limb, and the distal convoluted renal tubules (Riccardi *et al.,* 1996; Lee *et al.,* 1996; Yang *et al.,* 1997), i.e., in those renal segments that respond to PTH with an increase in cAMP accumulation (Chabardes *et al.,* 1975, 1980; Morel *et al.,* 1981).

The most important PTH-mediated actions in the kidney affect the synthesis of $1,25(OH)_2D_3$ from its precursor $25OHD_3$, the excretion of phosphate, and the reabsorption of calcium. The stimulation of 1α-hydroxylase activity, an action that appears to be at least partially cAMP-dependent (Garabedian *et al.,* 1972; Fraser and Kodicek, 1973; Horiuchi *et al.,* 1977; Kong *et al.,* 1999a; Fu *et al.,* 1997; Takeyama *et al.,* 1999), is largely restricted to the proximal convoluted tubule. The resulting increase in $1,25(OH)_2D_3$ production enhances the absorption of calcium and phosphate from the intestine. The PTH-dependent inhibition of renal tubular phosphate reabsorption has been extensively documented in a variety of *in vivo* and *in vitro* studies (for reviews, see Stewart and Broadus, 1987; Amiel *et al.,* 1998; Murer *et al.,* 1999; Silve and Friedlander, 2000). Thus, to increase renal phosphate excretion, PTH reduces the abundance of two type II sodium-phosphate cotransporters (Npt-2a and Npt-2c, also referred to as NaPi-IIa and NaPi-IIc) on the apical surface of proximal tubules; Npt-2a is expressed in the segments S1–S3, whereas Npt-2c is expressed only in the S1 segment (Segawa *et al.,* 2007). This effect of PTH is mediated by both cAMP-dependent and -independent mechanisms and is associated with an increased internalization and subsequent lysosomal degradation of Npt-2a, and similar mechanisms appear to apply to the regulation of Npt-2c (Segawa *et al.,* 2007; Bell *et al.,* 1972; Goldfarb *et al.,* 1978; Pfister *et al.,* 1998, 1999; Lotscher *et al.,* 1999; for review, see Muere *et al.,* 2000). PTH/PTHrP receptor protein expression has been demonstrated by immunohistochemical and immunoelectron microscopic analysis, and by functional studies, on both basolateral and luminal membranes in proximal tubular cells *in vitro* and in intact proximal tubules (Reshkin *et al.,* 1991; Kaufmann *et al.,* 1994; Amizuka *et al.,* 1997; Traebert *et al.,* 2000). Apical receptors may be preferentially coupled to cAMP-independent signaling pathway, whereas basolateral receptor activation initiates both cAMP-dependent and -independent effects. Recently, megalin, a multifunctional clearance receptor expressed on the apical surface of proximal tubular cells, has been shown to regulate the renal catabolism of PTH and to potentially antagonize PTH/PTHrP receptor activity (Hilpert *et al.,* 1999). In the distal convoluted tubule, PTH stimulates, possibly through second messengers other than cAMP, the reabsorption of calcium (Friedman *et al.,* 1996, 1999), which is associated with an increased expression of the calcium channel TRPV5 (Mensenkamp *et al.,* 2007). PTH also decreases the glomerular filtration, inhibits the proximal reabsorption of bicarbonate and of amino acids, and stimulates gluconeogenesis (Amiel *et al.,* 1998; Jaeger *et al.,* 1987). Consistent with the findings regarding the second messenger systems that mediate the proximal and distal actions of PTH, recently established clonal cell lines derived from proximal segments of rat renal tubules showed a PTH-dependent accumulation of cAMP accumulation and a rapid increase in intracellular free calcium. In contrast to the characteristics of these "proximal" tubular cells, where PTH stimulation led to a rapid release of intracellular calcium from intracellular stores, the increase in this second messenger in the "distal" tubular cells was dependent largely on extracellular calcium (Friedman *et al.,* 1996, 1999). These findings indicated that distinct portions of the nephron respond differently to challenge with PTH.

Similar to its renal tubular effects, the PTH-dependent actions on bone are complex and often difficult to study. As outlined later in more detail, the hormone can influence, either directly or indirectly, the proliferation and differentiation of several bone cell precursors. Furthermore, the effects resulting from PTH stimulation of mature osteoblasts appear to be different depending on the intensity and duration of the stimulus, the type of bone (trabecular versus cortical), and the hormonal millieu of bone. As a result, the hormonal effects observed *in vitro* often fail to reflect the conditions *in vivo*. For example, PTH stimulates both bone formation and osteoclastic bone resorption, however the continuous administration of PTH *in vivo* is thought to favor bone resorption over bone formation, whereas intermittent doses of the hormone results in net anabolic effects (Tam *et al.,* 1982; Lane *et al.,* 1998; Neer *et al.,* 2001; Finkelstein *et al.,* 2003; Horwitz *et al.,* 2005).

The initial, PTH-dependent effect on bone is a rapid release of calcium from areas of the matrix that allow rapid exchange with the extracellular fluid (ECF). These events are followed, after a delay of several hours, by PTH-induced changes in bone cell metabolism. After stimulating osteoblast activity and thus bone formation (Silve *et al.,* 1982), activated osteoblasts increase the activity of already present osteoclasts as well as the differentiation of osteoclast precursors into mature bone-resorbing cells. The coupling between bone-forming osteoblasts and bone-resorbing osteoclasts is thought to depend, at least in part, on the ability of PTH to induce changes in the synthesis and/or activity of several different osteoblast-specific proteins, including insulin-like growth factor 1 (IGF1; Canalis *et al.,* 1989). However, the most important of these "coupling" factors is RANKL (also referred to as osteoclast-differentiation factor (ODF), TRANCE, or osteoprotegerin ligand), a TNF-related protein that is anchored on the surface of osteoblasts. RANKL interacts with the receptor RANK, which is expressed on preosteoclasts and mature osteoclasts and facilitates the differentiation of precursor cells into osteoclasts and stimulates the activity of these bone-resorbing cells (Quinn *et al.,* 1998; Yasuda *et al.,* 1998; Simonet *et al.,* 1997; Kong *et al.,* 1999b). PTH thus acts on two distinct tissues, kidney and bone, to increase through different mechanisms the blood concentration of calcium and to thereby prevent significant hypocalcemia.

PARATHYROID HORMONE-RELATED PEPTIDE (PTHrP)

PTHrP was first discovered as the major cause of the humoral hypercalcemia of malignancy syndrome (Stewart *et al.,* 1980; Mosely *et al.,* 1987; Suva *et al.,* 1987; Strewler *et al.,* 1987; Mangin 1988). Within its amino-terminal portion, PTHrP shares partial amino acid sequence homology with PTH, and as a result of these limited structural similarities, amino-terminal fragments of both peptides have largely indistinguishable biological properties, at least with regard to the regulation of mineral ion homeostasis (Kemp *et al.,* 1987; Horiuchi *et al.,* 1987; Fraher *et al.,* 1992; Everhart-Caye *et al.,* 1996). Shortly after its initial isolation from several different tumors, PTHrP and its mRNA were found in a large variety of fetal and adult tissues, suggesting that this peptide has an important biological role throughout life (Ikeda *et al.,* 1988; Broadus and Stewart, 1994; Yang and Stewart, 1996). However, it was not until the generation of genetically manipulated mice that the major physiological roles of PTHrP became apparent. These roles include the regulation of chondrocyte proliferation and differentiation during the process of endochondral bone formation (Karaplis *et al.,* 1994; Amizuka *et al.,* 1994; Weir *et al.,* 1996; Lanske *et al.,* 1996; Vortkamp *et al.,* 1997; Kronenberg, 2003) and epithelial-mesenchymal interactions during organogenesis of certain epithelial organs, including skin, mammary gland, and teeth (Wysolmerski *et al.,* 1994, 1996, 1998; Philbrick *et al.,* 1998; Calvi *et al.,* 2004; Strewler, 2000). During fetal development, the expression of mRNA transcripts encoding PTHrP or the PTH/PTHrP receptor is closely linked, both spatially and temporally, implying that the ligand and its receptor are involved in paracrine/autocrine signaling events at these sites (Karaplis *et al.,* 1994; Kronenberg, 2003; Wysolmerski *et al.,* 1998; Philbrick *et al.,* 1998; Lee *et al.,* 1995). These observations are furthermore consistent with the hypothesis that the PTH/PTHrP receptor mediates most of the actions of PTHrP.

Other PTHrP-dependent effects are likely to involve mid-/carboxyl-terminal peptide fragments that are generated through alternative splicing and/or post-translational processing and involve distinct, only incompletely characterized cell surface receptors and/or direct interactions with the nucleus (Wu *et al.,* 1996; Kovacs *et al.,* 1996; Lanske *et al.,* 1999; Henderson *et al.,* 1995; Nguyen and Karaplis, 1998; Massfelder *et al.,* 1997; de Miguel *et al.,* 2001; Fiaschi-Taesch *et al.,* 2006). Although these non-amino-terminal PTHrP fragments are unlikely to be of biological importance for adult mineral ion metabolism, recent evidence suggests that large carboxyl-terminal fragments of PTH such as PTH(7–84) act, directly or indirectly, as antagonists of the calcemic actions of PTH(1–84) (Slatopolsky *et al.,* 2000; Nguyen-Yamamoto *et al.,* 2001; D'Amour *et al.,* 2005). Through yet undefined mechanisms, carboxyl-terminal fragments of PTH may thus contribute to the regulation of calcium homeostasis.

THE PTH/PTHrP RECEPTOR: A RECEPTOR FOR TWO DISTINCT LIGANDS

The isolation of cDNAs encoding the PTH/PTHrP receptor from several different species, including humans, and subsequent expression of these cDNAs in various mammalian cell lines has confirmed and extended three key observations: (1) the recombinant PTH/PTHrP receptor binds amino-terminal fragments of PTH and PTHrP with similar or indistinguishable affinity; (2) both ligands stimulate with similar potency the formation of at least two second messengers, cAMP and inositol phosphate; and (3) identical receptors are expressed in renal tubular cells and in osteoblasts (Jüppner *et al.,* 1991; Abou-Samra *et al.,* 1992; Schipani *et al.,* 1993). Furthermore, similar to the widely expressed PTHrP, the mRNA encoding the PTH/PTHrP receptor is found in a large variety of fetal and adult tissues (Tian *et al.,* 1993; Urena *et al.,* 1993; Riccardi *et al.,* 1996; Lee *et al.,* 1994, 1995,1996) and at particularly abundant concentrations in proximal tubular cells, in osteoblasts, and in prehypertrophic chondrocytes of metaphyseal growth plate (Karaplis *et al.,* 1994; Weir *et al.,* 1996; Lanske *et al.,* 1996; Vortkamp *et al.,* 1996).

PTH/PTHrP receptor belongs to the class B family of heptahelical G protein-coupled receptors (GPCR), which also comprises the receptors for secretin, calcitonin, glucagon, and several other peptide hormones (Jüppner *et al.,* 2005; Gardella *et al.,* 2002; Gensure *et al.,* 2005). These hormone receptors share no homology with other G protein-coupled receptors, for example the members of the class A and class C family of receptors, and the organization of genes encoding the latter receptors are distinctly different from encoding the class B receptors. All class B receptors are characterized by an amino-terminal, extracellular domain that comprises approximately 150 amino acids and by eight conserved extracellular cysteine residues, as well as several other conserved amino acids that are dispersed throughout the amino-terminal domain, the membrane-spanning helices, and the connecting loops.

The organization of the PTH/PTHrP receptor gene, which comprises in mammals 14 coding exons, appears to be similar in all vertebrates (Kong *et al.,* 1994; McCuaig *et al.,* 1994). The approximately 2.5-kb transcript encoding the full-length PTH/PTHrP receptor is the predominant mRNA species in most tissues (Tian *et al.,* 1993; Urena *et al.,* 1993), but several larger and smaller transcripts have also been detected, suggesting the presence of splice variants of the PTH/PTHrP receptor. In contrast to alternatively spliced mRNAs that encode functional calcitonin and CRF receptors lacking portions of the seventh membrane-spanning helix (Shyu *et al.,* 1996; Grammatopoulos *et al.,* 1999), no alternatively spliced, functionally active PTH/PTHrP receptors have yet been identified (Jobert *et al.,* 1996; Joun *et al.,* 1997).

Transcripts encoding identical PTH/PTHrP receptors are derived from at least three different promoters that are located upstream of one of three different untranslated exons (Kong *et al.,* 1994; McCuaig *et al.,* 1994; Bettoun *et al.,* 1997; Manen *et al.,* 1998). Little is known about the factors that control the activity of these promoters, some of which appear to be species- and tissue-specific (Amizuka *et al.,* 1997; Bettoun *et al.,* 1998), and their activity may be different throughout development and adult life (Bettoun *et al.,* 1998; Amizuka *et al.,* 1999). Transcripts derived from the P1 and the P2 promoter have been observed in rodents and humans (McCuaig *et al.,* 1994, 1995; Joun *et al.,* 1997). P1-derived transcripts are found mainly, at least in rodents, in vascular smooth muscles and in peritubular endothelial cells of the adult kidney (Amizuka *et al.,* 1997). In contrast, transcripts derived from ubiquitous P2 promoter can be detected in numerous fetal and adult tissues, including cartilage and bone (Amizuka *et al.,* 1999). The P2 promoter activity is equivalent in humans and mice, whereas the activity of the P1 promoter is prominent only in rodents and appears to be weak or absent in humans. A third promoter, P3, has been identified thus far only in humans, where it is thought to control PTH/PTHrP receptor expression in several different tissues, including kidney and bone (Manen *et al.,* 1998; Bettoun *et al.,* 1998). Methylation appears to play a role in controlling human PTH/PTHrP receptor gene promoter activity (Bettoun *et al.,* 2000). A differential regulation of P2 activity in osteoblasts and chondrocytes following vitamin D_3 administration has been demonstrated in rodents (Amizuka *et al.,* 1999).

A second PTH-receptor, termed type 2 PTH receptor or PTH2-receptor, which belongs to the same family of G protein-coupled receptors as the PTH/PTHrP receptor, is less broadly expressed, with little if any expression in kidney and bone (Usdin *et al.,* 1996, 1999). The human PTH2-receptor is efficiently activated by PTH, but not by PTHrP (Usdin *et al.,* 1995; Gardella *et al.,* 1996; Behar *et al.,* 1996), whereas the rat receptor homologue is not activated by either of these two ligands (Hoare *et al.,* 1999). Instead, both PTH2-receptor isoforms are efficiently and equally activated by TIP39, a recently identified hypothalamic peptide, which shows limited amino acid sequence homology with PTH and PTHrP. It is therefore likely that TIP39 represents the primary agonist for the PTH2-receptor (Usdin *et al.,* 1999; Usdin, 1997). Although TIP39 does not activate the PTH/PTHrP receptor, the full-length peptide as well as several fragments that are truncated at the amino-terminus can bind to the latter receptor and furthermore inhibit PTH- and PTHrP-stimulated cAMP accumulation (Hoare *et al.,* 2000a, 2000b; Jonsson *et al.,* 2001). It appears unlikely, however, that TIP39 has a role in the regulation of mineral ion homeostasis.

Role of PTHrP and the PTH/PTHrP Receptor in Endochondral Bone Formation

PTHrP was initially discovered as the cause of the humoral hypercalcemia of malignancy syndrome. However, its most prominent physiological role was revealed only through the homologous ablation of its gene in mice and through the development of transgenic animals that express PTHrP under the control of a growth plate-specific promoter (Karaplis *et al.,* 1994; Weir *et al.,* 1996; Kronenberg, 2003).

Homozygous PTHrP gene-ablated animals die during the perinatal period and show striking skeletal changes, which include domed skulls, short snouts and mandibles, and disproportionately short extremities, yet no obvious developmental defects in other organs (Fig. 2). These skeletal changes are caused by a dramatic acceleration of chondrocyte differentiation that lead to premature growth plate mineralization (Karaplis *et al*., 1994; Fig. 3). Heterozygous animals, lacking only one copy of the PTHrP gene, show normal growth and development and are fertile, but develop, despite apparently normal calcium and phosphorus homeostasis, mild osteopenia later in life (Amizuka *et al.,* 1996). Growth plate abnormalities that are, in many aspects, the opposite of those found in PTHrP-ablated mice are observed in animals that overexpress PTHrP under the control of the α1(II) collagen promoter (Weir *et al.,* 1996). Throughout life these animals are smaller in size than their wild-type litter mates and show a disproportionate foreshortening of limbs and tail, which is most likely due to a severe delay in chondrocyte differentiation and endochondral ossification. Thus, too little or too much PTHrP expression in the growth plate leads to short-limbed dwarfism, although through entirely different mechanisms.

From these and other studies, it is now well established that PTHrP facilitates the continuous proliferation of chondrocytes in the growth plate, and that it postpones their programmed differentiation into hypertrophic chondrocytes. Consistent with this role of PTHrP in endochondral bone formation, earlier *in vitro* studies had shown that PTH (used in these studies instead of PTHrP) affects chondrocyte maturation and activity (Lebovitz and Eisenbarth, 1975; Smith *et al.,* 1976). More recent studies confirmed these findings by showing that PTH and PTHrP stimulate, presumably through cAMP-dependent mechanisms (Jikko *et al.,* 1996), the proliferation of fetal growth plate chondrocytes, inhibit the differentiation of these cells into hypertrophic chondrocytes, and stimulate the accumulation of cartilage-specific proteoglycans that are thought to act as inhibitors of mineralization (Takano *et al.,* 1985; Koike *et al.,* 1990; Iwamoto *et al.,* 1994). In the absence of these cartilage-specific PTHrP effects, growth plates of homozygous PTHrP gene-ablated mice have a thinner layer of proliferating chondrocytes, whereas the layer of hypertrophic chondrocytes is relatively normal in thickness, but somewhat disorganized. Taken together, these findings suggested that the lack of PTHrP accelerates the normal differentiation process of growth plate chondrocytes, i.e., resting and proliferating chondrocytes undergo fewer cycles of cell division and differentiate prematurely into hypertrophic cells, which then undergo apoptosis before being replaced by invading osteoblasts.

The phenotypic changes in mice that are "null" for either PTHrP or the PTH/PTHrP receptor are similar, and current evidence indicates that the autocrine/paracrine

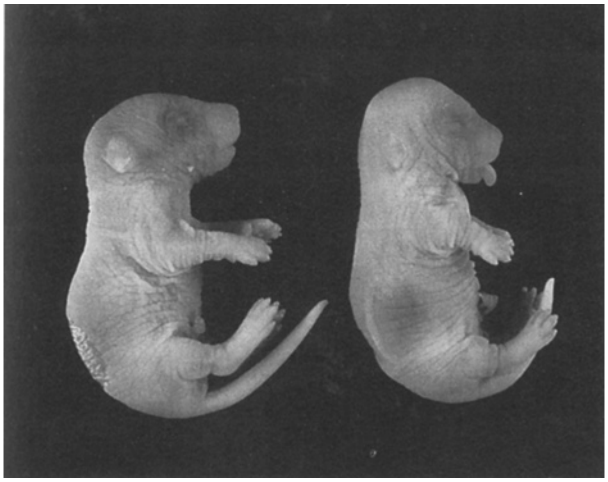

FIGURE 2 Phenotype of a homozygous PTHrP-ablated mouse (day 18.5 of embryonic development). Note the chondrodysplasia characterized by a domed skull, short snout and mandible, protruding tongue, narrow thorax, and disproportionately short limbs, From Karaplis *et al.* (1996), with permission.

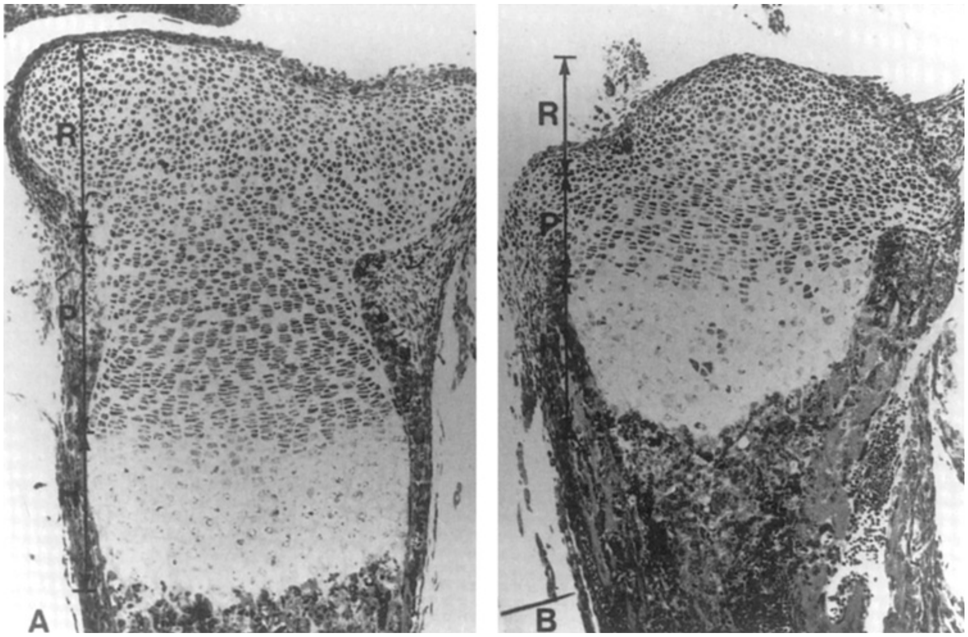

FIGURE 3 Low-magnification photomicrographs of the proximal tibiae of a wild-type mouse (**A**) and a homozygous PTHrP-ablated mouse (**B**) (E 18.5). Note that the tibial epiphysis of the PTHrP ablated mouse is shortened and the zones of reserve and proliferative chondrocytes are markedly reduced. From Amizuka *et al.* (1994), with permission.

actions of PTHrP within the growth plate are mediated through the PTH/PTHrP receptor (Karaplis *et al.,* 1994; Lanske *et al.,* 1996; Chung *et al.,* 1998). Furthermore, mice missing either PTHrP or its receptor are resistant to the actions of *Indian Hedgehog* (*Ihh*), a developmentally important protein that is most abundantly expressed in growth plate chondrocytes which are about to differentiate into hypertrophic cells. *Ihh* binds directly to *patched,* a membrane receptor, which interacts with *smoothened* and thereby suppresses the constitutive activity of the latter protein (Stone *et al.,* 1996; Marigo *et al.,* 1996). The ectopic expression of *Ihh* in the chicken wing cartilage stimulates the production of PTHrP and thereby blocks the normal chondrocyte differentiation program (Vortkamp *et al.,* 1996); whether PTHrP represses, as part of a feedback loop, the expression of *Ihh* remains to be established. PTHrP and *Ihh* are thus critically important components of normal bone growth and elongation (Lanske *et al.,* 1996; Vortkamp *et al.,* 1996). However, not all actions of PTHrP appear to be mediated through the PTH/PTHrP receptor, because the ablation of the PTHrP gene or the PTH/PTHrP receptor gene leads to subtle, but distinctly different, abnormalities in early bone development (Lanske *et al.,* 1999). This suggests that some actions of PTHrP in bone involve either distinct receptors or the peptide's direct nuclear actions (Henderson *et al.,* 1995; Nguyen and Karaplis, 1998; Massfelder *et al.,* 1997; de Miguel *et al.,* 2001; Fiaschi-Taesch *et al.,* 2006).

ROLE OF PTHrP IN REGULATING EPITHELIAL-MESENCHYMAL INTERACTIONS

Studies with transgenic mice, in which PTHrP expression is targeted through a human keratinocyte-specific promoter (K14) to the developing epidermis and mammary gland, demonstrated that PTHrP plays also a critical role in hair follicle development and branching morphogenesis of the mammary gland (Wysolmerski *et al.,* 1994, 1996). These conclusions were further supported by findings in PTHrP-null mice that had been rescued from neonatal death by targeting PTHrP expression to chondrocytes through the $\alpha1$(II) collagen promoter (Weir *et al.,* 1996). These rescued mice lack mammary epithelial ducts, because of a failure of the initial round of branching growth that is required for transforming the mammary bud into the primary duct system; ablation of both copies of the PTH/PTHrP receptor gene recapitulated the phenotype of PTHrP-ablated animals (Wysolmerski *et al.,* 1998).

Using similar approaches, it was furthermore demonstrated that PTHrP is required for normal tooth eruption. Teeth appeared to develop normally in rescued PTHrP knockout mice, but became trapped by the surrounding bone and underwent progressive impaction (Philbrick *et al.,* 1998). In these tissues, PTHrP mRNA was identified by *in situ* hybridization in epithelial cells, whereas the PTH/PTHrP receptor mRNA was found on mesenchymal or stromal cells. These observations led to the concept that the communication between epithelium and mesenchyme involves the PTH/PTHrP receptor, and that PTHrP signaling is essential for normal development of these tissues (Wysolmerski and Stewart, 1998).

JANSEN'S METAPHYSEAL CHONDRODYSPLASIA

JMC, first described in 1934 (Jansen, 1934), is a rare autosomal-dominant form of short-limbed dwarfism associated with laboratory abnormalities that are typically observed only in patients with either primary hyperparathyroidism or with the humoral hypercalcemia of malignancy syndrome (reviewed in Jüppner, 1996, and Parfitt *et al.,* 1996). These biochemical changes, i.e., hypercalcemia, renal phosphate wasting, and increased urinary cAMP excretion, occur despite low or undetectable concentrations of PTH in the circulation and PTHrP concentrations that are not elevated (Frame and Poznanski, 1980; Holt, 1969; Kessel *et al.,* 1992; Rao *et al.,* 1979; Silverthorn *et al.,* 1983; Schipani *et al.,* 1999; Kruse and Schütz, 1993). Severe hypercalcemia, which is often asymptomatic, and hypophosphatemia had been noted in Jansen's first patient (De Haas *et al.,* 1969) and in a subsequently described child with the same disorder (Cameron *et al.,* 1954). It was not until the description of a third patient, however, that the association between the abnormalities in endochondral bone formation and in mineral ion homeostasis was formally considered (Gram *et al.,* 1959). At that time the biochemical abnormalities could not be readily distinguished from those observed in primary hyperparathyroidism, but the surgical exploration of the patient revealed no obvious abnormalities of the parathyroid glands. It was therefore concluded that the changes in mineral metabolism were either "secondary to the underlying bone defect" or "related to an undefined metabolic disorder that gave rise to both metaphyseal and biochemical changes" (Gram *et al.,* 1959). Most reported cases of JMC are sporadic, but the description of two unrelated affected females that gave birth to affected daughters (Lenz, 1969; Holthusen *et al.,* 1975; Charrow and Poznanski, 1984) suggested an autosomal dominant mode of inheritance; this conclusion was subsequently confirmed for two families at the molecular level (Schipani *et al.,* 1996; Bastepe *et al.,* 2004).

At birth some patients with JMC have dysmorphic features, which can include high skull vault, flattening of the of the nose and forehead, low-set ears, hypertelorism, high-arched palate, and micro- or retrognathia (for a review, see Jüppner, 1996). Although body length is within normal limits at birth, growth becomes increasingly abnormal, eventually

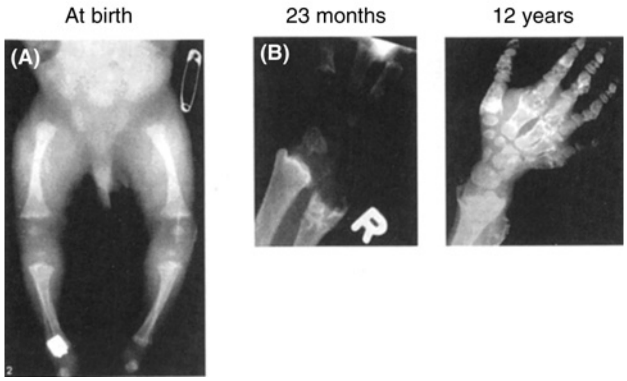

FIGURE 4 A patient with Jansen's disease and his radiological findings at different ages; at birth, 23 months, and 12 years of age. From Silverthorn *et al.* (1983), with permission.

leading to the development of short stature. Additional signs may include kyphoscoliosis with a bell-shaped thorax and widened costochondral junctions, metaphyseal enlargement of the joints, waddling gait, prominent supraorbital ridges, and frontonasal hyperplasia. The legs are usually bowed and short, whereas the arms are relatively long.

Radiological studies have shown considerable, age-dependent differences in the osseous manifestations of JMC. In younger patients, severe metaphyseal changes, especially of the long bones, are present (Fig. 4). The metaphyses are enlarged and expanded, giving a clublike appearance to the ends of the long bones with a wide zone of irregular calcifications. Patches of partially calcified cartilage that protrude into the diaphyses are also present and appear relatively radiolucent. These findings, which are characteristically observed throughout early childhood, are similar to the lesions observed in rickets. However, distinct from the findings in rickets, metacarpal and metatarsal bones are also involved.

Later in childhood, the changes are no longer reminiscent of rickets. Until the onset of puberty, almost all tubular bones show irregular patches of partially calcified cartilage that protrude into the diaphyses; the spine and vertebral bodies show no obvious abnormalities (Frame and Poznanski, 1980; Kessel *et al.,* 1992; Rao *et al.,* 1979; Silverthorn *et al.,* 1983; Schipani *et al.,* 1999; Kruse and Schütz, 1993; Cameron *et al.,* 1954; Charrow and Poznanski, 1984). After adolescence, the cartilaginous tissue in the metaphyses gradually disappears and turns into bone, leading to bulbous deformities (see Fig. 4). The ends of most tubular bones remain expanded, deformed and radiolucent, but a more normal trabecular pattern gradually emerges (Frame and Poznanski, 1980; Kessel *et al.,* 1992; Rao *et al.,* 1979; Silverthorn *et al.,* 1983; Scvhipani *et al.,* 1999; Kruse and Schütz, 1993; Cameron *et al.,* 1954; Charrow and Poznanski, 1984).

In addition, sclerosis and thickening of the base of the skull and of the calvaria is noted in most cases. The former changes are thought to be the cause of cranial auditory and optical nerve compression, which has been observed later in life in some affected individuals. Loss of the normal cortical outline, areas of subperiosteal bone resorption, and generalized osteopenia are reminiscent of the changes seen in hyperparathyroidism. Furthermore, there is an increased in trabecular bone volume and a thinning of cortical bone (Parfitt *et al.,* 1996). The two only reports that investigated the histological changes in the growth plates, described a severe delay in endochondral ossification of the metaphyses, including a lack of the regular columnar arrangement of the maturing cartilage cells, a lack of excess osteoid (which is usually indicative of active rickets or osteomalacia), little or no vascularization of cartilage, and no evidence for osteitis fibrosa (Cameron *et al.,* 1954; Jaffe, 1972).

One female patient with JMC was reported to be unable to breast-feed and to have, similar to her affected daughter, a dry and scaly skin (Schipani *et al.,* 1996). As discussed earlier, PTHrP and the PTH/PTHrP receptor are expressed in breast and skin, and this ligand/receptor system appears to have an important role in these two tissues. Tooth development and enamel formation appear normal in patients with JMC. Intelligence appears to be normal in all reported cases.

Most laboratory findings in JMC are reminiscent of those observed in patients with primary hyperparathyroidism or with the syndrome of humoral hypercalcemia of malignancy. In the newborn, blood phosphorus levels are typically at the lower end of the normal range, whereas alkaline phosphatase activity is almost invariably elevated. Hypercalcemia is usually absent at birth, but develops during the first months of life; it persists throughout life but is more pronounced during infancy and childhood. Hypercalciuria is usually present and can be associated with an increased incidence of nephrocalcinosis (Kessel *et al.,* 1992). 1,25$(OH)_2D_3$ levels have been reported to be normal or at the upper end of the normal range. Serum alkaline phosphatase activity and osteocalcin concentration are elevated throughout life, indicating that osteoblast activity is increased; compatible with an increased osteoclastic activity, urinary hydroxyproline excretion is elevated (Schipani *et al.,* 1999; Kruse and Schütz, 1993).

JANSEN'S DISEASE IS CAUSED BY ACTIVATING PTH/PTHrP RECEPTOR MUTATIONS

Because of the findings in the various genetically manipulated mice described earlier, and because of the abundant expression of the PTH/PTHrP receptor in the three organs that are most obviously affected in JMC, i.e., kidney, bone, and metaphyseal growth plates, activating receptor mutations were considered as a cause of this rare disease. Indeed, in several unrelated patients with this disorder, a heterozygous nucleotide exchange, which changes a histidine at position 223 to arginine, was identified in exon M2 of the PTH/PTHrP

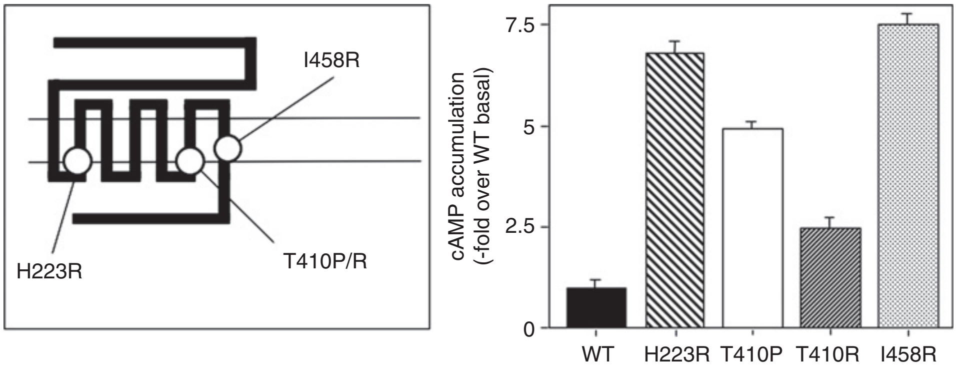

FIGURE 5 Schematic representation of the PTH/PTHrP receptor and basal, agonist-indepenent cAMP accumulation of wild-type ■ and mutant receptors. The approximate location of the three different missense mutations that were identified in patients with Jansen's disease are indicated (*left*). Basal cAMP accumulation of COS-7 cells expressing wild-type and mutant PTH/PTHrP receptors (H223R, ▧; T410P, □; T410R, ▨; I458R, ▒ ; (*right*). Modified from Schipani *et al*. (1996, 1999).

receptor gene (Schipani *et al.,* 1995, 1996, 1999; Minagawa *et al.,* 1997). In other patients, two additional heterozygous nucleotide exchanges were identified that change either a threonine at position 410 to proline (exon M5), or isoleucine at position 458 to arginine (exon M7; Schipani *et al.,* 1996, 1999; Fig. 5; also see Table I). The three mutated residues are predicted to be located at or close to the intracellular surface of the cell membrane and are strictly conserved in all mammalian members of this receptor family (Jüppner *et al.,* 2005; Gardella *et al.,* 2002; Gensure *et al.,* 2005), suggesting an important functional role for these three residues. With the exception of one family where a mother-to-daughter transmission of the H223R mutation was documented (Schipani *et al.,* 1996), each of the three mutations was excluded in the healthy parents and siblings, and in genomic DNA from a significant number of unrelated healthy individuals. This suggests that JMC is usually caused by *de novo* mutations. To date, the T410P and the I458R mutation have been found in one patient each, whereas the H223R mutation has been identified in eight patients and is thus the most frequent PTH/PTHrP receptor mutation in JMC.

To test *in vitro* the functional consequences of the identified missense mutations in JMC, each of the three different nucleotide exchanges was introduced into the cDNA encoding the wild-type human PTH/PTHrP receptor (Schipani *et al.,* 1995, 1996, 1999; Minagawa *et al.,* 1997). COS-7 cells transiently expressing PTH/PTHrP receptors with either the H223R, the T410P, or the I458R mutation showed significantly higher basal accumulation of cAMP than did cells expressing the wild-type PTH/PTHrP receptor (see Fig. 5). Cells expressing PTH/PTHrP receptors with either of the three point mutations showed no evidence for increased basal accumulation of IP3, indicating that this signaling pathway is not constitutively activated (Schipani *et al.,* 1995, 1996, 1999). Interestingly, the D578H mutation in the luteinizing hormone receptor, which is at a position equivalent to the T410P mutation in the PTH/PTHrP receptor, led to constitutive activity of both signaling pathways, cAMP and IP3 (Liu *et al.,* 1999). It is therefore plausible that the lack of constitutive IP3 generation by three activating mutations in the PTH/PTHrP receptor could be related to insufficient sensitivity of the methods that were used to explore this second-messenger system.

When challenged with increasing concentrations of either PTH or PTHrP, cells expressing the mutant H223R and T410P receptors showed, in comparison to cells expressing the wild-type PTH/PTHrP receptor, reduced maximal cAMP accumulation. In contrast, cells expressing the I458R mutant showed the same maximal cAMP accumulation as cells transfected with the wild-type receptor (Schipani *et al.,* 1999). Agonist-dependent IP accumulation was observed with COS-7 cells expressing the I458R and the T410P mutant, but not with cells expressing the H223R mutant. Despite the differences in the *in vitro* response to PTH or PTHrP, patients with either of the three PTH/PTHrP receptor mutations showed no obvious differences in their clinical and/or biochemical presentation.

More recently, a novel T410R mutation was identified in a less severe form of Jansen disease (Bastepe *et al.,* 2004; see Table I). Patients carrying this mutation show mild skeletal dysplasia with relatively normal stature, and serum calcium levels that are within the normal range. When tested *in vitro,* the degree of constitutive activity of the T410R mutant was significantly lower, when compared with the H223R, T410P, and I458R mutants, respectively. This finding provided the first evidence of an obvious correlation between severity of phenotypical features and degree of constitutive activity in Jansen disease.

Activating mutations in other G protein-coupled receptors have been implicated in several other human diseases, but none of these involve members of the class B receptor family. These disorders include rare forms of retinitis pigmentosa or congenital stationary blindness (activating mutations in rhodopsin; Robinson *et al.,* 1992; Dryja *et al.,* 1993), thyroid adenomas or non-autoimmune hyperthyroidism (activating TSH receptor mutations; Parma

TABLE I Mutations in the PTH/PTHrP receptor gene associated with different human disorders. The description of the sequence variant is based on the nomenclature described in the last updated version (June 2007) of den Dunnen JT and Antonarakis SE (211). LOF: loss of function; GOF: gain of function.

Category	Location	Nucleotide change	Amino acid change	Mutation type	Comment	Disease	Reference
LOF	E2	c.310C>T	p.R104X	Nonsense	Homozygous	Blomstrand	Hoogendam *et al.*, 2007
LOF	E3	c.395C>T	p.P132L	Missense	Homozygous	Blomstrand	Zhang *et al.*, 1998; Karaplis *et al.*, 1998; Hoogendam *et al.*, 2007
GOF (?)	G	c.448C>T	p.R150C	Missense	Heterozygous	Enchondromatosis	Hopyan *et al.*, 2002
GOF	M2	c.668A>G	p.H223R	Missense	Heterozygous	Jansen	Schipani *et al.*, 1995; Schipani *et al.*, 1996; Minagawa *et al.*, 1997; Schipani *et al.*, 1999
LOF	M4	c.+27C>T	p.G350fsX351	Splice donor	Homozygous	Blomstrand	Hoogendam *et al.*, 2007
LOF	E12	c.1093delG	p.V365CfsX141	Frameshift	Heterozygous	Blomstrand	Karperien *et al.*, 1999
LOF	M5	c.1148G>A	p.L373_R383del	Splice acceptor	Heterozygous	Blomstrand	Jobert *et al.*, 1998
GOF	M6-7	c.1228A>C	p.T410P	Missense	Heterozygous	Jansen	Schipani *et al.*, 1996; Schipani *et al.*, 1999
GOF	M6-7	c.1229C>G	p.T410R	Missense	Heterozygous	Jansen	Bastepe *et al.*, 2004
GOF	M7	c.1373T>G	p.I458R	Missense	Heterozygous	Jansen	Schipani *et al.*, 1996; Schipani *et al.*, 1999
Regulatory	T	c.1453C>T	p.R485X	Nonsense	Homozygous	Eiken	Duchatelet *et al.*, 2004

et al., 1993; Duprez *et al.*, 1994; Paschke *et al.*, 1994; Kopp *et al.*, 1995; Tonacchera *et al.*, 1996; Grüters *et al.*, 1998; Khoo *et al.*, 1999; Nogueira *et al.*, 1999; Russo *et al.*, 1999; Trultzsch *et al.*, 1999), gonadotropin-independent male precocious puberty (Shenker *et al.*, 1993; Latronico *et al.*, 1995; Kraaij *et al.*, 1995; Shenker, 1998), Leydig-cell tumors (activating mutations in the luteinizing hormone receptor; Liu *et al.*, 1999), and autosomal dominant forms of familial hypocalcemia (activating calcium-sensing receptor mutations; Pollak *et al.*, 1994; Pearce *et al.*, 1995, 1997; Pearce and Brown, 1996; Baron *et al.*, 1996; Brown *et al.*, 1998; Watanabe *et al.*, 1998; Okazaki *et al.*, 1999). Cell membrane receptors exhibiting constitutive signaling have also been described in the pathogenesis of Kaposi's sarcoma and primary effusion lymphomas (constitutive signaling of the Kaposi's sarcoma herpesvirus-G protein-coupled receptor (KSHV-GPCR) via activation of phosphoinositide-specific phospholipase; Burger *et al.*, 1999a, 1999b).

To prove that the growth plate abnormalities in Jansen's disease are indeed caused by constitutively active PTH/PTHrP receptors, transgenic mice were generated that express the H223R mutant under the control of the rat α1(II) collagen promoter, thereby targeting receptor expression to proliferating chondrocytes (Schipani *et al.*, 1997). Two transgenic mouse lines were established, both of which showed delayed mineralization and decelerated differentiation of proliferative chondrocytes into hypertrophic chondrocytes, a delay in vascular invasion, and a prolonged presence of hypertrophic chondrocytes (Fig. 6). In one of these mouse lines, the defect in endochondral bone formation was only apparent at the microscopic level,

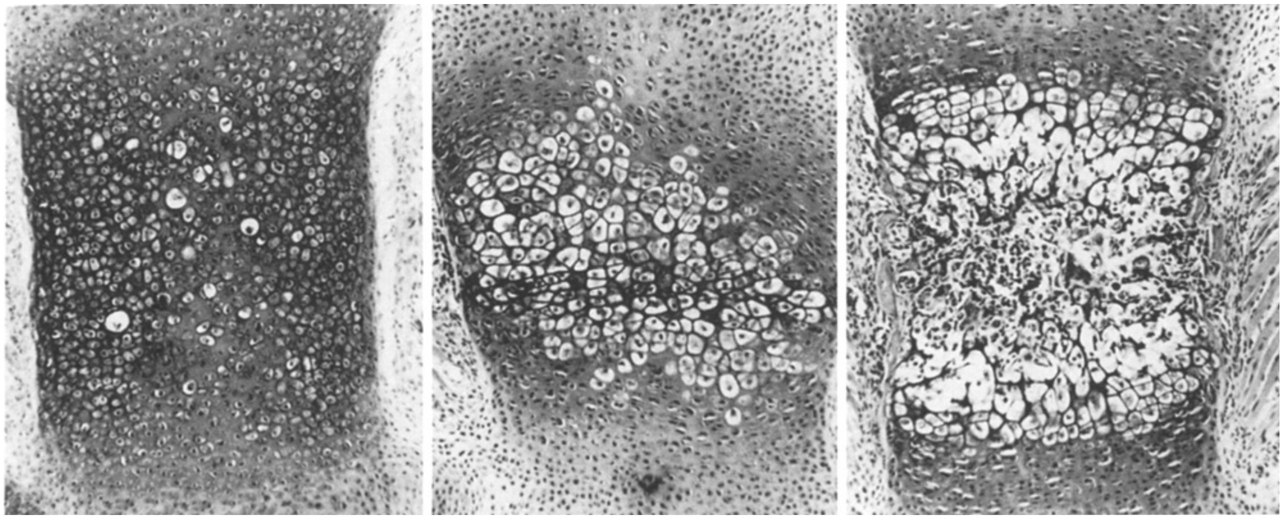

FIGURE 6 Histological sections, stained with hematoxylin and eosin, of decalcified sternum from a newborn wild-type mouse (*left*), and transgenic littermates that were heterozygous (*middle*) or homozygous (*right*) for expression of a constitutively active PTH/PTHrP receptor under the control of the type II collagen promoter. From Schipani *et al.* (1997).

whereas the second line showed shortened and deformed limbs that are reminiscent of the findings in patients with Jansen's disease. Based on these results in transgenic mice, it appears likely that the growth abnormalities in JMC are caused by the expression of mutant, constitutively active PTH/PTHrP receptor in growth-plate chondrocytes.

In an attempt to better understand how the PTH/PTHrP receptor can modulate bone development and turnover, transgenic mice were recently generated in which the human PTH/PTHrP receptor with the H223R mutation is expressed under the control of the type I collagen promoter. Constitutive activity of this receptor was thus targeted to mature osteoblasts and osteoblast precursors. When compared to control littermates, long bones of transgenic mice were reduced in length. The histological analysis of these bones revealed findings that are reminiscent of the skeletal abnormalities in patients with hyperparathyroidism and with Jansen's disease (Parfitt *et al.,* 1996), i.e., a thinner and more porous cortex in the diaphysis, an increased trabeculation of the metaphysis, and a reduction in bone marrow space (Fig. 7). Besides increased osteoblast function in trabecular bone and at the endosteal surface of cortical bone, osteoblastic activity in the periosteum was inhibited. Furthermore, mature osteoblasts as well as a heterogeneous population of preosteoblasts were increased in the trabecular compartment by a mechanism of increased proliferation and decreased apoptosis, and expression of the constitutively active PTH/PTHrP receptor in osteoblasts resulted in a dramatic increase in osteoclast number. The net effect of these actions was a substantial increase in trabecular bone volume and a decrease in cortical bone mass. These studies identified the PTH/PTHrP receptor as an important mediator of both bone-forming and bone-resorbing actions of PTH, and they point out the complexity and heterogeneity of the osteoblast population and/or their regulatory microenvironment (Calvi *et al.,* 2001).

Moreover, expression of a constitutively active PTH/PTHrP receptor in cells of the osteoblast lineage in the Cl2 mice led to a significant expansion of the hematopoietic stem cell (HSC) population, which very likely occurred through up-regulation of Notch-1 (Calvi *et al.,* 2003). These findings were confirmed in wild-type mice treated intermittent injection of PTH (Weber *et al.,* 2006; Adams *et al.,* 2007). PTH was also shown to promote mobilization of HCSs into circulation and to protect them from repeated exposure to chemotherapeutic agents (Adams *et al.,* 2007). Collectively, these data indicate that cells of the osteoblast lineage are critical components of the HSC niche in the bone marrow, and that activation of the PTH/PTHrP receptor can be an important pharmacological target for stem cell-based therapies.

BLOMSTRAND'S LETHAL CHONDRODYSPLASIA

Blomstrand's lethal chondrodysplasia is a recessive human disorder characterized by early lethality, advanced bone maturation and accelerated chondrocyte differentiation, and most likely severe abnormalities in mineral ion homeostasis. The first patient was described by Blomstrand and colleagues in 1985; descriptions of several other patients followed (Young *et al.,* 1993; Leroy *et al.,* 1996; Loshkajian *et al.,* 1997; den Hollander *et al.,* 1997; Oostra *et al.,* 1998, 2000; Galera *et al.,* 1999; Karperien *et al.,* 1999). The disorder was shown to occur in families of different ethnic backgrounds and appears to affect males and females equally. Most affected infants are born to consanguineous parents (only in one instance were unrelated parents reported to have two offspring that are both affected by Blomstrand's disease; Loshkajian *et al.,* 1997), suggesting that BLC is an autosomal recessive disease. Infants with

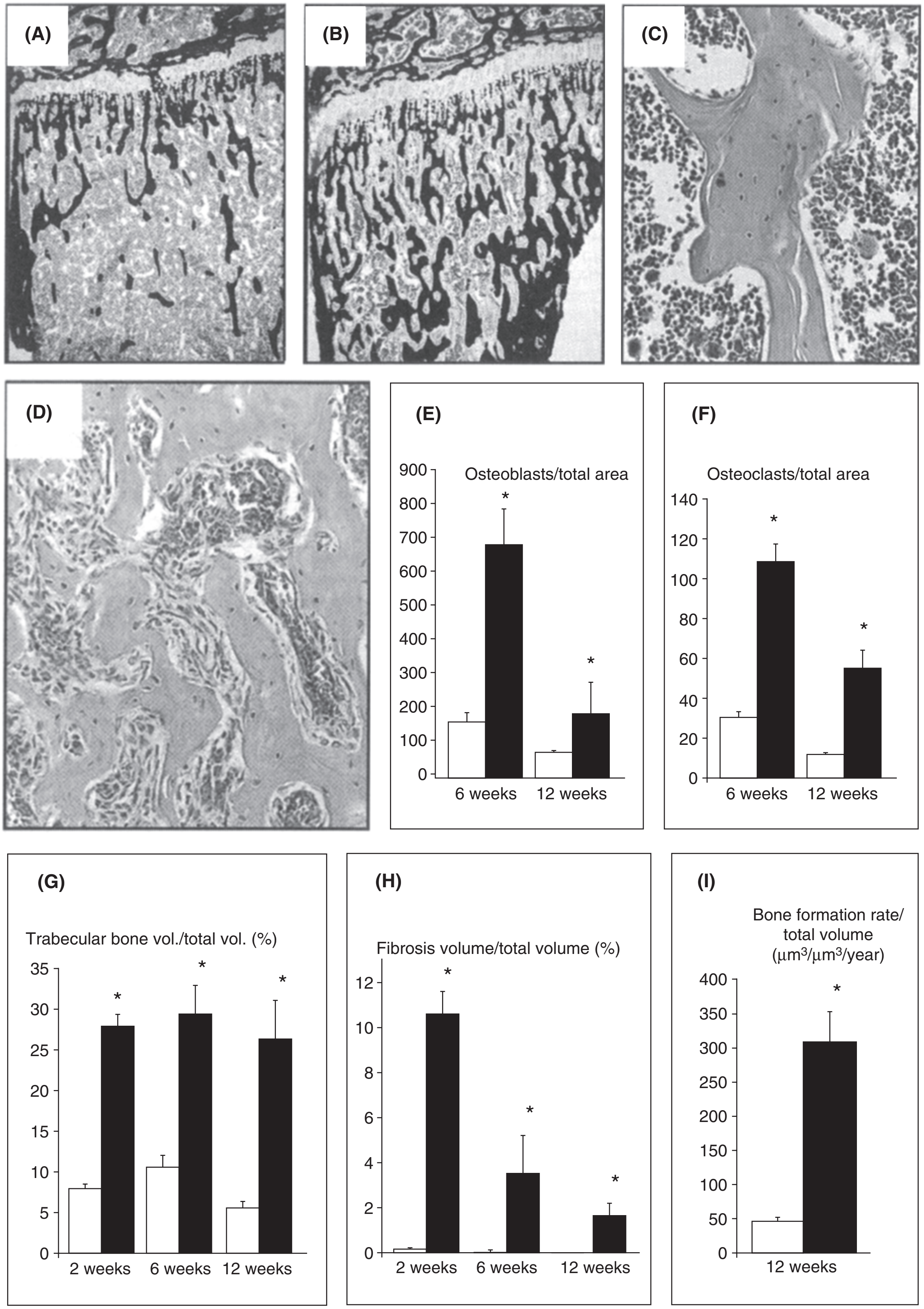

FIGURE 7 Histology and histomorphometric analysis of trabecular bone from wild-type and CL2 transgenic mice. Histologic sections of tibiae, stained by the method of von Kossa, from 12-week-old wild-type mice (**A**) and CL2 transgenic littermates (**B**). High-power light microscopy of decalcified sections, stained with hematoxylin and eosin, of the metaphyseal area from the proximal tibia of 12-week old wild-type (**C**) and CL2 transgenic littermates (**D**). Histomorphometric analysis performed in wild-type (white bars) and CL1 transgenic littermates (black bars) (E–I). Ages of animals are indicated on the *x*-axis; asterisks indicate a statistically significant difference between two groups of mice ($p < 0.05$); error bars represent the SEM. Modified from Calvi *et al*. (2001).

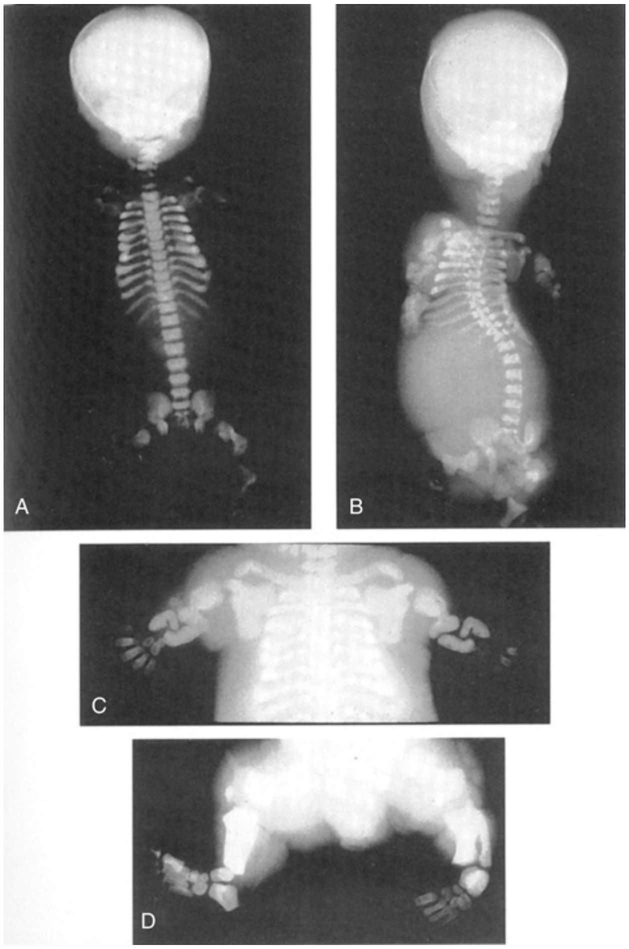

FIGURE 8 Radiological findings in two fetuses with Blomstrand's lethal chondrodysplasia (BLC). Anteroposterior (**A**) and lateral (**B**) views of a male fetus at 26 weeks of gestation; upper (**C**) and lower (**D**) limbs of a female fetus with BLC at 33 weeks of gestation. Particularly striking is the dramatic acceleration of endochondral bone formation of all skeletal elements. No secondary ossification centers of ossification are seen in the long bones. The limbs are coarsely shaped and extremely short, whereas carpal and tarsal bones have a comparatively normal shape and size. Note also that the clavicles are relatively long, but show abnormal bending. From Loshkajian *et al*. (1997), with permission.

BLC are typically born prematurely and die shortly after birth. Birth weight, when corrected for gestational age, appears to be normal, but may be overestimated because most infants are hydroptic; also, the placenta can be immature and edematous. Nasal, mandibular, and facial bones are hypoplastic; the base of the skull is short and narrow; the ears are low set; the thoracic cage is hypoplastic and narrow with short thick ribs and hypoplastic vertebrae. In contrast, the clavicles are relatively long and often abnormally shaped, the limbs are extremely short, and only the hands and feet are of relatively normal size and shape. Internal organs show no apparent structural or histological anomalies, but preductal aortic coarctation was observed in most published cases. The lungs are hypoplastic and the protruding eyes typically show cataracts. Defects in mammary gland and tooth development, previously overlooked, were demonstrated in two recently studied fetuses with BLC. In these fetuses, nipples were absent, and no subcutaneous ductal tissue could be identified by histochemical analysis. Tooth buds were present, but developing teeth were severely impacted within the surrounding alveolar bone, leading to distortions in their architecture and orientation (Wysolmerski *et al.,* 2001).

Radiological studies of patients with BLC reveal pronounced hyperdensity of the entire skeleton and markedly advanced ossification (Fig. 8). As mentioned earlier, the long bones are extremely short and poorly modeled, show markedly increased density, and lack metaphyseal growth plates. Endochondral bone formation is dramatically advanced and is associated with a major reduction in epiphyseal resting cartilage preventing the development of epiphyseal ossification centers (Fig. 9). The zones of chondrocyte proliferation and of column formation are lacking, and the zone that normally comprises the layer of hypertrophic chondrocytes is poorly defined, narrow and irregular (Oostra *et al.,* 2000). Cortical bone is thickened, and bone trabeculae are coarse with reduced diaphyseal marrow spaces. Capillary ingrowth, bone resorption, and bone formation are reported by some authors as being unaltered (Leroy *et al.,* 1996), whereas

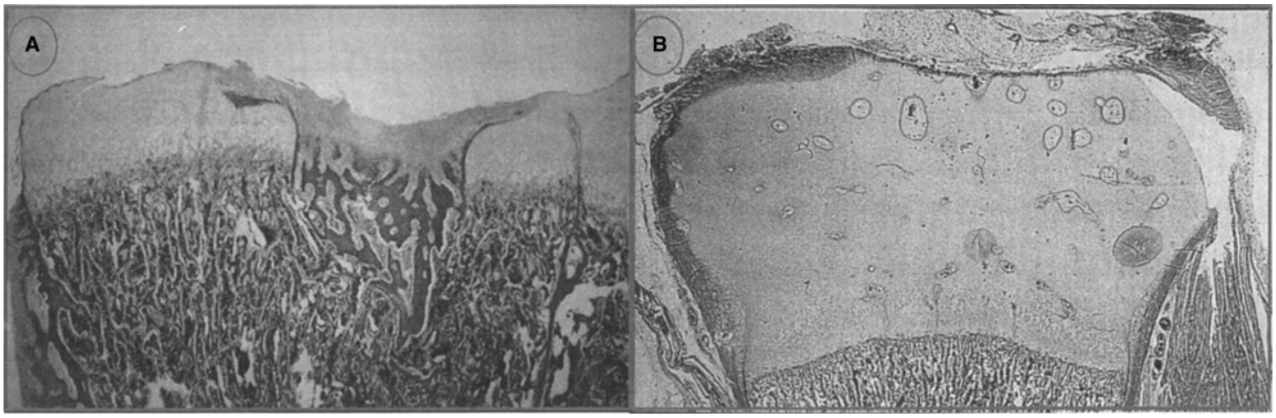

FIGURE 9 Section of the upper tibia end from a patient with BLC (**A**) and an age-matched control. Note the severely reduced size of the growth plate, the irregular boundary between the growth plate and the primary spongiosa, and the increased cortical bone thickness. From Loshkajian *et al*. (1997), with permission, and Anne-Lise Delezoide, personal collection.

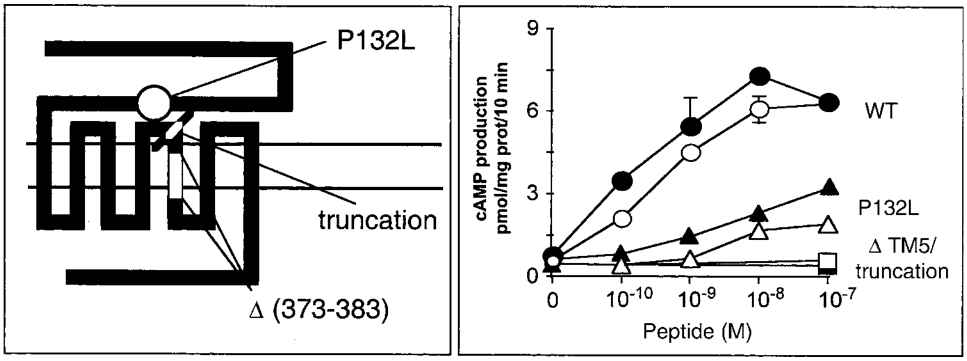

FIGURE 10 Schematic representation of the PTH/PTHrP receptor and functional evaluation of the wild-type and mutant receptors in COS-7 cells. Approximate location of loss-of-function mutation identified in patients with Blomstrand's disease (*left*) and cAMP accumulation in response to PTH (*right*, closed symbols) or PTHrP (open symbols) by wild-type and mutant PTH/PTHrP receptors. Modified from Karperien *et al.* (1999) and Zhang *et al.* (1998).

others describe these bone remodeling events as deficient (Loshkajian *et al.*, 1997).

BLOMSTRAND'S DISEASE IS CAUSED BY INACTIVATING PTH/PTHrP RECEPTOR MUTATIONS

Different defects in the PTH/PTHrP receptor gene have been described in genomic DNA from patients affected by BLC (see Table I). The first reported case, a product of nonconsanguineous parents, was shown to have two distinct abnormalities in the PTH/PTHrP receptor gene (Jobert *et al.*, 1998). Through a nucleotide exchange in exon M5 of the maternal PTH/PTHrP receptor allele, a novel splice acceptor site was introduced that led to a mutant mRNA encoding an abnormal receptor that lacks a portion of the fifth membrane-spanning domain (amino acids 373 to 383; Δ373–383). This receptor mutant fails, despite seemingly normal cell surface expression, to respond to PTH or PTHrP with an accumulation of cAMP (Fig. 10) and inositol phosphate (data not shown). For yet unknown reasons, the paternal PTH/PTHrP receptor allele from this patient is very poorly expressed, suggesting an unidentified mutation in one of the different promoter regions or in a putative enhancer element.

A second patient with BLC, the product of a consanguineous marriage, was shown to have a nucleotide exchange that leads to a proline to leucine mutation at position 132 (P132L; Zhang *et al.*, 1998; Karaplis *et al.*, 1998). This residue in the amino-terminal, extracellular domain of the PTH/PTHrP receptor is invariant in all mammalian members of this family of G protein-coupled receptors, indicating that the identified mutation is likely to have significant functional consequences. Indeed, COS-7 cells expressing this mutant PTH/PTHrP receptor showed, despite apparently normal cell surface expression, dramatically impaired binding of radiolabeled PTH and PTHrP analogues and greatly reduced agonist-stimulated cAMP accumulation (see Fig. 10), and the cells showed no measurable inositol phosphate response. It is important to note, however, that cells expressing the P132L mutant receptor showed some agonist-induced second-messenger response and showed little, but detectable, specific binding of radiolabeled PTHrP. To date the P132L mutation has been identified in two additional patients affected with BLC (Hoogendam *et al.*, 2007). Although not definitive, haplotype analysis performed on the genomic DNA of these patients with the P132L mutation is consistent with an ancient founder effect (Hoogendam *et al.*, 2007).

A homozygous deletion of G at position 1093 (from A of the ATG-translation initiation codon; exon EL2) was identified in a third case of BLC (Karperien *et al.*, 1999). This mutation led to a shift in the open reading frame, which resulted in a truncated protein that completely diverged from the wild-type receptor sequence after amino acid 364, and thus lacked transmembrane domains 5, 6, and 7, the connecting intra- and extracellular loops, and the cytoplasmic tail (Δ365–593). Functional analysis of the Δ365–593 recombinant mutant receptor in COS-7 cells demonstrated a total absence of PTH-stimulated accumulation of intracellular cAMP, which was confirmed in studies performed with the patient's dermal fibroblasts (see Fig. 10).

As for the other cases of BLC, these findings provided a plausible explanation for the severe abnormalities in endochondral bone formation. The abnormalities in mammary gland and tooth development furthermore support the conclusion that the PTH/PTHrP receptor has in humans and mice identical roles in the development of these organs. Compatible with the role of PTH/PTHrP receptor and PTHrP in organogenesis, both were demonstrated to be expressed in the developing breast and tooth of human control fetuses (Wysolmerski *et al.*, 2001). It is also worth noting that abnormalities in skeletal development in the fetuses carrying the P132L mutation, which inactivates the PTH/PTHrP receptor incompletely, are less severe than those observed in most cases, particularly with regard to the bones of the lower limbs (Young *et al.,* 1993; Karperien *et al.,* 1999; Oostra *et al.,* 2000). This led to the proposal that two forms of BLC can be distinguished clinically and on the basis of the *in vitro* characteristics of the mutant PTH/PTHrP receptors (Oostra *et al.,* 2000).

More recently, two additional homozygous mutations in the PTH/PTHrP receptor have been identified in fetuses affected BLC that further document the molecular basis for the two forms of BLC Hoogendam *et al.,* 2007; see Table I). A homozygous point mutation causing a premature stop codon at position 104 (R104X) and therefore resulting in a truncated completely inactive protein has been identified in a case affected with the severe (type I) form. A homozygous nucleotide change (intron M4 + 27C > T) creating a novel splice site has been identified in a case affected with the less severe form (type II). This novel splice site, which results in an aberrant transcript with a premature stop codon, was shown to be preferentially used in dermal fibroblasts, but the wild-type transcript remained expressed, albeit at low levels. Taken together the findings in patients with BLC suggested that this rare human disease is the equivalent of the mouse PTH/PTHrP receptor "knockout" (Lanske *et al.,* 1996).

Inactivating mutations have been described in several other G protein-coupled receptors (reviewed in Spiegel and Weinstein, 2004, and Tao, 2006). For example, genetic forms of growth hormone deficiency were shown to be caused by mutations in the growth hormone-releasing hormone receptor (Wajnrajch *et al.,* 1996; Godfrey *et al.,* 1993), mutations in the thyrotropin receptor are the cause of inherited hypothyroidism (Sunthornthepvarakui *et al.,* 1995), and mutations in the calcium-sensing receptor have been associated with familial hypocalciuric hypercalcemia and neonatal severe primary hyperparathyroidism (reviewed in Jüppner and Thakker, 2007).

PTH/PTHrP RECEPTOR MUTATIONS IN EIKEN FAMILIAL SKELETAL DYSPLASIA AND ENCHONDROMATOSIS (OLLIER'S DISEASE)

In addition to BLC and JMC, PTH/PTHrP receptor gene mutations have been associated to two other diseases, Eiken familial skeletal dysplasia (Duchatelet *et al.,* 2004) and enchondromatosis (Ollier's disease; Hopyan *et al.,* 2002).

Eiken Familial Skeletal Dysplasia

Eiken familial skeletal dysplasia has been described in a single consanguineous family (Eiken *et al.,* 1984). The disease is characterized by multiple epiphyseal dysplasia, with extremely retarded ossification, as well as by abnormal modeling of the bones in hands and feet, abnormal persistence of cartilage in the pelvis, and mild growth retardation. Serum calcium and phosphate levels have been normal in all the examined patients; serum PTH level was measured in only one patient and was found to be slightly elevated with a normal 1,25-$(OH)_2$ vitamin D level. A homozygous mutation in the PTH/PTHrP receptor, R485X, which leads to the truncation of the last 108 amino-acids of the PTH/PTHrP receptor, was identified in all affected patients, but it was not found in DNA from healthy controls (Duchatelet *et al.,* 2004; see Table I). The functional properties of the mutant PTH/PTHrP receptor have not been characterized *in vitro*. However, based on the properties of a receptor mutant with deletion after amino acid 480 (Iida-Klein *et al.,* 1995; Castro *et al.,* 2002), it appears plausible that the truncated receptor has an unbalance between the different signaling pathways that are activated by PTH. Why the deletion of the carboxy-terminal tail of the PTHR1 results in a bone phenotype, but no obvious abnormality in the regulation of mineral ion homeostasis, remains unclear.

Enchondromatosis (Ollier's Disease)

Enchondromatosis is usually a nonfamilial disorder characterized by the presence of multiple enchondromas. It is characterized by an asymmetric distribution of the cartilaginous lesions, which can be extremely variable (in terms of size, number, location, evolution of enchondromas, age of onset and of diagnosis, and requirement for surgery). Clinical problems caused by enchondromas include skeletal deformities, limb-length discrepancy, and the potential risk for malignant change to chondrosarcoma. The condition in which multiple enchondromatosis is associated with soft-tissue hemangiomas is also referred to as Maffucci's syndrome. The irregular distribution of the lesions in Ollier's disease strongly suggests that it is a disorder of endochondral bone formation that occurs due to a postzygotic somatic mutation that results in mosaicism. A mutant PTHR1 (R150C) was found to be expressed in the enchondromas from two of six unrelated patients with enchondromatosis (Hopyan *et al.,* 2002; see Table I). The mutation was found on one parental allele in one patient and his father, who presented with atypical mild skeletal dysplasia, but not with enchondromatosis. Neither the R150C mutation (26 tumors) nor any other mutation in the *PTHR1* gene (11 patients) could be identified in a subsequent study, suggesting heterogeneity of the molecular defect(s) leading to enchondromatosis (Rozeman *et al.,* 2005). Consistent with a role of the PTH/PTHrP receptor in some forms of Ollier's disease, novel heterozygous PTHR1 mutations were recently identified in several additional patients (Couvineau *et al.,* 2008).

The mutant PTHR1 (R150C) seems to constitutively activate the PTHrP-dependent pathway, thus decreasing chondrocyte differentiation, thereby leading to the formation of enchondromas (Hopyan *et al.,* 2002). Consistent with this conclusion, transgenic mice expressing the mutant PTHR1, but not the wild-type receptor, under the control of the collagen type II promoter develop tumors that are similar to those observed in human enchondromatosis. Because regulation of Ihh by PTHrP was found to be lost in these enchondromas, additional transgenic mice were generated that overexpress the Hedgehog (Hh) transcriptional regulator, Gli2. These mice develop ectopic cartilaginous islands similar to those observed in the mice expressing the mutant PTHR1. Thus, Ihh signaling seems to play a crucial role in the formation of enchondromas.

CONCLUSIONS

The findings in PTHrP- and PTH/PTHrP receptor-ablated mice, and in transgenic animals overexpressing PTHrP in the growth plate, predicted that human disorders caused by mutations in either of these two proteins would be associated with severe abnormalities in endochondral bone formation and in the regulation of mineral ion homeostasis. These insights from genetically manipulated animals led to the identification of activating and inactivating PTH/PTHrP receptor mutations in two rare genetic disorders, Jansen's and Blomstrand's disease, respectively. More recently, mutations in the PTH/PTHrP receptor gene were found in two other diseases, namely Eiken familial skeletal dysplasia and Ollier's disease. In addition to resolving the pathogenesis of puzzling human disorders, these naturally occurring PTH/PTHrP receptor mutations have provided important new insights into the importance of this G protein-coupled receptor in mammalian development. The availability of mutant, constitutively active PTH/PTHrP receptors has furthermore provided novel tools to study bone and cartilage development independent of PTH and PTHrP.

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Chapter 67

Diseases Resulting from Defects in the G Protein $G_s\alpha$

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OVERVIEW

$G_s\alpha$ is a ubiquitously expressed heterotrimeric G protein α-subunit that couples cell surface receptors for hormones and other extracellular ligands to adenylyl cyclase and mediates receptor-stimulated intracellular cyclic adenosine monophosphate (cAMP) generation. Upon ligand binding, receptors promote the release of GDP and binding of GTP to $G_s\alpha$, leading to its activation. The "turn-off" mechanism is an intrinsic GTPase activity of $G_s\alpha$ that hydrolyzes bound GTP to GDP. Genetic defects involving $G_s\alpha$ are associated with a wide variety of human disorders, which are summarized in Table I. Heterozygous inactivating $G_s\alpha$ mutations in the germline usually lead to Albright hereditary osteodystrophy (AHO) and, less often, to progressive osseous heteroplasia (POH). Paternal transmission of these mutations leads to the AHO phenotype alone (pseudopseudohypoparathyroidism, PPHP), whereas maternal transmission leads to AHO plus obesity and multihormone resistance (pseudohypoparathyroidism type IA, PHPIA). The clinical differences between PHPIA and PPHP result from the fact that $G_s\alpha$ is imprinted in a tissue-specific manner, being primarily expressed from the maternal allele in specific hormone target tissues. $G_s\alpha$ is encoded by a complex imprinted gene (*GNAS*), which encodes other gene products other than $G_s\alpha$. Patients with isolated renal PTH resistance without AHO (pseudohypoparathyroidism type IB, PHPIB) have a *GNAS*-imprinting defect that presumably leads to tissue-specific $G_s\alpha$ deficiency. Endocrine tumors, fibrous dysplasia of bone (FD), and the McCune–Albright syndrome (MAS) result from somatic $G_s\alpha$ mutations that are constitutively activating because of loss of GTPase activity.

$G_s\alpha$ STRUCTURE AND FUNCTION

Like all heterotrimeric G proteins, G_s is composed of a specific α-subunit ($G_s\alpha$) associated with tightly but noncovalently bound $\beta\gamma$ dimers. These heterotrimeric complexes reside in the inner leaflet of the plasma membrane as well as in intracellular membranes. $G_s\alpha$ is ubiquitously expressed and couples a wide variety of seven-transmembrane receptors, including those for glycoprotein and peptide hormones, biogenic amines, and neurotransmitters, to the stimulation of adenylyl cyclase and generation of the intracellular second messenger cAMP (Weinstein *et al.*, 2001, 2004). cAMP mediates many of its downstream effects by activating cAMP-dependent protein kinase (protein kinase A [PKA]), a serine/threonine protein kinase that phosphorylates many substrates, including enzymes involved in intermediary metabolism, and transcription factors such as the cAMP response element binding protein CREB (Montminy, 1997). cAMP also binds to and modulates the activity of other molecules (Richards, 2001), including ion channels (Sudlow *et al.*, 1993; Wainger *et al.*, 2001) and guanine nucleotide exchange factors for the small guanine nucleotide-binding protein Rap1 (cAMP-GEFs) (de Rooij *et al.*, 1998; Kawasaki *et al.*, 1998). Whereas in many tissues cAMP is antimitogenic, in many neuroendocrine cell types it is mitogenic through activation of Rap1, which stimulates various mitogenic pathways

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TABLE I Human Diseases Associated with GNAS Gene Defects

GNAS gene defect	Disease
$G_s\alpha$ null mutations (maternal)	PHPIA (AHO + multihormone resistance and obesity PHPIA + POH)
$G_s\alpha$ null mutations (paternal)	PPHP (AHO alone) POH
$G_s\alpha$ A366S mutation (maternal)	PHPIA + testotoxicosis
Loss of exon 1A maternal imprinting (sporadic or associated with *STX16* or NESP55 deletion) Paternal UPD of chrom 20q $G_s\alpha$ △I382 mutation (maternal)	PHPIB
$G_s\alpha$ activating mutations (somatic)	GH-secreting pituitary tumors Thyroid adenomas, carcinomas Leydig cell tumors Pheochromocytoma Parathyroid adenoma ACTH-secreting pituitary tumor McCune–Albright syndrome Fibrous dysplasia Skeletal muscle myxomas Premature thelarche

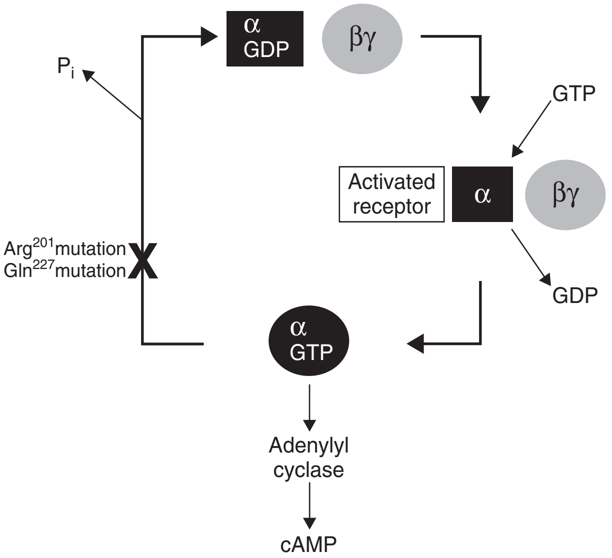

FIGURE 1 The Gs GTPase cycle. In the basal state GDP-bound $G_s\alpha$ associates with $\beta\gamma$ to form a heterotrimer. Activated (ligand-bound) receptors promote the exchange of GTP for GDP on $G_s\alpha$, which allows $G_s\alpha$ to attain an active conformation and dissociate from $\beta\gamma$. GTP-bound $G_s\alpha$ directly activates adenylyl cyclase to generate cAMP. $G_s\alpha$ is deactivated through an intrinsic GTPase activity that hydrolyzes bound GTP to GDP, allowing $G_s\alpha$ to reassociate with $\beta\gamma$. Mutation of Arg^{201} or Gln^{227} leads to constitutive activation by disruption of the GTPase activity.

including B-raf/mitogen-activated protein kinase, p70S6 kinase, and Akt (Cass and Meinkoth, 1998; Mei *et al.*, 2002; Miller, M. J. *et al.*, 1997; Vossler *et al.*, 1997; Wang *et al.*, 2006).

$G_s\alpha$ may also be activated by tyrosine kinase (growth factor) receptors through tyrosine phosphorylation (Krieger-Brauer *et al.*, 2000; Poppleton *et al.*, 1996; Sun *et al.*, 1997) and may activate other effectors, including cardiac Ca^{2+} channels (Mattera *et al.*, 1989; Yatani *et al.*, 1988) and Src tyrosine kinases (Ma *et al.*, 2000). In addition to its role as a signal transducer at the plasma membrane, $G_s\alpha$ may also play a role in the control of intracellular membrane and vesicle trafficking (Bomsel and Mostov, 1992; Zheng *et al.*, 2001).

Like all G proteins, G_s undergoes activation and deactivation through a "GTPase" cycle (Fig. 1). In the inactive state GDP is bound to $G_s\alpha$, which is localized to the inner leaflet of the plasma membrane associated with $\beta\gamma$. Ligand-bound receptors promote GDP release and binding of ambient GTP, allowing $G_s\alpha$ to attain an active conformation and to dissociate from $\beta\gamma$. Dissociation from $\beta\gamma$ as well as depalmitoylation results in $G_s\alpha$ being released from the plasma membrane upon activation (Allen *et al.*, 2005; Huang *et al.*, 1999; Wedegaertner *et al.*, 1996; Yu, J. Z. and Rasenick, 2002). GTP-bound $G_s\alpha$ directly activates its effectors including adenylyl cyclase. The turn-off mechanism is an intrinsic GTPase activity that hydrolyzes bound GTP to GDP, allowing $G_s\alpha$ to reassociate with $\beta\gamma$. An RGS (regulator of G protein signaling) protein that stimulates the GTPase activity of $G_s\alpha$ has been recently identified (Zheng *et al.*, 2001). Crystal structures have identified two highly conserved residues (Arg201 and Gln227 in the long form of $G_s\alpha$) that are critical for catalyzing the GTPase reaction (Coleman *et al.*, 1994; Graziano and Gilman, 1989; Landis *et al.*, 1989; Sondek *et al.*, 1994), and mutation or post-translational modifications of these residues leads to constitutive activation and human disease (see below).

Like all $G\alpha$'s, $G_s\alpha$ contains two structural domains, a Ras-like GTPase domain, which includes the sites for guanine nucleotide binding and effector interaction, and a helical domain (Sunahara *et al.*, 1997; Tesmer *et al.*, 1997). Alternative splicing leads to two long and two short forms of $G_s\alpha$ with helical domains of slightly differing length (Bray *et al.*, 1986; Kozasa *et al.*, 1988), which have only subtle biochemical differences between them (Jones *et al.*, 1990; Seifert *et al.*, 1998). Contacts between the helical and GTPase domains may be important for maintaining the binding of guanine nucleotide, which sits within a cleft between the two domains. (Mixon *et al.*, 1995; Warner *et al.*, 1998). GTP binding leads to an active conformation by altering the positions of three regions (named switches

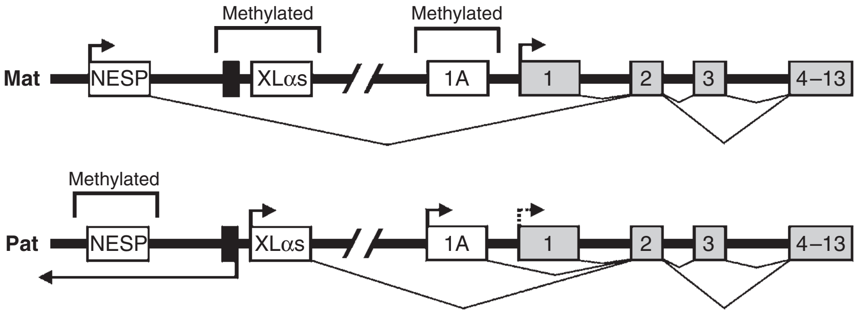

FIGURE 2 Organization and imprinting of *GNAS*. The maternal allele is shown above and paternal allele below (not to scale) indicating regions of DNA methylation, active promoters (arrows), and exon splicing. $G_s\alpha$ coding exons are depicted as gray boxes with exons 4–13 shown as a single box. Three other alternative first exons are shown as white boxes and the first exon of the antisense transcript is shown as a black box. The striped arrow for the paternal $G_s\alpha$ promoter indicates that the promoter is silenced in a tissue-specific manner. Long and short forms of $G_s\alpha$ result from alternative splicing of exon 3. (Reproduced from Weinstein (2004) "Molecular Basis of Inborn Errors of Development," Oxford University Press).

1, 2, and 3) within the GTPase domain (Lambright *et al.*, 1994; Noel *et al.*, 1993). In particular, switches 2 and 3 move toward each other and the active conformation is stabilized through mutual interactions between acidic and basic residues within these regions (Iiri *et al.*, 1997; Li and Cerione, 1997; Warner *et al.*, 1999). Interactions between switch 3 and helical domain residues may also be important for receptor-mediated activation (Grishina and Berlot, 1998; Marsh *et al.*, 1998; Warner *et al.*, 1998; Warner and Weinstein, 1999). The carboxyl terminus is the major site of interaction with receptors (Conklin *et al.*, 1996; Grishina and Berlot, 2000; Mazzoni *et al.*, 2000; Schwindinger *et al.*, 1994; Simonds *et al.*, 1989; Sullivan *et al.*, 1987).

THE $G_s\alpha$ GENE *GNAS*

GNAS, the gene encoding $G_s\alpha$, is located at 20q13.2–13.3 (Gejman *et al.*, 1991; Levine *et al.*, 1991; Rao, V.V. *et al.*, 1991). *GNAS* generates several gene products in addition to $G_s\alpha$ through the use of alternative promoters and first exons that splice onto a common exon (exon 2) (Fig. 2). In addition, *GNAS* is affected by genomic imprinting, an epigenetic phenomenon that leads to partial or complete suppression of gene expression from one parental allele (Reik and Walter, 2001). Allele-specific differences in gene expression are most likely the result allele-specific differences in DNA methylation, which are observed in all imprinted genes. Primary imprinting centers are regions of differential methylation in which DNA methylation is erased in primordial germ cells, reestablished in either male or female gametes, and maintained in all tissues throughout development. Differential gene expression may result from differential methylation of gene promoters (with the silenced allele methylated) or may result from more indirect mechanisms.

$G_s\alpha$ mRNA transcripts are generated from the most downstream *GNAS* promoter and span 13 exons (Kozasa *et al.*, 1988). Biologically active long and short forms of $G_s\alpha$ are generated by alternative splicing of exon 3, which encodes 15 amino acids within the helical domain (Bray *et al.*, 1986; Kozasa *et al.*, 1988). $G_s\alpha$ is imprinted in a tissue-specific manner, being primarily from the maternal allele in a few tissues (e.g., the pituitary, thyroid, and renal proximal tubules) and biallelically expressed in most tissues (Campbell *et al.*, 1994; Davies and Hughes, 1993; Germain-Lee *et al.*, 2002; Hayward *et al.*, 2001; Liu *et al.*, 2003; Mantovani *et al.*, 2002; Weinstein *et al.*, 2001; Yu, S. *et al.*, 1998). This imprinting is not associated with methylation of its promoter (Hayward *et al.*, 1998a; Kozasa *et al.*, 1988; Liu *et al.*, 2000b; Peters *et al.*, 1999). Rather, $G_s\alpha$ imprinting is controlled by a primary imprinting center located ~2 kb upstream of its promoter, which is methylated on the maternal allele (Liu *et al.*, 2000b, 2005a; Williamson *et al.*, 2004). This region contains a promoter and first exon (exon 1A or A/B) that splices to exon 2 to generate untranslated mRNA transcripts from the paternal allele (see Fig. 67.2) (Ishikawa *et al.*, 1990; Liu *et al.*, 2000a, 2000b). Maternal imprinting (methylation) of the exon 1A region is absent in PHPIB patients, as discussed in greater detail later.

Alternative promoters and first exons located 46 and 35 kb upstream of the $G_s\alpha$ promoter generate transcripts that encode the chromogranin-like protein NESP55 and the alternative $G_s\alpha$ isoform XLαs, respectively (Hayward *et al.*, 1998a, 1998b; Kelsey *et al.*, 1999; Peters *et al.*, 1999) (see Fig. 67.2). The NESP55 and XLαs promoter regions are oppositely imprinted: NESP55 is only expressed from the maternal allele and its promoter is methylated on the paternal allele, whereas XLαs is only expressed from the paternal allele and its promoter is methylated on the maternal allele. Both NESP55 and XLαs are expressed primarily in neuroendocrine tissues. NESP55 is unrelated to the Gα family and its coding sequence is restricted to its specific upstream exon. Its biological function is unknown and studies in mouse and humans suggest that loss of its expression leads to minimal phenotypic consequences (Liu *et al.*, 2000a;

Plagge *et al.*, 2005). Imprinting of NESP55 is not established until after implantation and depends on a primary imprinting center located within the promoter region of a paternally expressed antisense transcript that traverses the NESP55 promoter from the opposite direction (Liu *et al.*, 2000b; Williamson *et al.*, 2006). XLαs is a $G_s\alpha$ isoform in which the first 47 amino acids of $G_s\alpha$ are replaced with a long amino-terminal extension encoded by its specific first exon (Kehlenbach *et al.*, 1994; Pasolli *et al.*, 2000). XLαs is capable of signal transduction and activation of adenylyl cyclase (Bastepe *et al.*, 2002; Klemke *et al.*, 2000). Although XLαs is critical for postnatal feeding and metabolic regulation in mice (Plagge *et al.*, 2004; Xie *et al.*, 2006), its role in humans is less clear.

ALBRIGHT HEREDITARY OSTEODYSTROPHY (AHO)

Clinical Presentation

Patients with AHO may present with one or more of the following clinical features: short stature, round face, broad body habitus, brachydactyly, subcutaneous ossifications, depressed nasal bridge, hypertelorism, and mental deficits or developmental delay (Fig. 3) (Ringel *et al.*, 1996; Spiegel and Weinstein, 2001; Weinstein, 1998; Weinstein *et al.*, 2001). None of these features are completely specific for this disorder, but the presence of subcutaneous ossifications is a fairly specific sign for AHO. The severity of the phenotype varies greatly, and some patients who carry the genetic trait present with few or no clinical manifestations (Farfel and Friedman, 1986; Miric *et al.*, 1993; Shore *et al.*, 2002; Weinstein *et al.*, 1990). Although obesity has in the past been considered a general feature of AHO, a recent study shows that it is selectively present only in PHPIA patients, as discussed later (Long *et al.*, 2007).

Ectopic ossifications in AHO (osteoma or calcinosis cutis) are often one of the earliest features of the disease (Gelfand *et al.*, 2006; Poomthavorn and Zacharin, 2006) and can occur in any location. They present as punctate hard nodules or as subcutaneous calcifications on radiographs located within the dermis and subcutaneous tissue (Eyre and Reed, 1971; Goeteyn *et al.*, 1999; Prendiville *et al.*, 1992; Sethuraman *et al.*, 2006) which occasionally enlarge to form more plate-like lesions but do not invade into deeper tissues. These lesions result from intramembranous, as opposed

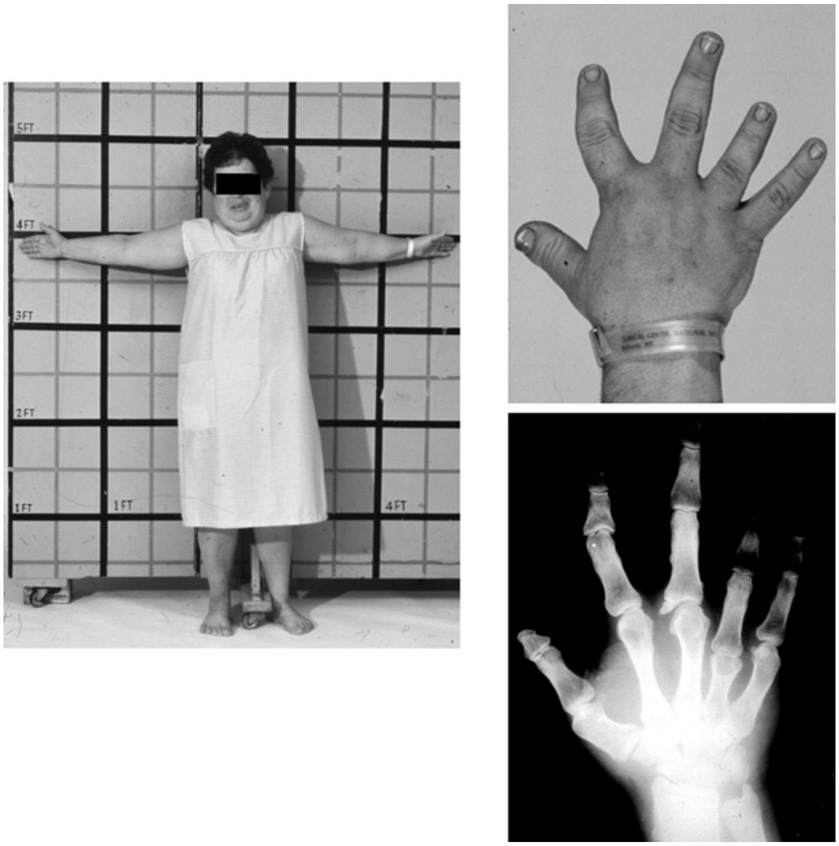

FIGURE 3 Albright hereditary osteodystrophy (AHO). (*Left*) AHO patient with short stature, obesity, and rounded face with depressed nasal bridge. (*Right*) Photograph (above) and radiograph (below) of hand with severe brachydactyly of the first, fourth, and fifth metacarpals. (Reproduced from Weinstein (2004) “Molecular Basis of Inborn Errors of Development,” Oxford University Press).

to endochondral, ossification, and may be surrounded by inflammation (Riepe *et al.*, 2005). These lesions result from a primary cellular defect, rather than a defect in mineral metabolism, because they are absent in patients with primary hypoparathyroidism and are present in PPHP patients, who are normocalcemic and have no evidence for parathyroid hormone (PTH) resistance.

Brachydactyly refers to shortening and widening of long bones in the hands and feet. Patients with AHO have a relatively specific pattern, which most often involves the distal thumb and third, fourth, and fifth metacarpals and is often asymmetric (de Sanctis, L., *et al.*, 2004; Graudal *et al.*, 1986; Poznanski *et al.*, 1977; Steinbach and Young, 1966). Brachydactyly is generally not observed in early childhood but becomes apparent later in the first decade of life because of premature growth plate closure with coning of the epiphysis (Gelfand *et al.*, 2006). Spinal cord compression occurs rarely, either from calcifications of longitudinal spinal ligaments (Chen, H. *et al.*, 2005a; Mak and Mok, 2005; Yamamoto, Y. *et al.*, 1997) or spinal canal narrowing (Goadsby *et al.*, 1991; Okada *et al.*, 1994).

Neurobehavioral abnormalities are also common in AHO patients, occurring in up to 50% of cases (Farfel and Friedman, 1986). There is no distinct neurocognitive syndrome associated with AHO, although psychomotor delay and mental retardation are relatively common. Psychiatric conditions and juvenile dementia have also been described in AHO patients (Maeda *et al.*, 2005). In rare cases, basal ganglia calcification resulting from the hypoparathyroid biochemical state may lead to Parkinsonian symptoms.

In addition to the physical features described earlier, AHO patients who inherit the disease from their mother (or have a *de novo* $G_s\alpha$ mutation on the maternal allele) also develop obesity and multihormone resistance, a condition known as PHPIA (Davies and Hughes, 1993; Long *et al.*, 2007; Weinstein *et al.*, 2001). In patients who inherit AHO from their father (or have a *de novo* mutation on the paternal allele) obesity and multihormone resistance is absent. This condition, in which patients present with only AHO, is also known as PPHP. Both PHPIA and PPHP often occur in the same kindred with the presentation of each case determined by parental inheritance. The multihormone resistance in PHPIA primarily involves PTH, thyroid-stimulating hormone (TSH), growth hormone-releasing hormone (GHRH), and the gonadotropins, all of which activate G_s pathways in their target tissues. There is no resistance, at least based on clinical parameters, to other hormones that also activate G_s, such as glucagon (Brickman *et al.*, 1986; Levine *et al.*, 1983), adrenocorticotropic hormone (ACTH) (Faull *et al.*, 1991; Levine *et al.*, 1983), isoproterenol (Carlson and Brickman, 1983), and vasopressin (Faull *et al.*, 1991; Moses *et al.*, 1986).

PHPIA patients develop renal PTH resistance during early childhood but may initially present with acute symptoms of hypocalcemia (seizures, tetany, numbness) at any time of life, often during early adolescence. In general, there is no evidence for PTH resistance at birth or even in the first year of life, but soon thereafter PTH levels rise followed by the development of hyperphosphatemia and finally hypocalcemia (Barr *et al.*, 1994; Gelfand *et al.*, 2006; Riepe *et al.*, 2005; Tsang *et al.*, 1984; Werder *et al.*, 1978; Yu, D. *et al.*, 1999). Over time patients develop chronic features of biochemical hypoparathyroidism, including basal ganglia calcifications, cataracts, and calcifications at other sites, and on rare occasions may develop rickets. Some PHPIA patients have elevated PTH levels but remain eucalcemic (Balachandar *et al.*, 1975; Breslau *et al.*, 1980; Drezner and Haussler, 1979). The PTH signaling defect is primarily localized to the renal proximal tubule, and this results in hyperphosphatemia owing to increased phosphate reabsorption and low or inappropriate normal serum 1,25 dihydroxyvitamin D [$1,25(OH)_2D$] levels (Braun *et al.*, 1981; Breslau and Weinstock, 1988; Lambert *et al.*, 1980; Miura *et al.*, 1990). Because the defect is limited to the proximal tubules, the ability of the distal tubules to reabsorb calcium in response to PTH remains intact, and therefore, PHP patients do not tend to develop hypercalciuria upon treatment as do patients with primary hypoparathyroidism. Consistent with the presence of a G_s defect, the acute PTH-stimulated urinary cAMP response is markedly blunted in PHPIA patients with PHPIA, whereas it is normal in PPHP patients (Chase *et al.*, 1969; Levine *et al.*, 1986). Although one bone sample from a PHPIA patient showed no defect in PTH-$G_s\alpha$ signaling (Ish-Shalom *et al.*, 1996), the occurrence of secondary skeletal effects of chronically elevated PTH is lower in PHPIA than in PHPIB, perhaps because of partial $G_s\alpha$ deficiency in osteoblasts from these patients.

TSH resistance is present in virtually all PHPIA patients and is often the first observed manifestation as TSH levels are generally elevated in neonatal screening (Gelfand *et al.*, 2006; Levine *et al.*, 1985; Weisman *et al.*, 1985; Yokoro *et al.*, 1990; Yu, D. *et al.*, 1999). TSH levels may normalize for several months before once again becoming elevated (Yu, D. *et al.*, 1999). Once TSH resistance is established, TSH levels are mildly or moderately elevated and thyroid hormone levels are normal or slightly low. Because the defect is at the level of TSH signaling, which also stimulates thyroid growth, goiter is usually absent. Antithyroid antibodies are usually absent.

Female PHPIA patients often present with signs of clinical hypogonadism, including delayed or incomplete sexual maturation, oligomenorrhea, or infertility, associated with mildly low estrogen levels (de Sanctis, C. *et al.*, 2003; Levine *et al.*, 1983; Namnoum *et al.*, 1998; Shapiro *et al.*, 1980; Shima *et al.*, 1988; Wolfsdorf *et al.*, 1978). Although it is assumed that these patients are resistant to gonadotropins, studies have not consistently demonstrated that they have elevated gonadotropin levels. It has been proposed that PHPIA patients may have a more subtle form of gonadotropin resistance that leads to an ovulatory defect owing to a poor ovulatory response to the midcycle luteinizing hormone surge (Namnoum *et al.*, 1998). Some PHPIA patients also have prolactin deficiency (Brickman *et al.*, 1981; Carlson *et al.*, 1977; Faull *et al.*, 1991; Kruse *et al.*, 1981; Levine *et al.*, 1983; Schuster *et al.*, 1993).

Many, although not all, PHPIA patients have growth hormone (GH) deficiency, presumably secondary to pituitary resistance to GHRH (de Sanctis, L. *et al.*, 2007; Faull *et al.*, 1991; Germain-Lee *et al.*, 2003; Mantovani *et al.*, 2003; Shima *et al.*, 1988). However, short stature in PHPIA does not correlate with GH deficiency and appears to result from a primary skeletal growth plate defect leading to premature maturation and closure. In fact, prior to puberty, PHPIA patients often are tall for their biological age and have advanced bone ages. Olfactory defects have also been described in PHPIA (but not PPHP) patients, although it is unclear whether this reflects a true sensory defect or a central neurological defect (Doty *et al.*, 1997; Henkin, 1968; Ikeda *et al.*, 1988; Weinstock *et al.*, 1986).

Molecular Genetics

AHO was initially shown to be associated with a G_s signaling defect by biochemical and cellular assays performed on membrane samples from various tissues. (Bourne *et al.*, 1981; Downs *et al.*, 1983; Farfel and Bourne, 1980; Farfel *et al.*, 1980, 1981, 1982; Heinsimer *et al.*, 1984; Levine *et al.*, 1980, 1986; Mallet *et al.*, 1982; Ong *et al.*, 1996; Spiegel *et al.*, 1982). In these assays G_s signaling activity was similarly reduced by ~50% in tissues from both PHPIA and PPHP patients, suggesting that these patients have similar genetic G_s defects resulting from a genetic defect in one parental allele. Consistent with these findings, these patients were shown to have similar reductions in $G_s\alpha$ mRNA (Carter *et al.*, 1987; Levine *et al.*, 1988) and protein (Patten and Levine, 1990), and to have heterozygous $G_s\alpha$ loss-of-function mutations (Ahmed *et al.*, 1998; Ahrens *et al.*, 2001; Aldred *et al.*, 2000; Aldred and Trembath, 2000; de Sanctis, C. *et al.*, 2003; Linglart *et al.*, 2002; Miric *et al.*, 1993; Oude Luttikhuis *et al.*, 1994; Patten *et al.*, 1990; Rickard and Wilson, 2003; Shapira *et al.*, 1996; Spiegel and Weinstein, 2001; Thiele *et al.*, 2007; Weinstein *et al.*, 1990; Weinstein, 2004). All AHO patients within the same kindred (both those with PHPIA and PPHP) have identical mutations, confirming that AHO is an autosomal dominant disorder (Fischer *et al.*, 1998; Mantovani *et al.*, 2000; Nakamoto *et al.*, 1998; Walden *et al.*, 1999; Weinstein *et al.*, 1990; Wilson *et al.*, 1994; Yu, D. *et al.*, 1999). Whether an individual has PHPIA or PPHP is determined by parental inheritance, as discussed in the section on pathogenesis.

$G_s\alpha$ mutations in AHO are spread throughout the coding exons and in the majority of cases are unique to each kindred, although some identical mutations or mutations affecting the same amino acid have been identified in more than one kindred (Ahmed *et al.*, 1998; Ahrens *et al.*, 2001; Aldred and Trembath, 2000; Farfel *et al.*, 1996; Iiri *et al.*, 1994; Miric *et al.*, 1993). One 4-bp deletion in exon 7 has been identified in many families (Ahrens *et al.*, 2001; Aldred and Trembath, 2000; de Sanctis, C. *et al.*, 2003; Linglart *et al.*, 2002; Mantovani *et al.*, 2000; Shore *et al.*, 2002; Yu, S. *et al.*, 1995) as this site appears prone to *de novo* mutation owing to pausing of DNA polymerase and slipped strand mispairing (Yu, S. *et al.*, 1995). In most cases, there is no genotype–phenotype correlation as the mutations completely disrupt $G_s\alpha$ mRNA expression (frame shift, nonsense, or splice junction mutations) or $G_s\alpha$ protein stability (Warner *et al.*, 1997, 1998, 1999).

Several missense mutations have specific effects on $G_s\alpha$ function. For example, mutations at the carboxyl terminus uncouple $G_s\alpha$ from receptors (Linglart *et al.*, 2002, 2006; Schwindinger *et al.*, 1994; Wu, W.I. *et al.*, 2001). Mutation of the either Arg^{231} within the switch 2 region or Glu^{259} within the switch 3 region lead to a receptor activation defect by destabilizing interactions between the two switch regions that are necessary to maintain the active conformation (Iiri *et al.*, 1997; Warner *et al.*, 1999). The $Arg^{258}Trp$ mutation results in decreased GDP binding in the basal state as well as a receptor-activation defect owing to increased GTPase activity (Warner *et al.*, 1998; Warner and Weinstein, 1999). The $Ala^{366}Ser$ mutation results in PHPIA plus male gonadotropin-independent precocious puberty (testotoxicosis) (Iiri *et al.*, 1994). This mutation leads to increased GDP release in a temperature-sensitive manner. At core body temperature, the mutant protein has thermolability owing to an inability to maintain GDP binding, which results in PHPIA. At the lower testicular temperature, the GDP-binding defect is more subtle, allowing the protein to remain stable. However, testotoxicosis results from constitutive activation of $G_s\alpha$ owing to increased GDP/GTP exchange in the absence of receptor stimulation. but resulting in constitutive activation owing to GDP/GTP exchange independent of activation by receptor.

Diagnosis and Management

Although PTH stimulation of urinary cAMP (Ellsworth–Howard test) is the classical test for confirming the presence of PTH resistance (Chase *et al.*, 1969), the PTH analogue is no longer clinically available and with modern PTH assays the diagnosis can usually be made based on baseline hormonal measures once nutritional vitamin D deficiency and renal dysfunction are ruled out. Patients with a blunted urinary cAMP response to PTH are classified as having PHPI. PHPI is further subclassified as PHPIA or PHPIB based on the presence or absence of AHO and multihormone resistance, respectively. Rarely, patients have the PHPIA phenotype without evidence for a $G_s\alpha$ defect, and are classified as PHPIC. Patients who have a normal urinary cAMP response but a defective phosphaturic response to PTH are classified as PHP type II (Rao, D.S. *et al.*, 1985).

Because many of the features of AHO (brachydactyly, obesity, neurobehavioral abnormalities) are nonspecific, the diagnosis should not be made based on the presence of these features alone, especially in the absence of family history, multihormone resistance, or subcutaneous ossifications, which is a more specific feature of AHO. These features are also present in other known genetic syndromes (e.g., Prader–Willi, brachydactyly syndromes, Turner's syndrome, Rubinstein–Taybi syndrome) and have also been found in association with chromosomal deletion of 2q37 (Phelan *et al.*, 1995; Wilson *et al.*, 1995). Therefore, in order to confirm the diagnosis of PHPIA or PPHP, one must confirm the presence of a $G_s\alpha$ defect by biochemical or genetic studies.

The most direct way to confirm the presence of a $G_s\alpha$ defect is by *GNAS* mutation, which is commercially available but only has a sensitivity of ~70%. Alternatively one can assay for G_s signaling activity in erythrocyte membrane sample, but this is only available in a few research laboratories and is more technically difficult. In at least some cases patients have been diagnosed as having PHPIC because a nonhydrolyzable GTP analogue was used to stimulate G_s in the biochemical assay. Because this bypasses the necessity for receptor stimulation, it will give a normal result even when the mutation produces a specific defect in receptor-stimulated signaling (Iiri *et al.*, 1997; Linglart *et al.*, 2002, 2006). For this reason, biochemical assays should be performed using a receptor ligand (e.g., isoproterenol) so that these $G_s\alpha$ biochemical defects are not missed.

There is no specific therapy for the physical and neurocognitive manifestations of AHO. The subcutaneous ossifications rarely require surgical excision to relieve discomfort or disfigurement. PTH resistance in PHPIA (and PHPIB) patients should be aggressively managed with oral calcium and vitamin D (either 50 to 100,000 IU/day of ergocalciferol or cholecalciferol or 0.5 to 2 μg/day of calcitriol). The goal is to try to normalize calcium and PTH, if possible, in order to prevent the skeletal effects of chronically elevated PTH and the development of autonomous (tertiary) hyperparathyroidism. Treatment should be initiated when patients develop high PTH levels even if normocalcemic. If autonomous parathyroid tumors develop, surgical excision may be required to reverse hypercalcemia, but management of the underlying PTH resistance will still be required postoperatively. PHP patients generally do not develop hypercalciuria upon treatment because the PTH defect is localized to the renal proximal tubule and the distal tubules can still increase calcium reabsorption in response to circulating PTH (Mizunashi *et al.*, 1990; Stone *et al.*, 1993). However, patients still need to be periodically monitored for hypercalciuria while on therapy.

Levothyroxine should be prescribed to normalize TSH levels and hypogonadism can be managed with oral contraceptives in females and testosterone in males. GH testing should be performed in children with PHPIA and GH therapy may be instituted if GH deficiency is diagnosed (de Sanctis, L. *et al.*, 2007; Germain-Lee *et al.*, 2003; Mantovani *et al.*, 2003). The more general use of GH in AHO patients to maximize height is under investigation.

Pathogenesis

The first clue that *GNAS* imprinting may be important for the clinical variability of AHO (PHPIA vs. PPHP) came from that observation that maternal inheritance produces offspring with PHPIA whereas paternal inheritance produces offspring with PPHP (Davies and Hughes, 1993; Nakamoto *et al.*, 1998; Wilson *et al.*, 1994). Molecular studies in mice and humans have confirmed that $G_s\alpha$ is imprinted in a tissue-specific manner, being expressed primarily from the maternal allele in pituitary, thyroid, gonads, and renal proximal tubules (Germain-Lee *et al.*, 2002; Hayward *et al.*, 2001; Liu *et al.*, 2003; Mantovani *et al.*, 2002; Yu, S. *et al.*, 1998). In these tissues mutation of the active maternal allele will result in $G_s\alpha$ deficiency and impaired hormone signaling, whereas mutation of the inactive paternal allele would have little effect on $G_s\alpha$ expression or hormone action (Fig. 4). Therefore, $G_s\alpha$ imprinting in renal proximal tubules would account for the fact that PTH-stimulated urinary cAMP is markedly reduced in PHPIA but is normal in PPHP (Chase *et al.*, 1969; Levine *et al.*, 1986). In most other nonendocrine tissues $G_s\alpha$ is biallelically expressed (Campbell *et al.*, 1994; Hayward *et al.*, 1998a, 1998b), which accounts for a similar ~50% reduction in $G_s\alpha$ expression observed in erythrocytes and other tissues in both PHPIA and PPHP patients (Levine *et al.*, 1986).

In renal proximal tubules PTH normally stimulates G_s/cAMP signaling pathways, which leads to decreased phosphate reabsorption and increased $1{,}25(OH)_2D$ production by induction of the 1α-hydroxylase gene. Therefore $G_s\alpha$ deficiency in PHPIA (and PHPIB) leads to decreased $1{,}25(OH)_2D$ production and hyperphosphatemia owing to low urinary phosphate excretion. Hyperphosphatemia also acts to inhibit $1{,}25(OH)_2D$ production. Hypocalcemia results from the combined effects of hyperphosphatemia and low $1{,}25(OH)_2D$ levels, because the latter results in decreased intestinal calcium absorption and skeletal calcium mobilization (Drezner *et al.*, 1976; Drezner and Haussler, 1979; Epstein *et al.*, 1983). Hypocalcemia, hyperphosphatemia, and low $1{,}25(OH)_2D$ levels all contribute to secondary hyperparathyroidism. Likewise, hypothyroidism, hypogonadism, and GH deficiency in PHPIA likely results from resistance to TSH, gonadotropins, and GHRH in their respective target tissues (thyroid, gonads, pituitary). (Germain-Lee *et al.*, 2003; Mallet *et al.*, 1982;

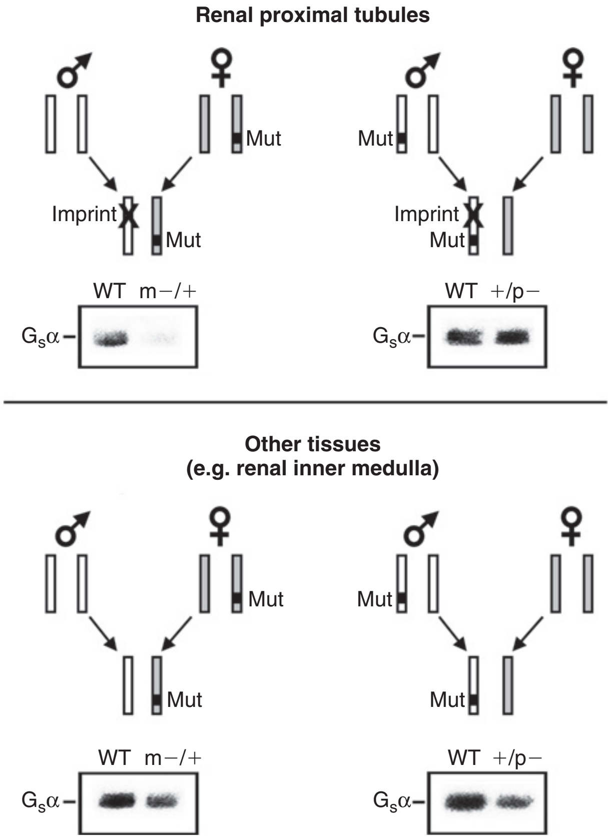

FIGURE 4 The role of tissue-specific $G_s\alpha$ imprinting in the pathogenesis of PHPIA and PPHP. (*Top*) $G_s\alpha$ is paternally imprinted (denoted with an X) in renal proximal tubules and therefore mutation (Mut) of the active maternal allele (gray) results in loss of $G_s\alpha$ expression and PTH resistance (*left*), whereas mutations in the inactive paternal allele (white) do not effect $G_s\alpha$ expression or hormone signaling (*right*), as shown below in immunoblots of renal cortical membranes from wild-type (WT) mice and mice with a $G_s\alpha$ mutation on the maternal or paternal allele (m −/+ or +/p−, respectively) using a $G_s\alpha$-specific antibody (Yu *et al.*, 1998). (*Bottom*) In other tissues $G_s\alpha$ is not imprinted and therefore maternal and paternal $G_s\alpha$ mutations both lead to ~50% loss of $G_s\alpha$ expression, as shown below in immunoblots of renal inner medullary membranes from the same mice (Yu *et al.*, 1998). (Reproduced from Weinstein (2004) "Molecular Basis of Inborn Errors of Development," Oxford University Press).

Mantovani *et al.*, 2003; Namnoum *et al.*, 1998). In all of these tissues $G_s\alpha$ has been shown to be imprinted, and therefore, maternal $G_s\alpha$ mutation leads to severe $G_s\alpha$ deficiency.

A likely explanation for why PHPIA patients do not show clinical resistance to other hormones that also activate $G_s\alpha$ in their target tissues (e.g., ACTH or vasopressin) is that $G_s\alpha$ is not imprinted in these target tissues and therefore $G_s\alpha$ expression is only reduced to ~50% in these tissues (Weinstein *et al.*, 2000). This partial $G_s\alpha$ deficiency may be insufficient to produce clinical hormone resistance, either because $G_s\alpha$ is not rate limiting for generating the cAMP second messenger, or because the cAMP levels, although reduced, are still sufficient to elicit a normal physiological response (Brickman *et al.*, 1986; Carlson *et al.*, 1985; Weinstein *et al.*, 2000). This would also explain why PTH resistance is evident in the renal proximal tubule, but not in the distal tubule or in the skeleton (Ish-Shalom *et al.*, 1996; Stone *et al.*, 1993).

Obesity is also a feature specific for PHPIA and therefore is also a consequence of severe $G_s\alpha$ deficiency in a tissue in which $G_s\alpha$ expression is normally affected by imprinting (Long *et al.*, 2007). This is confirmed by mouse studies showing that germline disruption of $G_s\alpha$ expression on the maternal allele leads to severe obesity and insulin resistance, whereas disruption of the paternal allele leads to a much less severe metabolic phenotype (Chen, M. *et al.*, 2005b). One potential mechanism for the pathogenesis of obesity in PHPIA would be $G_s\alpha$ deficiency in adipocytes leading a decreased ability of the sympathetic nervous system activity and hormones to stimulate lipolysis in these cells of these cells, which has been documented in these patients (Carel *et al.*, 1999; Kaartinen *et al.*, 1994). Moreover, genetic manipulations in mice that increase or decrease cAMP or its downstream effectors can lead to a lean or obese phenotype, respectively (Cummings *et al.*, 1996; Martinez-Botas *et al.*, 2000; Valet *et al.*, 2000). However $G_s\alpha$ has been shown not to be imprinted in adipose tissue (Mantovani *et al.*, 2004a) and mice with adipose tissue-specific $G_s\alpha$ deficiency do not develop obesity (Min Chen, L. Weinstein, unpublished results). Rather, the primary defect is probably in the central nervous system as norepinephrine levels, which reflect sympathetic nervous system activity are low in both PHPIA patients (Carel *et al.*, 1999) and mice with disruption of the $G_s\alpha$ maternal allele (Yu, S. *et al.*, 2000). This is confirmed by results showing that disruption of the $G_s\alpha$ maternal (but not paternal) allele in the central nervous system leads to severe obesity in mice (Min Chen, L. Weinstein, unpublished results). Presumably $G_s\alpha$ is imprinted in one or more sites in the central nervous system that are critical for energy balance. One potential site is the hypothalamus, because this is a site where α-melanocyte-stimulating hormone is known to act through $G_s\alpha$ pathways to decrease food intake and increase sympathetic active and energy expenditure. Recent studies have shown that odorant receptor/$G_s\alpha$/cAMP pathways may be important for axon guidance of odorant neurons, but whether this can explain the olfactory defect observed in PHPIA is at present unknown (Imai *et al.*, 2006).

The other AHO features that are present in both PHPIA and PPHP patients are also most likely to be caused by $G_s\alpha$ deficiency rather than NESP55 or $XL\alpha s$ deficiency, because these gene products would only be disrupted by maternal or paternal mutations, respectively, and AHO is absent in PHPIB patients in whom NESP55 is not expressed because of biallelic methylation of its promoter (Liu *et al.*, 2000a). Brachydactyly is likely the result of $G_s\alpha$ deficiency in growth plate chondrocytes, which normally mediates the paracrine effects of PTH-related peptide (PTHrP) to inhibit hypertrophic differentiation of chondrocytes (Kronenberg, 2003; Vortkamp *et al.*, 1996). Mice with disruption of the gene encoding PTHrP (Karaplis *et al.*, 1994) or its receptor (Jobert *et al.*, 1998; Lanske *et al.*, 1996) develop severe skeletal dysplasia with stunted long bone growth resulting from accelerated chondrocyte differentiation. Other studies in mice have confirmed that $G_s\alpha$ deficiency in growth plate chondrocytes leads to a similar cellular and whole animal phenotype (Bastepe *et al.*, 2004; Sakamoto *et al.*, 2005). Osteoma cutis appears to result from the fact that $G_s\alpha$ deficiency in mesenchymal cells promotes osteoblast differentiation, as discussed in more detail in the sections on POH and MAS/FD. The exact mechanisms by which some AHO patients develop mental deficits are not clear, but may also be related to $G_s\alpha$ deficiency, as cAMP and PKA are known to be important for learning and memory (Abel *et al.*, 1997; Levin *et al.*, 1992; Rotenberg *et al.*, 2000; Wu, Z.L. *et al.*, 1995) and for neurite outgrowth and differentiation (Jessen *et al.*, 2001; Vossler *et al.*, 1997).

PROGRESSIVE OSSEOUS HETEROPLASIA

Clinical Features

POH is a more severe form of the ectopic ossification observed in AHO in which dermal and subcutaneous ossifications coalesce to form large plaques and invade deep connective tissues and skeletal muscle, leading to joint stiffness and bone deformity (Kaplan and Shore, 2000). Its onset is generally in early childhood, although one case of adult-onset POH has been reported in which a $G_s\alpha$ mutation was not identified (Seror *et al.*, 2007). POH may occur sporadically or in a familial manner. In many cases there are no other features of AHO, although POH has been reported to coexist with other features of AHO or multihormone resistance (Eddy *et al.*, 2000; Gelfand *et al.*, 2007). There is no specific therapy for the ossifications. A report of one case suggested that intravenous pamidronate may have slowed the progression of new lesions, although it did not affect the lesions that already had developed (Hou, 2006).

Genetics

In most cases POH is associated with heterozygous germline $G_s\alpha$ loss-of-function mutations that are similar, and in some cases identical, to those observed in patients with PHPIA and PPHP (Ahmed *et al.*, 2002; Eddy *et al.*, 2000; Gelfand *et al.*, 2007; Shore *et al.*, 2002; Yeh *et al.*, 2000). Although it has been reported that POH only occurs with paternal inheritance of *GNAS* mutations (Shore *et al.*, 2002), POH has been shown to occur upon maternal transmission of mutations in two cases, one of which also had features of PHPIA (Ahmed *et al.*, 2002; Eddy *et al.*, 2000). In one of these cases, the proband had POH whereas his affected sister presented with PHPIA without POH (Ahmed *et al.*, 2002). Moreover, another case showed features consistent with POH, AHO, and multihormone resistance (PHPIA) (Gelfand *et al.*, 2007). Although neither parent had the mutation, the presence of hormone resistance in the proband strongly suggests that the mutation was on the maternal allele. In one family, paternal transmission of a $G_s\alpha$ mutation resulted in five cases of POH in one generation, but maternal transmission of the mutation resulted in AHO (PHPIA vs. PPHP not determined) in the next generation (Shore *et al.*, 2002).

Pathogenesis

As in AHO, the ectopic ossifications in POH form by intramembranous ossification. Given that AHO and POH patients have similar mutations that completely disrupt $G_s\alpha$ expression, the severity of ectopic ossification in an individual patient is most likely determined by other factors, including differences in genetic background. Other genes that may modify the severity of ossification include those that may affect $G_s\alpha$ expression from the unaffected allele or those that encode other proteins involved in $G_s\alpha$ signaling or bone formation. It remains unclear why many POH patients do not also have other features of AHO. Although it has been reported that POH is only associated with mutation on the paternal allele (Shore *et al.*, 2002), several reports have contradicted this conclusion (Ahmed *et al.*, 2002; Eddy *et al.*, 2000; Gelfand *et al.*, 2007).

Although activation of $G_s\alpha$ mutations appears to inhibit osteoblast differentiation in FD, as discussed later, decreased $G_s\alpha$ expression promotes osteoblast differentiation in cultured cells with increased expression of the osteogenic transcription factor Cbfa1/RUNX2 and other osteoblast-specific genes (Lietman *et al.*, 2005). The effect of reduced cAMP on cellular levels of Cbfa1/RUNX2 protein may be, in part, the result of reduced degradation (Tintut *et al.*, 1999). This regulation may be bidirectional, because one study showed that Cbfa1/RUNX2 suppresses the expression of $G_s\alpha$ in a cultured osteoblastic cell line (Bertaux *et al.*, 2006). $G_s\alpha$ deficiency in mesenchymal precursor cells may promote osteoblast differentiation, leading to ectopic ossification in AHO and POH. Consistent with this, Cbfa1/RUNX2 mRNA was shown to be expressed in dermal fibroblasts from a POH patient (Yeh *et al.*, 2000).

PSEUDOHYPOPARATHYROIDISM TYPE IB

Clinical Features

PHPIB patients present with renal PTH resistance in much the same way as PHPIA patients, with diminished urinary cAMP response to administered PTH or its analogues, but do not exhibit the AHO phenotype or resistance to most other hormones (Levine *et al.*, 1983). Consistent with the absence of AHO, G_s bioactivity is unaffected in erythrocyte membranes derived from PHPIB patients (Levine *et al.*, 1983; Silve *et al.*, 1986). About half of the patients show evidence of borderline TSH resistance, with slightly elevated TSH levels that in some cases may be intermittent (Bastepe *et al.*, 2001a; Foppiani *et al.*, 2006; Liu *et al.*, 2003). Some PHPIB patients present with elevated serum alkaline phosphatase, cortical bone resorption, osteitis fibrosa cystica, or slipped capital femoral epiphysis, presumably owing to the skeletal effects of chronically elevated PTH levels (Agarwal *et al.*, 2006; Costello and Dent, 1963; Kolb and Steinbach, 1962) and can rarely present with a rachitic picture. These complications seem to be relatively specific for PHPIB, as this is not a feature of PHPIA. Management of the PTH and TSH resistance is similar to that discussed for PHPIA, except that normalization of PTH levels may be more critical to prevent skeletal manifestations of elevated PTH levels and the development of autonomous (tertiary) hyperparathyroidism. In one kindred PHPIB was associated with increased uric acid excretion and hypouricemia (Laspa *et al.*, 2004), whereas in another kindred the affected patients initially presented with paroxysmal dyskinesia (Mahmud *et al.*, 2005).

Genetics

Most cases of PHPIB are sporadic, but some are familial. In familial cases the PTH resistance only occurs upon maternal transmission of the trait, similar to the inheritance pattern in PHPIA (Juppner *et al.*, 1998). Those who inherit the trait paternally are asymptomatic carriers. Although mapping studies linked familial PHPIB to the vicinity of *GNAS* on 20q13 (Juppner *et al.*, 1998), the presence of normal erythrocyte G_s function in PHPIB patients ruled out the possibility that the disease is caused by mutations that directly disrupt the $G_s\alpha$-coding exons (Levine *et al.*, 1983; Silve *et al.*, 1986). Rather, PHPIB is associated with a *GNAS*-imprinting defect in which maternal-specific imprinting (methylation) of the exon 1A-imprinting control region is absent in virtually all cases, resulting in a paternal-specific imprinting pattern (unmethylated) on both parental alleles (Bastepe *et al.*, 2001b; Jan de Beur *et al.*, 2003; Liu *et al.*, 2000a, 2005b). In sporadic cases the upstream NESP55, XLαs, and antisense promoters may also be abnormally imprinted, although imprinting of these upstream regions is unaffected in most familial cases (Liu *et al.*, 2005b). PTH resistance specifically correlates with loss of exon 1A maternal imprinting, and there is no phenotypic correlation with the imprinting status of NESP55, XLαs, or the antisense promoter. Paternal UPD of chromosome 20 has been reported in one patient with PTH and TSH resistance associated with other abnormalities (Bastepe *et al.*, 2001a).

Familial PHPIB in almost all cases is associated with a deletion within the linked STX16 gene (Bastepe *et al.*, 2003 ; Linglart *et al.*, 2005; Liu *et al.*, 2005b). Upon maternal transmission the deletion results in loss of maternal exon 1A methylation and PHPIB, but paternal transmission has no effect on exon 1A imprinting because the region is normally unmethylated on this allele and does not produce a phenotype. Although this region is presumed to have one or more *cis*-acting elements required for the establishment or maintenance of exon 1A methylation, deletion of the orthologous region in mice did not result in the exon 1A methylation defect or PTH resistance (Frohlich *et al.*, 2007). In two kindreds PHPIB was associated with maternal deletion of the NESP55 region and loss of maternal imprinting throughout the rest of the *GNAS* locus (Bastepe *et al.*, 2005). In another kindred PHPIB resulted from maternal, but not paternal, inheritance of a 3-bp deletion that removes the carboxyl-terminal residue Ile^{382} in $G_s\alpha$ (Wu, W.I. *et al.*, 2001). The original report showed evidence that this mutation resulted in selective uncoupling of the G protein from the PTH/PTHrP receptor, but a subsequent study showed evidence that the mutation leads to uncoupling from other receptors as well (Linglart *et al.*, 2006).

Pathogenesis

Loss of exon 1A imprinting in PHPIB strongly suggests that this region, which is a primary imprinting control center (Liu *et al.*, 2000b), is required for tissue-specific imprinting of $G_s\alpha$. A model has been proposed in which the exon 1A region contains one or more *cis*-acting regulatory elements that inhibit the activity of the $G_s\alpha$ promoter in a methylation-sensitive and tissue-specific manner (Weinstein *et al.*, 2001). In the model shown in Fig. 5, the exon 1A region contains a silencer element that binds a *trans*-acting repressor protein that is only expressed in specific tissues such as renal proximal tubules. Normally the repressor binds to the paternal allele and inhibits the paternal $G_s\alpha$ promoter but is unable to bind to the maternal allele because of methylation, allowing the maternal $G_s\alpha$ promoter to remain active. In PHPIB both parental alleles are unmethylated and therefore the repressor can bind to and suppress $G_s\alpha$ expression from both alleles, resulting in renal PTH resistance. In most other tissues the repressor is not expressed and therefore $G_s\alpha$ is expressed from both parental alleles. Moreover, the absence of the repressor would predict that loss of maternal exon 1A methylation in PHPIB patients would have no effect on $G_s\alpha$ expression, which is consistent with the fact that $G_s\alpha$ is unaffected in erythrocyte membranes from these patients. The absence of the AHO phenotype in PHPIB patients presumably is due

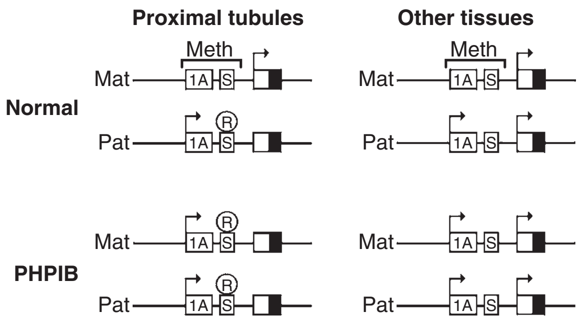

FIGURE 5 Model for tissue-specific $G_s\alpha$ imprinting and the pathogenesis of PHPIB. The model shown predicts that the exon 1A differentially methylated region has a *cis*-acting silencer element (S) that inhibits the paternal $G_s\alpha$ promoter (shown as half-filled box) in a tissue-specific manner by binding a repressor (R) that is expressed in a tissue-specific manner. In renal proximal tubules (*left*) the repressor can bind to and silence $G_s\alpha$ on the paternal allele but is unable to bind to or silence $G_s\alpha$ on the maternal allele because the binding site is methylated. In most other tissues (*right*) the repressor is not expressed and therefore $G_s\alpha$ is biallelically expressed. In PHPIB, methylation of the maternal allele is absent, allowing the repressor to bind to and silence $G_s\alpha$ from both alleles in renal proximal tubules, leading to $G_s\alpha$ deficiency and renal PTH resistance. In most other tissues $G_s\alpha$ expression is unaffected because the repressor is not expressed. This would explain why PHPIB patients have normal $G_s\alpha$ expression in erythrocytes and why they do not develop AHO. (Adapted from Weinstein (2004) "Molecular Basis of Inborn Errors of Development," Oxford University Press).

to the fact that $G_s\alpha$ expression levels are unaffected in the vast majority of tissues where $G_s\alpha$ is normally expressed equally from both parental alleles. Because $G_s\alpha$ is not imprinted in bone, this tissue would be expected to have normal $G_s\alpha$ levels and PTH responsiveness, and therefore be prone to the adverse effects of chronically elevated PTH levels (Murray *et al.*, 1993).

This model is supported by the observation that deletion of the exon 1A region from the paternal allele in mice leads to loss of tissue-specific $G_s\alpha$ imprinting (Liu *et al.*, 2005a; Williamson *et al.*, 2004). In fact, these mice have markedly reduced circulating PTH levels, presumably because of increased PTH sensitivity in renal proximal tubules owing to $G_s\alpha$ overexpression. Borderline TSH resistance in PHPIB results from the fact that $G_s\alpha$ expression from the paternal allele is normally only partially suppressed (Liu *et al.*, 2003).

FIBROUS DYSPLASIA OF BONE AND THE MCCUNE–ALBRIGHT SYNDROME

Clinical Features

Fibrous dysplasia of bone (FD) is a benign skeletal disorder with a broad spectrum of severity. It may involve a single bone (monostotic fibrous dysplasia, MFD), multiple bones (polyostotic fibrous dysplasia, PFD), or in rare circumstances, the entire skeleton (panostotic fibrous dysplasia) (Bianco *et al.*, 2003; Collins and Bianco, 2006; Collins, 2006; Lichtenstein and Jaffe, 1942). In a minority of cases FD (usually PFD) is found in association with characteristic skin spots and hyperfunctioning endocrinopathies, and this triad is known as the McCune–Albright syndrome (MAS) (Albright *et al.*, 1937; McCune, 1936). The skin lesions (café-au-lait spots) have a characteristic appearance of jagged borders (resembling the cartographic appearance of the coast of Maine), and tend to follow the developmental lines of Blaschko (Fig. 6). The hyperfunctioning endocrinopathies, in order of decreasing prevalence, include gonadotropin-independent precocious puberty, thyroid nodules with hyperthyroidism, growth hormone excess (acromegaly/gigantism), and/or adrenal hyperplasia with glucocorticoid excess (Cushing's syndrome) (Collins and Shenker, 1999; Danon and Crawford, 1987; Ringel *et al.*, 1996). In each case the associated pituitary trophic hormones (gonadotropins, thyrotropin, ACTH) are undetectable, indicating a primary defect in each endocrine gland. Parathyroid gland involvement has been reported to be part of the syndrome, but it is questionable if this is really a feature of the disease. In the one reported case of hyperparathyroidism associated with FD/MAS in which a mutation in the hyperplastic gland was sought, it was not present. Some of the suspected cases may in fact be affected with hyperparathyroidism jaw tumor syndrome (HJTS), in which fibro-osseous lesions (somewhat reminiscent of FD at the clinical, radiographic, and histopathological level) arise in the gnathic bones in association with hyperparathyroidism (Carpten *et al.*, 2002). The misdiagnosis of HJTS (a familial disease) as FD has led to some cases reported as "familial FD/MAS"(Osundwa *et al.*, 2001).

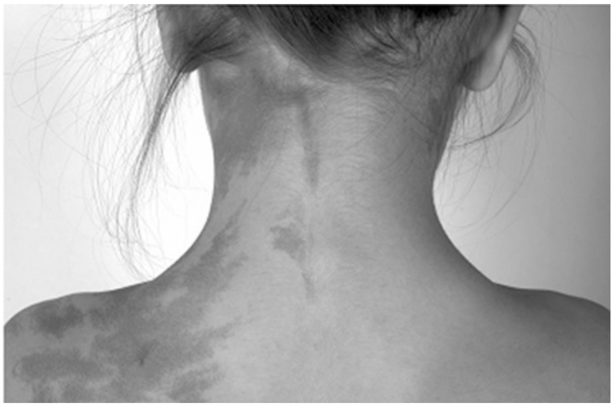

FIGURE 6 (A typical café-au-lait spot in a patient with McCune–Albright syndrome. Note the relationship of the skin lesions to the midline, the jagged (coast of Maine) borders, and the relationship to the developmental lines of Blashko. The linear patch at the nape of the neck is seen quite commonly, either in isolation or in association with the larger skin lesions.

FD was originally described by Donovan McCune and Fuller Albright in association with café-au-lait spots and endocrine hyperfunction, with Albright labeling the disease as osteitis fibrosa dessiminata (Albright *et al.*, 1937; McCune, 1936). This name was assigned because of the resemblance, especially at the histopathological level, to the bone disease of hyperparathyroidism, osteitis fibrosa cystica. In the original cases Albright described, the patients had extensive (disseminated) PFD, thus dessiminata. It was Lichtenstein who labeled the disease FD (Lichtenstein, 1938), and Lichtenstein and Jaffe were the first to report extensively on the spectrum, radiographic appearance, and histopathology of FD (Lichtenstein and Jaffe, 1942). FD in the long bones usually presents in childhood as a limp, pain, or pathological fracture (DiCaprio and Enneking, 2005; Leet and Collins, 2007). FD in the skull may first appear as subtle facial asymmetry, or a "lump," and is sometimes associated with pain (Riminucci *et al.*, 2002). Fractures (especially low trauma, pathological fractures) are more common in childhood, but can persist into adulthood (Leet *et al.*, 2004a). Pain, sometimes severe, even in the absence of fractures or trauma is a common feature of FD, particularly in adults (Kelly *et al.*, 2007a). The extent of the disease (skeletal disease burden score) can be quantified with the use of nuclear medicine bone scans (Collins, *et al.*, 2005), and the skeletal disease burden score directly correlates with fracture rate and number, but surprisingly not with the extent of pain. The skeletal disease burden score also has a direct correlation with the extent to which FD has a deleterious effect on the health-related quality of life (Kelly *et al.*, 2005), with the predominant factor affecting quality of life being the extent to which the skeletal disease burden impacts on the ability to ambulate without assistance. Renal phosphate wasting leading to hypophosphatemia, rickets, and osteomalacia is also seen in cases of FD or MAS with extensive skeletal involvement (Collins, *et al.*, 2001; Riminucci *et al.*, 2003a).

FD affects the spine commonly and can be associated with scoliosis (Leet *et al.*, 2004b) (Fig. 7). The FD-associated scoliosis usually appears in childhood and can progress rapidly, even into adulthood, and rarely can be lethal. An uncommon finding in association with FD can be intramuscular myxomas, known as Mazabraud's syndrome (Mazabraud and Girard, 1957). Isolated intramuscular myxomas, in the absence of FD, are also caused by the same $G_s\alpha$ mutations (Okamoto *et al.*, 2000). Rarely (<1% of cases), FD can undergo malignant transformation. Cancer will usually present as the rapid expansion of a preexisting FD lesion in association with pain. On imaging studies there will be a disruption of the cortex in association with a soft-tissue mass. Bone cancers have been reported in 123 cases in two large case series (Ruggieri, M. *et al.*, 1999; Schwartz and Alpert, 1964) and in two large reviews (Lopez-Ben *et al.*, 1999; Yabut *et al.*, 1988), wherein it was found that approximately half of the cases of cancer arose in MFD and half in PFD. When one considers that MFD is more common, cancer is more likely to occur in PFD. The region/bones most commonly involved are the craniofacial bones, the femur, and the tibia. In these series, the most commonly seen histological types were osteosarcoma, fibrosarcoma, and chondrosarcoma. There are reports suggesting that malignant transformation may be more common in patients with Mazabraud's syndrome (Jhala *et al.*, 2003; Lopez-Ben *et al.*, 1999). However, this is likely not the case, but rather represents an ascertainment bias, as the actual prevalence of intramuscular myxomas in association with FD may be more common than expected because most intramuscular myxomas are asymptomatic and go undetected.

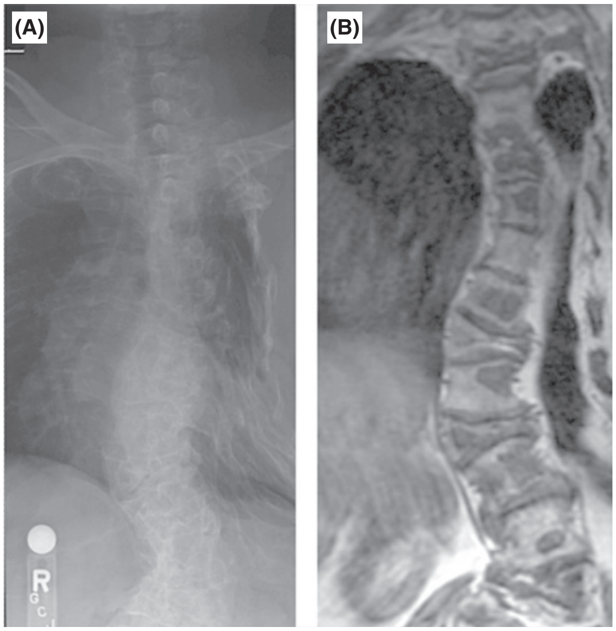

FIGURE 7 *Scoliosis in FD.* Scoliosis of the spine is demonstrated in an x-ray (**A**) and MRI of the spine (**B**) in a 54-year-old woman with FD. The extent of the involvement of the vertebral columns with FD is demonstrated in the MRI where large parts of the vertebrae are shown to be replaced with FD. Surprisingly, and typically, the woman had neither pain nor any evidence of neurological compromise, despite extensive disease and multiple compression fractures.

Genetics

FD/MAS is the result of activating mutations in $G_s\alpha$ (Schwindinger *et al.*, 1992; Weinstein *et al.*, 1991). The vast majority of the mutations (>95% in our series, unpublished data, and 100% in a large published series [Lumbroso *et al.*, 2004]) occur at Arg^{201}, and are divided roughly equally between either Arg^{201} to His or Arg^{201} to Cys. However, a recent report from a large series of patients with FD has found three cases (5%) in which the patients had Gln^{227} to Leu mutations (Idowu *et al.*, 2007). The lack of vertical transmission of FD or MAS, the fact that the skin lesions appear to roughly follow the developmental lines of

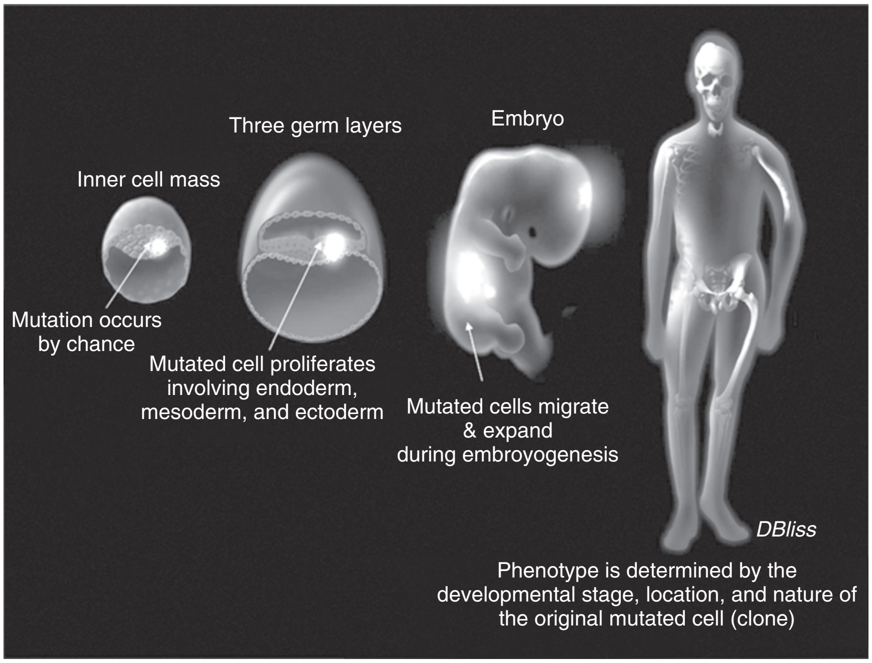
Inner cell mass
Three germ layers
Embryo
Mutation occurs by chance
Mutated cell proliferates involving endoderm, mesoderm, and ectoderm
Mutated cells migrate & expand during embroyogenesis
DBliss
Phenotype is determined by the developmental stage, location, and nature of the original mutated cell (clone)

FIGURE 8 Developmental explanation for FD and MAS. For involvement of tissues that derive from all three germ layers (skin, bone, and thyroid, for example), the mutation must have occurred in the inner cell mass at the blastocyst stage. The clone in which the mutation occurs is propagated into the three germ layers and the cells migrate and proliferate, leading to the final phenotype.

Blaschko, and the fact that the skeletal lesions often tend to affect one side of the body has led to the widely held concept that FD and MAS are the manifestation of somatically acquired *GNAS* mutations and that patients are mosaics. The concept, first proposed by Happle (1986), is that if germline, the *GNAS* mutations that cause FD and MAS, were lethal, the mutation would only "survive" because the somatic, mosaic nature of the mutation. The implication of this line of reasoning is that, for a patient with MAS whose disease is manifested by, for example, café-au-lait skin spots (a tissue of ectodermal origin), FD of the long bones (a tissue of mesodermal origin), and hyperthyroidism (a tissue of endodermal origin), the mutation must have occurred very early in development at the inner cell mass stage, prior to the development of the three germ layers (Fig. 8). Although the clinical data appear to support this model, it has yet to have been proven experimentally. Because $G_s\alpha$ is imprinted in a tissue-specific manner, the clinical presentation of individuals may also be affected by the parental allele that harbors the mutation, particularly with respect to the presence or absence of acromegaly (Mantovani *et al.*, 2004b).

Pathogenesis

FD is the result of $G_s\alpha$ activating mutations in bone marrow stromal cells, which are the skeletal stem cell that gives rise to osteogenic cells, including chondrocytes, osteoblasts, and osteocytes, as well as bone marrow adipocytes and hematopoietic supportive stromal cells (Bianco and Robey, 1999). Lichtenstein was the first to speculate that FD is the result of the "perverted activity of the specific bone-forming mesenchyme" (Lichtenstein, 1938) and Bianco and colleagues were the first to prove that FD results specifically from bone marrow stromal cells harboring the activating $G_s\alpha$ mutations (Bianco *et al.*, 1998; Bianco and Robey, 1999; Riminucci *et al.*, 1997). In addition, it has been shown that the ability of cells harboring the $G_s\alpha$ mutation to produce an FD lesion depends on the presence of both mutated and nonmutated cells (Bianco *et al.*, 1998). It is the fate of the original clone in which the mutation occurs that dictates the phenotype of an individual patient (see Fig. 8). If the mutation occurs very early in development in a clone in the inner cell mass there will be widespread tissue involvement, perhaps involving all bones and multiple endocrine glands as well. If it occurs later in development, perhaps lateral to the midline, there may be only skeletal tissue on one side of the body involved. The "map" of affected tissue is established early in life, with the majority of FD lesions established by the age of 5 years and with virtually no new lesions occurring after the age of 15 (Hart *et al.*, 2007). Developmentally, the skeleton forms normally, in that all bones are present in the appropriate anatomical location and relative size, growth plates form normally, and the cortex of bones is intact and normal (at least initially). FD first appears in the bone marrow space of an anatomically normal bone. The fibroosseous tissue that makes up FD replaces the normal bone marrow. It can, and often does expand within the bone marrow space, increasing the relative size of the bone marrow compartment, deforming the segment of the involved bone in an expansile fashion and thinning the bone cortices. Although entire bones, or even the entire skeleton, can be involved, the disease only involves the bone marrow compartment. Because of the fibrous nature of the tissue and the thinning of the cortex, the weight-bearing bones, especially the femurs, will often bow, giving rise to the "shepherd's crook" deformity of the proximal femur and the "windswept" deformity of the lower extremities (Fig. 9).

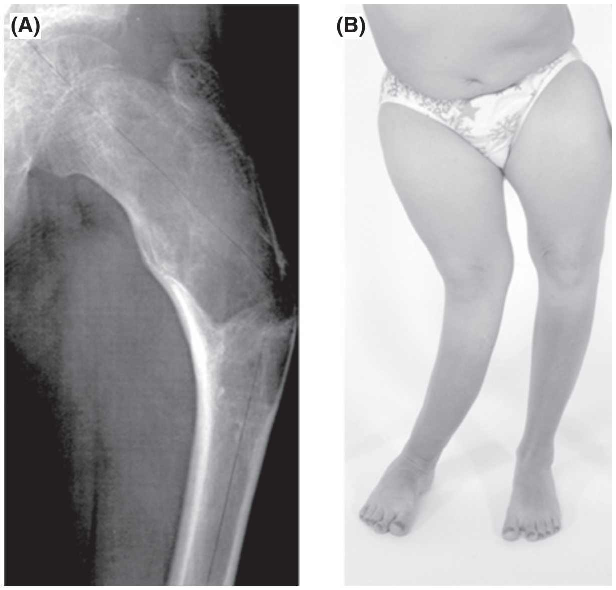

FIGURE 9 Classic deformities associated with FD. The shepherd's crook deformity (**A**) of the proximal femur and, the "windswept" deformity often seen in severe bilateral lower extremity disease (**B**) are shown.

The genesis of a FD lesion, from a nascent, virtually undetectable collection of bone marrow stromal cells harboring the $G_s\alpha$ mutation and residing in the marrow of an as yet clinically unaffected young child, to the full blown, clinically significant lesion giving rise to the shepherd's crook or windswept deformity involves a complex process that involves all steps in bone development and maintenance, including modeling, bone deposition, mineralization, and remodeling (Riminucci *et al.*, 2007). These processes give rise to a fibroosseous bone disease with a distinct and unique histopathology (Fig. 10). The details of the histopathology are complex and are detailed elsewhere (Bianco *et al.*, 1998; Bianco and Robey, 1999; Bianco *et al.*, 2000; Riminucci *et al.*, 1997, 1999., 2003b, 2006, 2007), but the essentials include a bone marrow space that is replaced with a "fibrous" tissue that is composed of cells that express markers of osteogenic cells. The islands of bone within the fibrous tissue are abnormal in that this tissue is immature, woven bone and has increased numbers of osteocytes. In some cases the lesions may also include islands of chondrocytic cells. There are two hallmarks of FD histopathology: one is the presence of retracted, stellate-shaped osteoblastic cells, which is because of the effect of excess cAMP (Miller, S.S. *et al.*, 1976; Riminucci *et al.*, 1997); and the second is the arrays of collagen bundles running perpendicular to the trabecular surface, known as Sharpey fibers (Riminucci *et al.*, 1997), which are a normal feature at sites of tendon and ligament insertion into bone. In addition, FD, which is created by the intrinsic genetic defect in the bone marrow stromal cell, is further impacted by the abnormal extrinsic hormonal milieu that can exist as part of the McCune–Albright syndrome.

It was an understanding of the signaling pathways in the endocrine tissues affected in MAS patients that led to the discovery of the molecular defect underlying FD. The

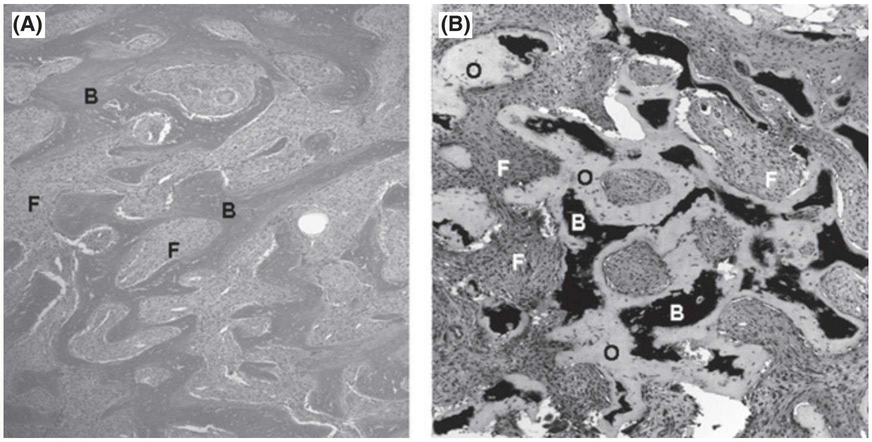

FIGURE 10 Histopathological appearance of FD. (**A**) H&E staining of a demineralized section of FD showing the classic "Chinese characters" made of trabeculae bone (labeled **B**) with fibrous tissue interwoven between (labeled F). (**B**) Von Kossa stain of an undemineralized, plastic-embedded section of bone, useful for inspecting the degree of mineralization, shows that the trabeculae of bone are composed of bone (black, labeled B) as well as osteoid (labeled O). The fibrous tissue is labeled (F). The presence of the unmineralized bone confirms the osteomalacic nature of the lesions.

realization that in each endocrine tissue growth and hormonal hypersecretion occurs in the absence of pituitary trophic hormones suggested that the defect was downstream of the cell surface receptors for these hormones and may involve the G proteins that are coupled to cell surface receptors in the generation of downstream signaling (Schwindinger, *et al.*, 1992; Weinstein *et al.*, 1991). The activating mutations in $G_s\alpha$ that give rise to FD lead to ligand-independent generation of intracellular cAMP by inhibiting its intrinsic GTPase activity, leading to activation of downstream signaling pathways (Fig. 11) (Spiegel and Weinstein, 2004). In osteogenic cells increased stimulation of protein kinase A by cAMP leads to phosphorylation of the cAMP-response element-binding protein (CREB) and the related cAMP-response element modulator proteins (CREM). CREB phosphorylation induces the c-*fos* gene, leading to increased expression of Fos protein, a component of the activator protein 1 (AP1) transcription complex (Gaiddon *et al.*, 1994; Sassone-Corsi, 1995). AP1 is more abundant in proliferating osteogenic precursors and its expression markedly decreases upon the onset of differentiation (Stein and Lian, 1993). Fos is overexpressed in FD lesions (Candeliere *et al.*, 1995) and Fos overexpression in osteogenic cells leads to lesions reminiscent of FD in mice (Rüther *et al.*, 1987). Consistent with this, cells in FD lesions have an increased proliferative rate and only express early markers of osteoblast differentiation (Marie *et al.*, 1997; Riminucci *et al.*, 1997). Phosphorylated CREB and AP1 also lead to induction of the interleukin 6 (IL6) gene, and increased IL6 production leads to osteoclast recruitment and cortical bone resorption, allowing concentric expansion of the FD lesion (Motomura *et al.*, 1998; Yamamoto, T. *et al.*, 1996).

Another metabolic syndrome that is commonly seen in association with FD is renal phosphate wasting (Collins *et al.*, 2001). Whereas it was originally thought that the phosphaturia, with resultant hypophosphatemia, was the result of the presence of the $G_s\alpha$ mutation in the kidney (Zung *et al.*, 1995), it has more recently been shown that phosphaturia is caused by oversecretion of the recently described phosphaturic hormone fibroblast growth factor 23 (FGF-23) by FD bone cells (Riminucci *et al.*, 2003a). As such, the degree of renal phosphate wasting is directly correlated with the extent of the FD skeletal disease burden (Collins *et al.*, 2005). Of the associated extrinsic endocrine/metabolic syndromes associated with FD, it is hypophosphatemia that probably has the most profound effect on FD. Hypophosphatemia will exacerbate the intrinsic

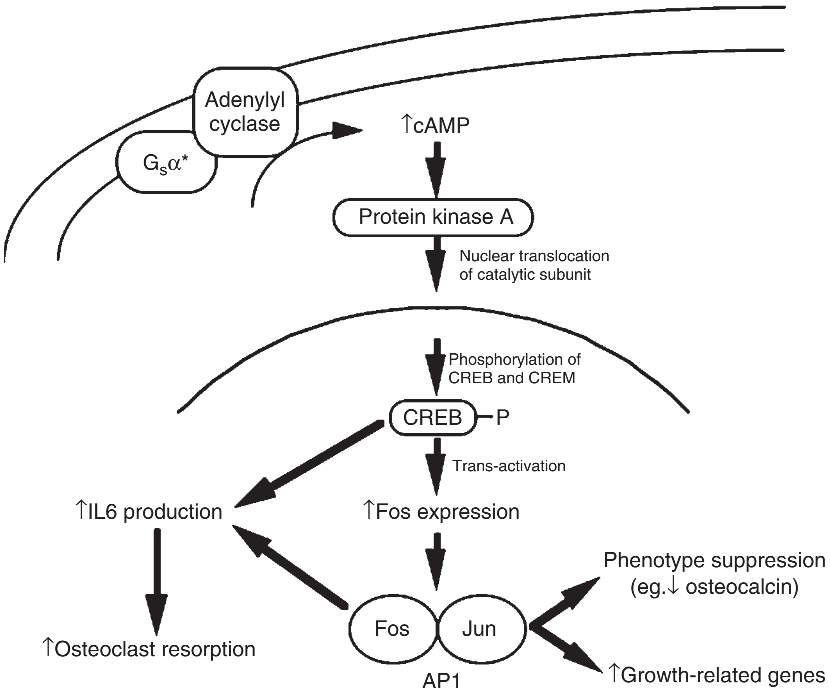

FIGURE 11 Possible mechanisms by which activated G_s in osteogenic cells may lead to FD. G_s activation increases the activity of the effector adenylyl cyclase, leading to increased intracellular cAMP levels. Binding of cAMP to the regulatory subunits of cAMP-dependent protein kinase (protein kinase A) allows release of its catalytic subunits, which translocate to the nucleus and phosphorylate proteins such as CREB and CREM. Phosphorylated CREB binds to promoters of cAMP-responsive immediate-early genes (e.g., c-*fos*) and increases their expression. Fos, the product of c-*fos*, binds with Jun to form AP-1. AP-1 is a transcription factor that increases the expression of growth-related genes and decreases the expression of osteoblast-specific genes, such as osteocalcin (phenotype suppression). Phosphorylated CREB and AP-1 also stimulate the transcription of the IL-6 gene. IL-6 may be important in recruiting osteoclasts and stimulating osteoclastic bone resorption.

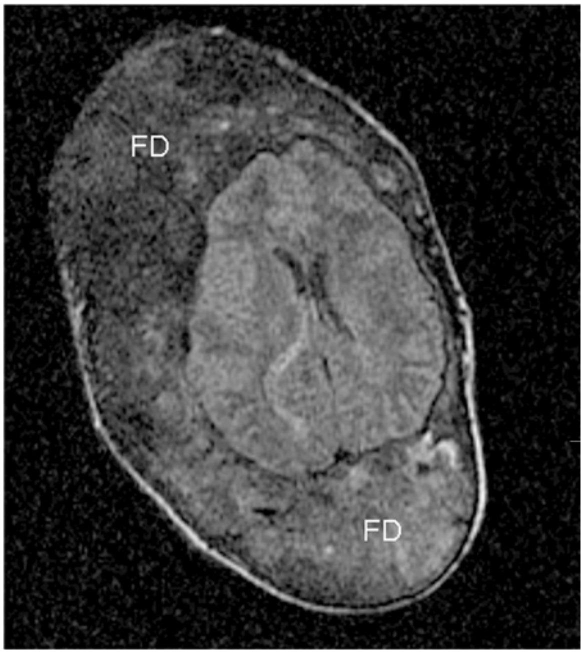

FIGURE 12 Craniofacial FD complicated by growth hormone excess. Areas of FD that have undergone massive expansion under the stimulation of excess GH/IGF-1 in a patient with McCune–Albright syndrome are shown. The craniofacial bones are the only bones to undergo such expansion in patients with FD and GH excess.

hypomineralization that exists in FD lesions (Bianco *et al.*, 2000; Corsi *et al.*, 2003) and is associated with greater degree of deformity and an increased number of fractures that occur at a younger age (Leet *et al.*, 2004a). Growth hormone excess worsens craniofacial fibrous dysplasia and may lead to bone overgrowth that can be dramatic (Lee *et al.*, 2002; Uwaifo *et al.*, 2001) (Fig. 12) and lead to vision and hearing loss owing to nerve compression (Cutler *et al.*, 2006).

Diagnosis and Management

Clinically significant disease is usually diagnosed in childhood—the more significant, the earlier the presentation. The initial presentation of disease in the long bones is usually a limp, pain, or fracture through an FD lesion. Disease in the craniofacial region usually presents as a "lump," which in recollection by the parents is often felt to have occurred as the result of minor, everyday trauma of childhood, but which never resolved. X-rays usually have a characteristic "ground glass" appearance (Fig. 13A). However, this classic ground glass appearance is, to an extent, age-dependent. In fact, in very young children, FD is not even apparent on x-rays and is only detected on a nuclear medicine bone, and when it does appear on an x-ray it has an inhomogeneous "streaked" appearance, rather then the ground glass appearance (see Fig. 13B). This streaked appearance is sometimes mistaken for the "dripping candle wax" appearance of Ollier's disease. In older patients, sclerosis can appear at the edges of FD lesions, representing the reaction of the adjacent normal bone to the FD, and indicating less active disease (see Fig. 13C). The same is true of FD in the craniofacial region. By CT scanning, the modality of choice for imaging craniofacial FD, the appearance is homogeneous and ground glass in young children (see Fig. 13D). With time, the lesions develop a mottled appearance, in which the lesions are an admixture of typical fibroosseous tissue with islands of what is essentially soft tissue (see Fig. 12E). This appearance is often referred to as "cystic," which is a useful descriptor, but it must not be mistaken for true, fluid-filled cysts, which occur uncommonly in FD. Fluid-filled cysts, with pathognomonic fluid levels, which are best seen on MRI scans, occur in fewer than 5% of the cases of FD. These truly cystic lesions often behave aggressively, rapidly expanding into adjacent tissue, destroying cortex, and when they are adjacent to vital structures, especially in the skull, can have significant morbidity. These lesions require immediate surgical attention. Fluid-filled cysts in the craniofacial region can be distinguished from soft tissue "cystic" lesions on CT scanning by measuring the Houndsfield units; fluid-filled lesions will be = 10 HU, while cystic lesions will be more than 10 HU (Collins and Bianco, 2006).

When FD lesions occur in the setting of either typical café-au-lait spots or hyperfunctioning endocrinopathies, the diagnosis is straightforward. However, if there is confusion, a biopsy demonstrating the typical histopathological changes cited earlier can aid in the diagnosis. Finally, if confusion still exists, mutation analysis on affected tissue can confirm the diagnosis if positive for a $G_s\alpha$ mutation. Another factor that can aid in the diagnosis is consideration of the bones involved and/or the distribution of the disease. FD most commonly involves the proximal femur and skull base, and in polyostotic disease at least one of these two areas will always be involved. When multiple bones are involved, asymmetry is the rule, and the presence of bilateral, symmetric disease is suggestive of another process. A nuclear medicine bone scan is a useful tool for assessing the extent of disease and distribution. In addition, measurement of skeletal disease burden by bone scan can quantify the extent of the disease and aid in prognostication in terms of potential future morbidity (Collins *et al.*, 2005).

In evaluating and caring for patients with FD, it is important to screen for and treat associated endocrinopathies. There often is not a direct correlation between the extent of the skeletal disease and the extent of endocrine involvement, so one cannot assume that minimal skeletal disease automatically excludes the presence of significant endocrine disease. A careful history, physical examination, evaluation of the growth chart, and focused laboratory testing is necessary. The evaluation should assess for precocious puberty, hyperthyroidism, hypophosphatemia (with a low serum 1,25(OH)$_2$-vitamin D and renal phosphate

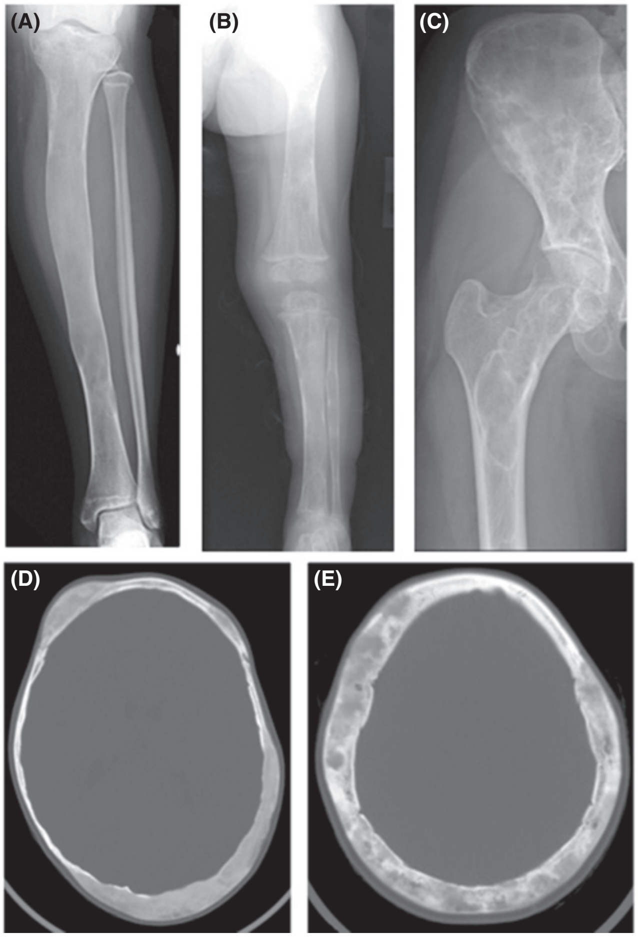

FIGURE 13 Radiographic appearance of FD. The classic description of the radiographic appearance of FD is "ground glass," which is depicted in the tibia (**A**). In very young children, the appearance has more of a streaked appearance (**B**), and in older patients with less active disease there is often a sclerotic rim around FD lesions (**C**). On CT scanning in young children the appearance is, again, homogeneous and ground glass (**D**), but with age, the appearance is inhomogeneous and mottled, and is sometimes reported to be "cystic" (**E**).

wasting), and growth hormone excess. Glucocorticoid excess can also occur, usually in the neonatal period. The altered phosphate and vitamin D metabolism is caused by overproduction of the phosphaturic factor FGF-23 by FD bone, so measurement of serum FGF-23 (which is not currently commercially available) can aid in the diagnosis (Collins *et al.*, 2001; Riminucci *et al.*, 2003a). There is a direct correlation between the risk for hypophosphatemia and the FD skeletal burden, so FGF-23-mediated renal phosphate wasting is only seen in cases of fairly extensive FD.

Surgery remains the mainstay of treatment for FD of the appendicular skeleton. Yet, there is no consensus to date as to the timing or indications and which techniques are best for which indication and at which age. There is an emerging consensus that, when their use is possible, intramedullary rods are better than side plates and screws.(Ippolito *et al.*, 2003; Leet and Collins, 2007; Stanton, 2006). Although bone grafting (filling FD lesions with grafting material— often bone bank, cadaveric bone) was at one time considered useful, a consensus is emerging, especially considering children, that this is not the best practice.

The skull base, through which the optic nerves pass, is the most common site for FD involvement, and it was believed that optic nerve encasement was progressive and visual impairment the rule if left untreated (Chen, Y. R. *et al.*, 1997; Moore *et al.*, 1985). For this reason, prophylactic decompression of the optic nerves was recommended. However, recent evidence has shown that this not the case (Lee *et al.*, 2002). In fact, the vast majority of patients with skull base FD, despite extensive disease, will have normal vision throughout their lives. It has been further shown that the one group of patients who do suffer functional morbidity owing to craniofacial FD (blindness and deafness) are the subgroup of patients with growth hormone excess (Cutler *et al.*, 2006). This point emphasizes the importance for screening and treatment of growth hormone excess in patients with craniofacial FD.

Another aspect of the disease that needs to be noted because of the possibility of significant morbidity, and even mortality, is that of scoliosis associated with FD of the vertebral column. FD in the spine was thought to be an uncommon occurrence, probably because it is difficult to detect on plain radiographs. However, it has been shown recently that when the sensitive detection technique of ^{99}Tc-MDP bone scanning is used, FD in the spine can be seen in up to 63% of the patients with FD, and that in 64% of the patients who had FD in the spine there was associated scoliosis (Leet *et al.*, 2004b). Importantly, although scoliosis can be progressive and associated with significant morbidity and mortality, it is also amenable to surgical correction, and the surgical correction is effective and lasting— more lasting than most other surgical interventions in FD.

Bisphosphonates are a useful adjuvant in the treatment of FD. Early reports contained great enthusiasm for the use of bisphosphonates in having a significant effect on the natural history of the disease (Chapurlat *et al.*, 1997; Liens *et al.*, 1994; Parisi *et al.*, 2001; Zacharin and O'Sullivan, 2000). However, subsequent studies and longer follow-up have not substantiated this benefit (Chan and Zacharin, 2006; Plotkin *et al.*, 2003). Yet, it is clear that bisphosphonates are quite effective in the relief of pain associated with FD (Chapurlat, 2006; Lala *et al.*, 2006; Plotkin *et al.*, 2003). Pain is one of the most significant causes of disease-related loss of quality of life in FD (Kelly *et al.*, 2005, 2007b). The pain can often be debilitating and require the use of narcotic analgesics. Quite often this pain can be controlled with the use of high-dose intravenous bisphosphonates. However, it has been our experience that the pain of craniofacial FD is often less amendable to treatment with bisphosphonates. Other medical treatments have been tried, including calcitonin (Bell *et al.*, 1970), glucocorticoids(Di Figlia, 1951), and external beam radiation (Tanner *et al.*, 1961), but with no benefit. In fact, it has been demonstrated that external beam radiation is probably associated with an increased rate of sarcomatous transformation (Ruggieri, P. *et al.*, 1994).

In summary, FD is a complicated disease that results from somatic mutations in *GNAS* that occur early in embryogenesis and lead to a specific mosaic pattern of involved bones that is established early in development. The intrinsic genetic defect can be impacted by associated endocrine dysfunction that can occur as part of MAS, and highlights the need for screening for and treatment of the associated endocrine diseases. Although a consensus on surgical treatment is lacking, progress has been made. To date medical treatment appears to be palliative, with bisphosphonates offering significant benefit in terms of pain control. Improvement in both medical and surgical care is needed, and given the relative rarity of the disease, will only come from multicenter, international cooperative studies.

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Chapter 68

Renal Osteodystrophy

Pathogenic Mechanisms and Therapeutic Options

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INTRODUCTION

Renal osteodystrophy has been considered traditionally to encompass all of the disorders of mineral metabolism that occur among persons with chronic kidney disease (CKD) (Coburn and Slatopolsky, 1990; Goodman *et al.*, 2003a; Lentz *et al.*, 1978; Malluche and Faugere, 1990). These include most prominently disturbances in calcium, phosphorus, and magnesium metabolism, in vitamin D metabolism, and in parathyroid gland function (Cozzolino *et al.*, 2005; Llach and Massry, 1985; Mawer *et al.*, 1973; Slatopolsky and Bricker, 1973). Each is affected adversely to a degree that varies when the excretory and metabolic functions of the kidney become impaired as renal function declines. Either alone or together, these abnormalities lead to discrete types of metabolic bone disease that are described collectively as the renal bone diseases (Coburn and Slatopolsky, 1990; Goodman *et al.*, 2003a; Lentz *et al.*, 1978; Malluche and Faugere, 1990).

Apart from untreated patients with CKD, certain therapeutic interventions such as the use of vitamin D sterols to treat secondary hyperparathyroidism (HPT) and the use of phosphate-binding agents to manage phosphorus retention influence bone metabolism both directly and indirectly through effects on mineral homeostasis systemically (Goodman *et al.*, 1994; Indridason and Quarles, 2000). They too contribute to skeletal abnormalities in some patients with CKD whether or not treatment with dialysis is required (Hernandez *et al.*, 1994).

Information is now available that provides a better understanding about the relationships among the renal bone diseases, the abnormalities in mineral metabolism associated with them, and the occurrence of soft-tissue and vascular calcification among patients with CKD (Blacher *et al.*, 2001; Goodman *et al.*, 2004; London *et al.*, 2002). Vascular calcification, specifically arterial calcification, is common in CKD (Kramer *et al.*, 2005). This has been known for many years, but recent evidence indicates that vascular calcification has substantial and specific adverse consequences on the cardiovascular system (Blacher *et al.*, 2001; London *et al.*, 2002). It may contribute to the very high rates of morbidity and mortality from cardiovascular causes among patients with CKD (Blacher *et al.*, 2003; London *et al.*, 2003; London, 2003).

Vascular calcification has long been considered to be one consequence of the broader problem of soft-tissue calcification that occurs among patients with CKD (Kuzela *et al.*, 1977; Parfitt, 1969). The disorder was thought to arise primarily from the passive, dystrophic deposition of mineral in soft tissues owing largely to alterations in calcium and phosphorus metabolism. A growing body of evidence indicates, however, that arterial calcification in CKD is a regulated process that is affected, at least in part, by certain genes and proteins that are normally involved in the regulation of bone metabolism (Giachelli, 2004; Shanahan *et al.*, 1999; Shanahan, 2005). Similar relationships have been documented in the calcification of atherosclerotic plaques and cardiac valves among persons from the general population (Bostrom *et al.*, 1993; Levy *et al.*, 1983; Shanahan *et al.*, 1994).

Because bone disease has been implicated as a contributor to cardiovascular calcification among patients with CKD, it has been suggested that vascular calcification be included as an integral component of renal osteodystrophy as understood traditionally (Moe *et al.*, 2006). In this context, renal bone disease is considered to be only one part of a broader disease entity or syndrome that encompasses the various clinical, biochemical, and radiographic consequences of abnormal mineral metabolism among patients with CKD that includes alterations in bone mass and/or bone density. Soft-tissue and cardiovascular calcification

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TABLE I A Suggested Framework for the Classification of the Syndrome of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD)[a]

Type	Laboratory abnormalities (L)[b]	Bone disease (B)[c]	Vascular or soft-tissue calcification (C)[d]
L	Present	Absent	Absent
LB	Present	Present	Absent
LC	Present	Absent	Present
LBC	Present	Present	Present

[a] *Adapted from* Moe *et al.*, 2006.

[b] L, *abnormal values for serum or plasma concentrations of for calcium, phosphorus,* PTH, *alkaline phosphatase, or metabolites of vitamin* D.

[c] B, *bone disease as documented by abnormalities in of bone turnover* (T), *mineralization* (M), *or volume* (V) *using bone histomorphometry, reductions in linear growth, or evidence of reductions in bone strength.*

[d] C, *calcification in vascular or other soft tissues.*

represent important components of this newly defined clinical syndrome (Moe *et al.*, 2006).

The term Chronic Kidney Disease-Mineral and Bone Disorder, or CKD-MBD, has recently been introduced to displace the traditional definition of renal osteodystrophy as summarized in a position statement from the Kidney Disease Improving Global Outcomes (KDIGO) initiative (Moe *et al.*, 2006). A framework for classifying patients with CKD-MBD has also been proposed based on the presence or absence of three key elements of this newly defined clinical syndrome (Table I). These elements are biochemical abnormalities as measured by selected laboratory parameters of mineral metabolism, bone disease as determined by bone morphometry, and evidence of vascular and/or soft-tissue calcification.

Within the context of the syndrome of CKD-MBD, the term renal osteodystrophy is now defined more narrowly to refer solely to the bone pathology that affects patients with CKD (Moe *et al.*, 2006). Renal bone disease thus represents but one aspect of CKD-MBD. It is judged by laboratory assessments of bone morphology using bone histomorphometry to characterize three key components of skeletal health, specifically bone turnover (T), bone mineralization (M), and bone volume (V) (Fig. 1).

In quantitative terms, bone turnover can be normal, reduced, or elevated, whereas mineralization may be either normal or impaired (Table II). The assessment of each parameter requires measurements obtained in bone biopsy samples evaluated using the technique of double-tetracycline labeling. Bone volume is determined separately, and values may be normal, diminished, or elevated as judged by static measures of bone morphology, which provide information about the volume of bone per unit volume of skeletal tissue (see Table II). Such findings are crucial to understanding the impact of the renal bone diseases on bone mass, the relationship between various types of renal osteodystrophy and osteoporosis as defined traditionally, and their aggregate effects on the structural integrity of bone and on the risk of skeletal fracture among patients with CKD (Martin *et al.*, 2004; Moe *et al.*, 2006).

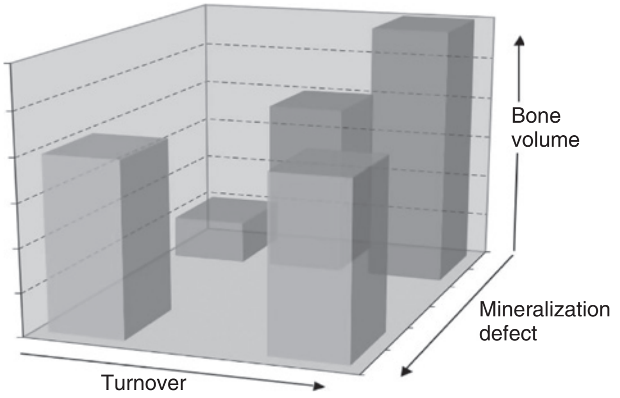

FIGURE 1 The classification of renal osteodystrophy based upon measurements of bone turnover (T), mineralization (M), and bone volume (V) as measured by bone histomorphometry. (From reference (Moe *et al.*, 2006)).

OVERVIEW OF THE RENAL BONE DISEASES

From one perspective, the renal bone diseases represent a set of discrete pathological entities when assessed by bone histomorphometry in biopsy specimens obtained from patients with CKD (see Fig. 1). This categorical method has been used for many years, and it serves to highlight

TABLE II A System for Classifying Renal Osteodystrophy, or Renal Bone Disease, Based on Measurements of Bone Turnover (T), Mineralization (M), and Volume (V) Using Bone Histomorphometry

Turnover	Mineralization	Volume
High		High
Normal	Normal	Normal
Low	Abnormal	Low

Adapted from Moe et al., 2006.

TABLE III Classification of Chronic Kidney Disease (CKD)

Stage of CKD	GFR range (mL/min/1.73 m^2)
1	≥90
2	60–89
3	30–59
4	15–29
5	<15, dialysis

From Eknoyan et al., 2003.

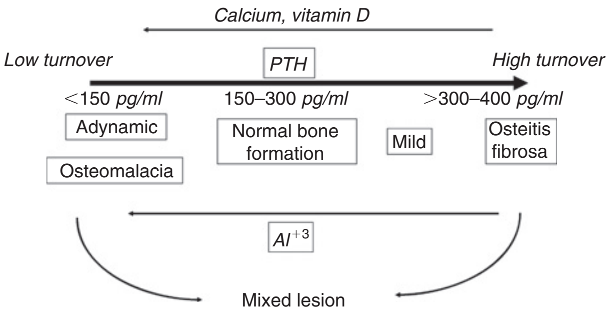

FIGURE 2 The spectrum of renal osteodystrophy. (From reference (Salusky and Goodman, 1995)).

the importance of certain pathogenic factors for some skeletal lesions (Malluche and Faugere, 1990; Sherrard *et al.*, 1993). The approach may understate, however, the role of other key elements such as variations in parathyroid gland function that account for the development and progression of several of the renal bone diseases and the importance of common pathogenic mechanisms among them. An integrated view of renal osteodystrophy thus places somewhat greater emphasis on mechanisms that account for transitions among various skeletal lesions over time (Fig. 2). Both approaches are informative and they share central common elements (Goodman *et al.*, 2003a; Salusky and Goodman, 1995).

Hyperparathyroidism

Histological manifestations of hyperparathyroidism in bone occur in most patients with CKD who have persistently elevated plasma parathyroid hormone (PTH) levels (Quarles *et al.*, 1992; Salusky *et al.*, 1988; Sherrard, 1986; Sherrard *et al.*, 1993; Wang *et al.*, 1995). The extent and severity of such changes correspond generally to the magnitude of the increase in plasma PTH. The skeletal response to PTH differs substantially, however, between patients classified as stage 5 CKD, who require treatment with dialysis, and those with less advanced CKD, that is, CKD stages 1 to 4 (Table III). Plasma PTH levels that are four to five times higher than the upper limit of the normal reference range are required before the skeletal features of hyperparathyroidism develop among patients undergoing dialysis (Qi *et al.*, 1995; Quarles *et al.*, 1992; Salusky *et al.*, 1988; Sherrard, 1986; Sherrard *et al.*, 1993; Wang *et al.*, 1995). In contrast, the skeletal changes owing to hyperparathyroidism are evident when plasma PTH values exceed only modestly the upper limit of normal among patients with stage 3 or stage 4 CKD (Kanis *et al.*, 1979). Tissue resistance to the biological actions of PTH probably accounts for this difference, but the mechanisms responsible are not understood fully (Massry *et al.*, 1973, 1979).

Apart from biochemical assessments of the severity of secondary HPT as judged by plasma PTH measurements, bone histology is useful for differentiating subjects with less advanced skeletal lesions, or mild secondary hyperparathyroidism, from those with overt disease, or osteitis fibrosa cystica (Table IV) (Salusky *et al.*, 1988). Measurements of the rates of bone formation and bone remodeling using the technique of double-tetracycline labeling are often required to discriminate adequately between the two disorders and to distinguish patients with mild lesions of secondary HPT from normal (Goodman *et al.*, 1994; Salusky *et al.*, 1988, 1998; Sanchez *et al.*, 1998).

Bone formation and turnover rates in most patients with secondary HPT exceed those of persons with normal renal and parathyroid gland function (Quarles *et al.*, 1992; Salusky *et al.*, 1988; Sherrard, 1986; Sherrard *et al.*, 1993; Wang *et al.*, 1995). By using the suggested classification scheme for renal osteodystrophy as defined recently in the context of CKD-MBD, bone turnover (T) is high in hyperparathyroidism owing to CKD, whereas mineralization (M) is normal (see Table IV) (Moe *et al.*, 2006). Assessments of bone volume (V) may differ, however, according to the skeletal site chosen and the method of measurement. Bone volume may be normal or high in cancellous bone when evaluated by histomorphometry, but values are normal

TABLE IV Histological Features of High-Turnover Renal Osteodystrophy

	Mild lesion of secondary HPT	Osteitis fibrosa
Bone volume		
Trabecular bone volume	Normal	Normal or high
Bone formation		
Osteoid volume	Normal or high	Normal or high
Osteoid seam thickness	Normal or high	Normal or high
Osteoid surface	Normal	Normal or high
Number of osteoblasts	High	Very high
Bone formation rate	High	Very high
Mineralization lag time	Normal	Normal
Bone resorption		
Eroded surface	High	Very high
Number of osteoclasts	High	Very high
Marrow fibrosis	Absent	Present

TABLE V Histological Features of Low-Turnover Renal Osteodystrophy

	Adynamic	Osteomalacia
Bone volume		
Trabecular bone volume	Normal or low	Variable Low, normal, or high
Bone formation		
Osteoid volume	Normal or low	High or very high
Osteoid seam thickness	Normal or low	High or very high
Osteoid surface	Normal or low	High
Number of osteoblasts	Low	Low
Bone formation rate	Low or immeasurable	Low or immeasurable
Mineralization lag time	Normal*	Prolonged
Bone resorption		
Eroded surface	Normal or low	Low
Number of osteoclasts	Low	Low or normal, but may be high
Marrow fibrosis	Absent	Absent

**As measured by conventional histomorphometric methods, the mineralization lag time (Mlt), which reflects the average value for all osteoid seams, may be prolonged in adynamic renal osteodystrophy. In contrast, the osteoid maturation time (O.mt), which represents values for osteoid seams that are undergoing active mineralization as judged by the uptake of tetracycline into bone, is normal in the adynamic lesion. The disparity between values for Mlt and O.mt is attributable to the lower proportion of osteoid seams undergoing active mineralization at any given point in time.*

or low when measurements are obtained in cortical bone either by histomorphometry or by radiographic imaging methods (Parfitt, 2003; Schober *et al.*, 1998).

Hypoparathyroidism and Adynamic Bone

Dialysis patients who have plasma PTH levels substantially lower than those seen typically in cases of established secondary HPT often display histological features in bone that are more characteristic of persons with hypoparathyroidism, a disorder known as adynamic renal osteodystrophy (Hercz *et al.*, 1993; Salusky *et al.*, 1988; Sherrard, 1986). Unlike hyperparathyroidism, bone turnover (T) is reduced, and this is a cardinal feature of the adynamic lesion. Mineralization (M) is normal, and bone volume (V) is usually maintained (Table V). Bone volume is reduced, however, if osteoporosis is the underlying cause. Decreases in cancellous bone volume may also occur in adynamic bone owing to aluminum toxicity (Faugere *et al.*, 1986; Faugere and Malluche, 1986).

Adynamic bone can arise from a variety of causes (Table VI). In many but not all, parathyroid gland function is impaired and plasma PTH levels are uncharacteristically low, particularly among patients with stage 5 CKD, but this aspect of the disorder has not been assessed fully (Hercz *et al.*, 1993; Pei *et al.*, 1993; Sanchez *et al.*, 1995). Causal factors for adynamic bone include diabetes, aluminum-related bone disease, steroid-induced osteoporosis, immobilization, and sustained reductions in plasma PTH levels owing to treatment with vitamin D sterols and/or calcium-containing compounds or after parathyroidectomy (Hercz *et al.*, 1989, 1993; Pei *et al.*, 1993; Salusky and Goodman, 2001; Wang *et al.*, 1995). Some causes of adynamic bone can be corrected, whereas others can not. Racial and gender differences in bone turnover may also account for adynamic renal osteodystrophy in some patients with CKD (Garabedian *et al.*, 1981; Parfitt *et al.*, 1997; Sawaya *et al.*, 2003).

Unfortunately, the prevalence of these various factors, and their importance as causes of adynamic renal osteodystrophy in the current dialysis population, has not been studied systematically. Even less is known about such relationships among patients with less advanced CKD. Certain causes of adynamic bone are unique to patients

TABLE VI Causes of Adynamic Bone

Sustained	Reversible
Hypoparathyroidism	Vitamin D therapy
After parathyroidectomy	Exogenous calcium loading • Oral/dietary • Ca-based phosphate binders • Dialysate
Steroid-induced osteoporosis	Immobilization
Osteoporosis • Estrogen deficiency • Aging	Bone aluminum toxicity
Diabetes	

with stage 5 CKD who require treatment with dialysis (see later) (see Table VI).

The histological features of adynamic renal osteodystrophy do not differ substantially from those of postmenopausal or age-related osteoporosis (Goodman *et al.*, 2003a; Sherrard, 1986; Sherrard *et al.*, 1996). It is thus difficult to distinguish between adynamic bone that arises solely from factors unique to CKD and adynamic bone owing to osteoporosis among older women, or even older men, who are treated with dialysis regularly. Histomorphometric assessments of bone volume per unit volume of bone tissue and tetracycline-based measurements of bone turnover may be informative in this regard, but they are often not definitive. Bone volume is low in osteoporosis, but values are often normal in adynamic bone from other causes (Goodman *et al.*, 2003a; Sherrard, 1986; Sherrard *et al.*, 1996).

Additional information is needed to better understand the role of age-related factors such as osteoporosis that are distinct from the renal bone diseases *per se* as contributors to the very high rates of skeletal fracture, not only among patients undergoing dialysis, but also among those with less advanced CKD (Danese *et al.*, 2006; Leinau and Perazella, 2006; Stehman-Breen *et al.*, 2000). The observation that more than half of the current dialysis population in the United States are 65 years of age or older only further highlights the importance of this issue (United States Renal Data System: Annual Data Report, 2005).

Osteomalacia

Osteomalacia is a skeletal disorder characterized by the inadequate mineralization of newly formed bone collagen, or osteoid, that leads to the accumulation of excess amounts of unmineralized osteoid within cortical and cancellous bone (Eastwood *et al.*, 1977; Frame and Parfitt, 1978; Malluche *et al.*, 1979). Such changes compromise the structural integrity of bone and increase the risk of skeletal fracture (Pierides, 1978). Skeletal mineralization (M) is impaired and bone turnover (T) is reduced (see Table V). Bone volume (V), which includes both mineralized tissue and unmineralized osteoid, may be normal, but values are often elevated in trabecular bone when assessed by histomorphometry (Coburn *et al.*, 1980; Llach *et al.*, 1984).

Osteomalacia is classically associated with nutritional vitamin D deficiency, but other causes may be found among patients with CKD (Frame and Parfitt, 1978; Llach *et al.*, 1984; Ott *et al.*, 1983). Sustained reductions in serum calcium and/or phosphorus concentrations overextended periods to levels that are insufficient to adequately support skeletal mineralization account for osteomalacia in some cases of CKD (Baker *et al.*, 1974; Eastwood *et al.*, 1976; Frame and Parfitt, 1978; Sherrard *et al.*, 1974). Others are unique to patients managed by dialysis. Specific examples include the accumulation in bone of excess amounts of metals such as aluminum, iron, and strontium that directly and adversely affect skeletal mineralization (D′Haese *et al.*, 2000; Hodsman *et al.*, 1981, 1982).

Mixed Renal Osteodystrophy

Assessments of bone histology in some patients with CKD demonstrate changes of both hyperparathyroidism and osteomalacia (Malluche *et al.*, 1976; Ritz *et al.*, 1978; Sherrard *et al.*, 1974). The disorder is best described as the mixed lesion of renal osteodystrophy, and it represents the combined result of two distinct pathogenic processes (Sherrard *et al.*, 1974). Patients typically have biochemical evidence of secondary HPT with elevated plasma PTH levels, changes that account for the histological manifestations of hyperparathyroidism in bone. Other disturbances, however, are responsible for the concurrent defect in skeletal mineralization. The same pathogenic factors discussed previously as causes of osteomalacia are usually responsible for impaired mineralization in mixed renal osteodystrophy.

Bone turnover (T) in the mixed lesion reflects the integrated result of hyperparathyroidism, which promotes skeletal remodeling, and osteomalacia, which diminishes it. Turnover is elevated if hyperparathyroidism is the dominant component, whereas turnover will be low if osteomalacia predominates. Mineralization (M) is suboptimal, leading to osteoid accumulation both in cortical and in cancellous bone. Bone volume (V) may be normal, but values are elevated if the mineralization defect is pronounced and the accumulation of osteoid is extensive (Schultz *et al.*, 1984; Sherrard *et al.*, 1974).

Renal Bone Disease as a Dynamic Disorder

Results from studies that have used bone histomorphometry to assess biopsy samples obtained sequentially provide evidence that the histological features of renal bone osteodystrophy do not remain static but rather evolve over time (Goodman and Salusky, 1991; Hercz *et al.*, 1988; Hercz *et al.*, 1994; Salusky *et al.*, 1998; Sanchez *et al.*, 1998). The skeletal changes of secondary HPT thus diminish or revert toward normal as plasma PTH levels decrease during successful medical management (Keeting *et al.*, 1992). Adynamic bone can develop, however, if plasma PTH levels are lowered excessively or if values remain suppressed for sustained periods during treatment with vitamin D sterols or after surgical parathyroidectomy (Goodman *et al.*, 1994). Conversely, the histological changes of secondary HPT worsen as plasma PTH rise progressively in untreated patients or in those who do not respond adequately to medical therapy (Goodman and Salusky, 1991; Salusky *et al.*, 1998).

In this regard, the skeletal lesions of secondary HPT and adynamic bone can be considered to represent different portions of a spectrum of disorders determined largely by differences in parathyroid gland function among patients with CKD (see Fig. 2) (Goodman *et al.*, 2003a; Salusky and Goodman, 1995). Transitions from one skeletal lesion to another along this continuum are determined largely by interval changes in plasma PTH levels. Because PTH is such an important determinant of bone formation and skeletal remodeling among patients with CKD, the presence or absence of secondary HPT, and its severity, is a pivotal mediator of the development, progression, and resolution of certain types of renal bone disease, specifically hyperparathyroidism and adynamic bone (Salusky and Goodman, 1995).

Apart from PTH, other factors influence bone formation and skeletal remodeling more directly, and these can account for the development and resolution of renal bone disease by PTH-independent mechanisms. Corticosteroid therapy, bone aluminum toxicity, and osteoporosis all affect bone metabolism adversely, diminish bone formation, and can account for adynamic skeletal lesions (Avioli, 1984; Coburn *et al.*, 1986; Freundlich *et al.*, 2004; Kim *et al.*, 2006; Schot and Schuurs, 1990; Taal *et al.*, 1999). Vitamin D deficiency and bone aluminum deposition disrupt skeletal mineralization and lead to osteomalacia (Coburn *et al.*, 1986; Frame and Parfitt, 1978). These skeletal disorders are largely unrelated to alterations in parathyroid gland function, and different strategies are required to address them therapeutically.

PATHOGENIC MECHANISMS IN RENAL BONE DISEASE

Hyperparathyroidism: High-Turnover Renal Bone Disease

A detailed discussion of the pathogenesis of secondary HPT owing to CKD is beyond the scope of the current chapter. The matter has been considered elsewhere in this volume and it has been reviewed comprehensively by several authors (Goodman and Quarles, 2007; Silver *et al.*, 2002; Slatopolsky and Delmez, 1994). Several points, however, deserve comment. Secondary HPT develops early during the course of progressive CKD as an adaptive response to maintain serum calcium concentrations and to preserve calcium homeostasis systemically. It arises primarily because of reductions in the synthesis of calcitriol, or 1,25-dihydroxyvitamin D, by the diseased kidney, and it can be aggravated by inadequate dietary calcium intake.

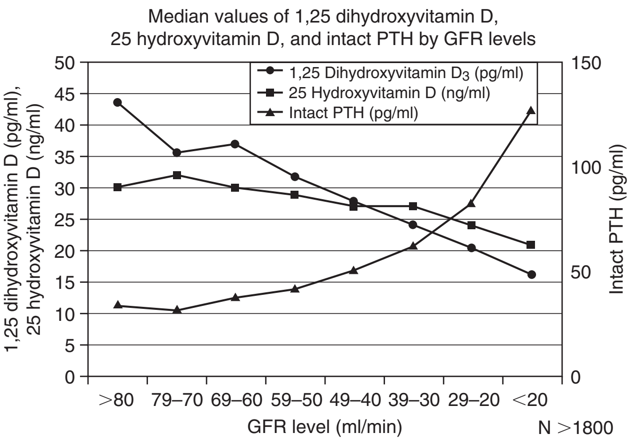

FIGURE 3 The serum levels of 1,25-dihydroxyvitamin D and parathyroid hormone (PTH) according to estimated glomerular filtration rate among patients with chronic kidney disease. (From reference (Levin *et al.*, 2007)).

Calcitriol is produced normally in cells of the proximal nephron (Nykjaer *et al.*, 1999). It is then released into the blood where it circulates in plasma bound to vitamin D-binding protein, or DBP, and interacts with its receptor, the vitamin D receptor, or VDR, in various tissues (Holick, 2006, 2007). Calcitriol functions systemically as a calcium-regulating hormone, and it serves as a key determinant of intestinal calcium absorption by regulating the expression of several proteins that mediate calcium transport in epithelial cells (Hoenderop *et al.*, 2005).

Serum calcitriol levels fall gradually as CKD progresses owing largely to a loss of renal parenchyma (Fig. 3) (De Boer *et al.*, 2002). Subtle changes in phosphorus metabolism and increases in the circulating levels of fibroblast growth factor 23 (FGF23), an important phosphate-regulating hormone, may further impede renal calcitriol production (Gutierrez *et al.*, 2005). Low levels of calcitriol in the circulation adversely affect vitamin D-dependent intestinal calcium transport and are largely responsible for impaired intestinal calcium absorption in CKD (Brickman *et al.*, 1975; Coburn *et al.*, 1973b). Such changes also explain the hypocalciuria that is a cardinal feature of untreated patients with mild to moderate CKD (Coburn *et al.*, 1973a, 2004).

As in other forms of secondary HPT where intestinal calcium absorption is impaired, such as that owing to mild to moderate vitamin D deficiency, increases in PTH

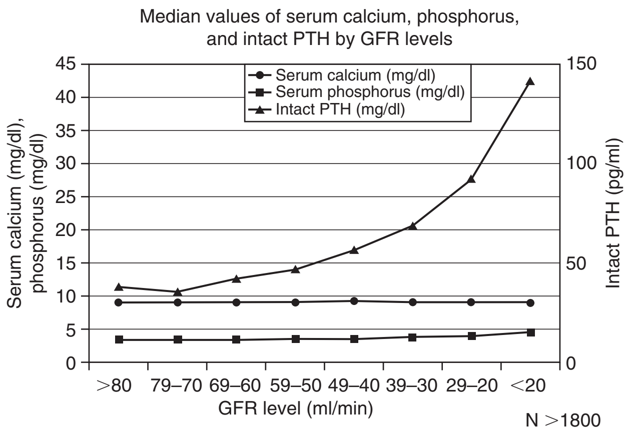

FIGURE 4 The serum concentrations of calcium and phosphorus and plasma parathyroid hormone (PTH) levels according to estimated glomerular filtration rate among patients with chronic kidney disease. (From reference (Levin *et al.*, 2007)).

synthesis and secretion by the parathyroid glands represent an appropriate physiological response to maintain serum calcium levels. Plasma PTH levels thus rise progressively when serum calcitriol levels and intestinal calcium absorption decline as renal function deteriorates, and values exceed the upper limit of normal in the majority of those with an estimated glomerular filtration rate below 30 to 40 mL/min (see Fig. 3) (De Boer *et al.*, 2002). This adaptive response is sufficient to prevent measurable decreases in serum calcium concentration in most patients (Fig. 4) (Goodman and Quarles, 2007). Overt hypocalcemia develops only when these compensatory responses no longer prove adequate. As such, correcting disturbances in calcium and vitamin D metabolism that arise owing to CKD represent important therapeutic measures for the treatment and prevention of secondary HPT.

Phosphorus retention owing to decreases in phosphorus excretion in the urine leads ultimately to the development of hyperphosphatemia among patients with advanced CKD, and serum phosphorus levels are elevated in nearly all patients undergoing dialysis regularly (Levin *et al.*, 2007). Hyperphosphatemia aggravates secondary HPT by contributing to parathyroid gland hyperplasia (Dusso *et al.*, 2001). It also renders treatment of the disorder quite challenging because vitamin D sterols cannot be used safely to lower plasma PTH levels when serum phosphorus concentrations are elevated because of the risks of soft-tissue and vascular calcification (Coburn *et al.*, 1991).

Separately, secondary HPT *per se* can worsen hyperphosphatemia among dialysis patients who have little or no residual renal function. Serum phosphorus levels decrease consistently when plasma PTH levels are lowered in such patients during treatment with calcimimetic agents, compounds that do not promote intestinal phosphorus transport (Goodman *et al.*, 2000a, 2002a). In contrast, serum phosphorus levels remain elevated or increase further when vitamin D sterols are used to lower plasma PTH levels because these agents enhance intestinal phosphorus absorption (Andress *et al.*, 1989; Frazao *et al.*, 2000; Martin *et al.*, 1998). Ongoing PTH-mediated phosphorus release from bone thus contributes materially to the persistent hyperphosphatemia that affects many dialysis patients with secondary HPT.

Once established, secondary HPT is a progressive disorder that increases in severity as a function of the duration of CKD and/or the number years of treatment with dialysis (Chertow *et al.*, 2000; Kestenbaum *et al.*, 2004; Malberti *et al.*, 2001). The likelihood of surgical parathyroidectomy also rises progressively as the duration of renal replacement therapy increases (Foley *et al.*, 2005; Malberti *et al.*, 2001). Parathyroid gland hyperplasia is a key determinant not only of disease severity but also of disease progression. Monoclonal proliferation of subpopulations of parathyroid cells can be demonstrated in more than half of parathyroid glands removed surgically from patients with secondary HPT occurring in both the diffuse and nodular forms of parathyroid gland hyperplasia (Arnold *et al.*, 1995). There is little evidence that the hyperplastic process can be reversed or that the size of enlarged parathyroid glands diminishes with medical treatment or after successful kidney transplantation.

The manifestations of secondary HPT in bone reflect the known biological actions of PTH on skeletal tissue (Parisien *et al.*, 1990a, 1990b). The disorder is characterized by increases in the number of sites of active bone remodeling by basic multicellular units, or BMUs, both in cancellous and in cortical bone (Parfitt, 1976). Bone reabsorption by osteoclasts and bone formation by osteoblasts are both abnormally high, and the proportion of skeletal tissue undergoing remodeling at any given point in time is greater than normal. Such changes lead ultimately to bone loss and to reductions in bone mass because the amounts of mineral removed during the reabsorption phase slightly exceed the amounts replaced during the formation phase with each remodeling cycle (Parfitt, 1983).

Parathyroid hormone enhances bone reabsorption by promoting the differentiation of precursor cells of the monocyte-macrophage lineage into mature osteoclasts (Boyle *et al.*, 2003; Teitelbaum, 2000). The receptor activator of nuclear factor kappa B (RANK), its ligand (RANK-L), and the soluble decoy receptor osteoprotegerin (OPG) serve as key regulators of this process. Both PTH and 1,25-dihydroxyvitamin D enhance the recruitment and differentiation of osteoclasts via this pathway.

Various cytokines and growth factors also affect osteoclastic maturation in an autocrine/paracrine fashion within bone and the adjacent bone marrow (Hruska and Teitelbaum, 1995). They serve indirectly to mediate some of the actions of PTH on osteoclastic bone reabsorption. In this regard, the type 1 PTH receptor, or PTH1R, is expressed exclusively in osteoblasts and osteocytes but

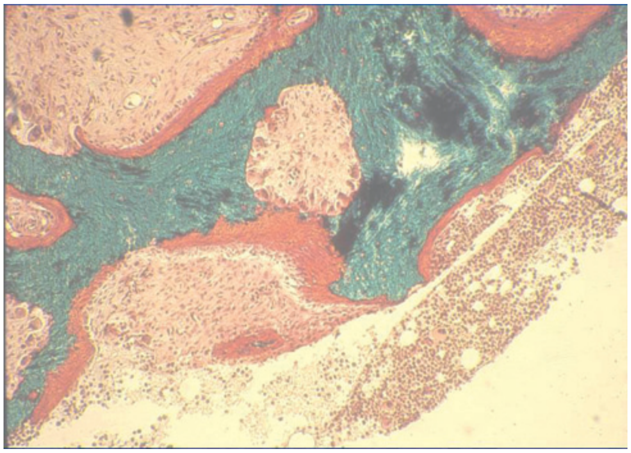

FIGURE 5 A Goldner stained section of undecalcified bone from a hemodialysis patient with osteitis fibrosa; magnification, 50×. Mineralized bone appears green and osteoid appears red. Fibrous tissue has accumulated within the marrow space immediately adjacent to bone, and the serrated margins along the bone surface represent sites of osteoclastic bone resorption. (See plate section)

not in osteoclasts (Jüppner *et al.*, 1991; Kronenberg *et al.*, 1998).

Both the number and size of osteoclasts is greater than normal in hyperparathyroidism, and the fraction of cancellous bone surfaces undergoing reabsorption is increased when assessed by bone histomorphometry (see Table IV). Fibrous tissue often accumulates immediately adjacent to individual bony trabeculae, a change described as peritrabecular fibrosis. More extensive deposits of fibrous tissue may be found throughout the marrow space, and partial or complete fibrous replacement of trabecular structures can occur in advanced cases (Fig. 5).

As noted previously, bone formation rates are elevated in hyperparathyroidism owing to increases in the number of active remodeling sites both in cortical and in cancellous bone. Static histological indices of bone formation such as the fraction of trabecular bone surfaces covered by osteoid seams, the volume of osteoid per unit volume of bone, and the number of osteoblasts along trabecular bone surfaces are thus greater than normal.

The maturation of newly formed bone matrix may be disrupted in overt secondary HPT owing in part to high rates of synthesis of type I collagen by osteoblasts and to the rapid deposition of collagen fibrils as new bone matrix in the extracellular space (Malluche *et al.*, 1976, 1979; Sherrard *et al.*, 1983). Under these circumstances, collagen molecules that are normally arranged tightly and aligned closely in parallel become spatially less well organized. The newly formed osteoid and the subsequently mineralized bone thus have a coarse, hatched appearance microscopically that is described as woven osteoid or woven bone, respectively. The pattern is similar to that of a straw basket. The disordered arrangement of collagen fibrils within bone alters its material properties and adversely affects its structural integrity.

The histological changes of hyperparathyroidism in bone in secondary HPT owing to CKD are generally more pronounced and they are often much more severe than in primary HPT (Goodman *et al.*, 2003a). Among patients receiving dialysis, such differences are most likely caused by the very high plasma PTH levels that characterize these individuals. In contrast, overt histological manifestations of HPT are common among patients with stage 3 or stage 4 CKD who have plasma PTH levels that are substantially lower than those typical of dialysis patients with secondary HPT and not markedly different from those seen in primary HPT (Hamdy *et al.*, 1995). Lesser increases in plasma PTH levels are thus required to invoke the skeletal changes of hyperparathyroidism in persons with mild to moderate CKD compared with those with advanced CKD who require treatment with dialysis.

The reasons for this disparity are not understood. Differences in serum calcitriol levels and variations in the levels of expression of the PTH1R, vitamin D receptor (VDR), and calcium-sensing receptor (CaR) in osteoblasts and other bone cells may be involved (Korkor, 1987; Ureña *et al.*, 1994). Peptide fragments of PTH(1–84) that interact with a putative carboxyl-terminal PTH receptor (C-PTH receptor), distinct from the PTH1R, could also play a role (D′Amour *et al.*, 2005; Divieti *et al.*, 2002, 2005; Nguyen-Yamamoto *et al.*, 2001). There is evidence to support such a mechanism, but its importance will remain uncertain until the receptor is identified and cloned and the signal transduction pathways associated with it are characterized adequately.

Patients with CKD who have less pronounced histopathological changes of hyperparathyroidism in bone are categorized as mild lesions of secondary HPT (see Table IV) (Salusky *et al.*, 1988; Sherrard *et al.*, 1983). Plasma PTH levels are elevated, but values are generally not as high as in patients with overt secondary HPT (Salusky *et al.*, 1994; Sherrard *et al.*, 1993). Tetracycline-based measurements of bone formation and other histomorphometric criteria are needed to distinguish patients with mild skeletal lesions of hyperparathyroidism from those with normal bone histology and bone turnover and from others with adynamic skeletal lesions (see Table IV) (Salusky *et al.*, 1988; Sherrard *et al.*, 1983).

Among patients with CKD that progresses over several or many years before dialysis becomes necessary, secondary HPT represents an important and potentially preventable cause of bone loss (Rix *et al.*, 1999). Bone mass is reduced when renal replacement therapy is begun in a substantial proportion of patients with CKD, in particular, when measurements are obtained at skeletal sites in the appendicular skeleton that are composed mainly of cortical bone (Parfitt, 1998, 2003). Two factors account for this finding.

First, the presence of a large number of bone-remodeling sites, or Haversian systems, within cortical bone increases its porosity among patients with hyperparathyroidism. Additionally, the mineral content of newly completed osteons is less than that of fully mature osteons in older quiescent bone. Both changes diminish the volumetric density of cortical bone as a tissue and lower its mineral content. Second, bone is lost disproportionately from the endosteal surface of long bones in hyperparathyroidism owing to subcortical tunneling by osteoclasts. As a result, cortical bone along endosteal surfaces is transformed structurally to become partially trabecular in nature, thus contributing to reductions in cortical thickness, a common radiographic finding among patients with secondary HPT owing to CKD (Parfitt, 2003).

The Low-Turnover Skeletal Lesions of Relative or Absolute Hypoparathyroidism: Adynamic Bone

The histological features of adynamic renal osteodystrophy differ markedly from those of hyperparathyroidism (Goodman *et al.*, 2003a; Sherrard *et al.*, 1983, 1993). Indeed, they are more consistent with the skeletal changes of hypoparathyroidism from a variety of causes. The volume of osteoid and the width of osteoid seams are either normal or reduced, resorption surfaces are diminished, and few osteoblasts or osteoclasts are seen (Fig. 6). Peritrabecular and marrow fibrosis are distinctly absent. Static and dynamic indices of bone formation are subnormal, and the rate of bone formation often cannot be measured using the technique of double-tetracycline labeling (see Table V) (Andress *et al.*, 1986; Salusky *et al.*, 1988; Sherrard *et al.*, 1983).

Adynamic bone was first described as a discrete entity in dialysis patients with bone aluminum toxicity (Table VI) (Andress *et al.*, 1986). In the past, aluminum-related bone disease owing to aluminum retention was common among patients managed with dialysis (Coburn *et al.*, 1986). Key sources of aluminum exposure included aluminum-based phosphate-binding agents and dialysis solutions that contained aluminum. The use of aluminum-free compounds to manage phosphorus retention and the widespread application of effective methods of water purification in dialysis facilities has markedly diminished these risks. Aluminum-related bone disease is now an infrequent cause of either adynamic bone or osteomalacia (see later) among patients managed with dialysis.

The adynamic lesion arising from bone aluminum toxicity may represent a histological forerunner of overt aluminum-related osteomalacia (Goodman, 1984, 1985). Bone aluminum levels are thus not as high among patients with adynamic bone as in those with osteomalacia when aluminum retention is the cause (Hodsman *et al.*, 1982). The adverse effects of aluminum on osteoblasts are mediated, at least in part, by the uptake of transferrin-bound aluminum by osteoblasts via the transferrin receptor (Kasai *et al.*, 1990, 1991). Several metals such as aluminum, gallium, and iron itself adversely affect the proliferation rate and differentiated function of cells when excess amounts enter the cytoplasm via this pathway.

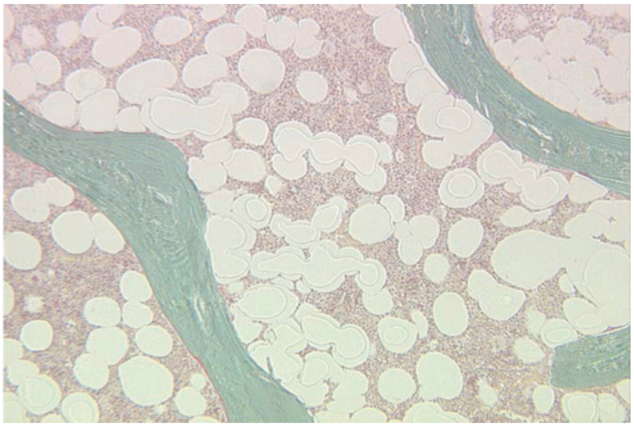

FIGURE 6 A Goldner stained section of undecalcified bone from a hemodialysis patients with adynamic renal osteodystrophy, magnification 50×. Mineralized bone appears green and osteoid appears red. The total amount of osteoid is less than normal, osteoid seams are thin, the fraction of the trabecular bone surface that is covered with osteoid is diminished, and there are no eroded surfaces. (See plate section)

Although aluminum-related bone disease is now uncommon, adynamic lesions still account for 10% to 40% of cases of renal bone disease in the current dialysis population. The overall prevalence of adynamic renal osteodystrophy is even higher now than in the past. Other causes of the disorder probably account for this finding, most notably diabetes and ageing (see Table VI). In this regard, the increased prevalence of adynamic bone in the current dialysis population in the United States corresponds temporally to the progressive rise in the proportion of diabetic patients and persons older than 65 years undergoing renal replacement therapy (United States Renal Data System: Annual Data Report, 2005).

Nearly half of patients receiving dialysis regularly in the United States have diabetes. Reductions in bone formation and turnover, which are key features of adynamic bone, are common among diabetic subjects. Such changes may reflect either insulin resistance or insulin deficiency at the level of bone as a tissue, but subtle alterations in parathyroid gland function consistent with hypoparathyroidism may also contribute (Goodman and Hori, 1984; Hough *et al.*, 1981).

The average age of the dialysis population in the United States, and elsewhere around the world, continues to rise. More than half of patients currently receiving dialysis in the United States are 65 years of age or older (United States Renal Data System: Annual Data Report, 2005). Postmenopausal osteoporosis and age-related osteoporosis both represent potential causes of adynamic bone among older women with CKD (Cunningham *et al.*, 2004).

Estrogen deficiency may also explain the occurrence of adynamic lesions among younger women receiving dialysis because partial or complete amenorrhea is common in such individuals.

Little is known about the role of aging as a contributor to bone disease among older men with CKD. Male osteoporosis is likely to occur, however, in men who require treatment with dialysis just as it does in the general population (Jackson and Kleerekoper, 1990). Men with advanced CKD often have biochemical evidence of gonadal insufficiency as judged by reductions in serum testosterone levels. Whether such changes adversely affect bone remodeling and/or bone mass or account for the development of adynamic bone in male dialysis recipients has not been determined (Khosla *et al.*, 2005).

Additional causes of adynamic renal osteodystrophy among patients with CKD include previous parathyroidectomy and the use of large intermittent doses of vitamin D sterols to manage secondary HPT either alone or together with large oral doses of calcium-containing, phosphate-binding agents (see Table VI) (Andress *et al.*, 1985, 1989; Charhon *et al.*, 1985; Goodman *et al.*, 1994). Treatment-induced decreases in plasma PTH levels, often to values that are quite low, are largely responsible (Goodman *et al.*, 1994). Intermittent, or pulse, calcitriol therapy has been reported to diminish bone formation markedly even when plasma PTH levels remain elevated, a change that may reflect direct inhibitory effects of calcitriol on osteoblasts or on other bone cells (Goodman *et al.*, 1994). Whether similar changes occur with the use of new vitamin D analogues such as paricalcitol or doxercalciferol is not known. The combined use of large intravenous doses of vitamin D sterols and large oral doses of calcium to manage secondary HPT among patients undergoing hemodialysis may thus account for the high prevalence of adynamic bone in the current dialysis population (Pei *et al.*, 1992).

The long-term consequences of adynamic renal osteodystrophy remain uncertain. If bone aluminum toxicity is the cause, bone pain and fractures are prominent features (Coburn *et al.*, 1986). The risk of skeletal fracture has been reported to be greater among dialysis patients with low plasma PTH than those with higher values, but the results available are not conclusive (Atsumi *et al.*, 1999; Coco and Rush, 2000). The prevalence and extent of vascular calcification was found to be greater among hemodialysis patients with low-turnover skeletal lesions as documented by bone biopsy regardless of the cause, which included previous parathyroidectomy, aluminum-related bone disease, and exogenous calcium loading from oral medications (London *et al.*, 2004). Additional work is required to determine whether these findings are attributable to alterations in the regulation of mineral metabolism systemically or to tissue-specific mechanisms that have yet to be characterized. Reductions in linear growth have been described by some but not by others in prepubertal children with adynamic renal osteodystrophy (Kuizon *et al.*, 1998; Schmitt *et al.*, 2003).

Patients with certain glomerular diseases and others with systemic vasculitis, such as systemic lupus erythematosus, are often treated with corticosteroids. These compounds are recognized widely to adversely affect bone metabolism. They not only diminish bone formation markedly, in part, by increasing apoptosis in osteoblasts, but also enhance bone reabsorption (Kim *et al.*, 2006; Weinstein *et al.*, 1998). Together such changes can lead to marked reductions in bone mass. Steroid-induced osteoporosis thus represents another important cause of adynamic renal osteodystrophy.

Osteomalacia: The Other Major Type of Low-Turnover Renal Bone Disease

Osteomalacia was a common type of renal bone disease in the past, but the disorder is seen less often in the current dialysis population. As noted previously, the cardinal feature of osteomalacia is the accumulation of excess amounts osteoid in bone owing to a primary defect in skeletal mineralization (Goodman *et al.*, 2003a). Osteoid seams along trabecular bone surfaces and within cortical Haversian systems are thickened, and they have multiple lamellae. The fraction of trabecular bone surfaces covered with osteoid is increased, often markedly (see Table V). Few cells with features that characterize active osteoblasts are seen adjacent to osteoid seams and most have the flattened, spindle-like profile of resting or inactive osteoblasts. Bone formation rates are subnormal, and measurements often cannot be obtained (see Table V). Although the rates of deposition of new bone matrix and its subsequent mineralization are both reduced, mineralization lags behind matrix formation. The disparity causes osteoid seams to widen and osteoid to accumulate (Goodman *et al.*, 2003a).

Before calcitriol became available for use clinically, hypocalcemia was a common biochemical finding among patients with CKD and in those receiving dialysis (Sherrard *et al.*, 1983). It still occurs in some who are not receiving oral calcium supplements or treatment with vitamin D sterols. Persistently low serum calcium concentrations compromise the mineralization of bone and account for the development of osteomalacia in some patients with CKD (Sherrard *et al.*, 1983; Stauffer *et al.*, 1973). Longstanding hypophosphatemia, although uncommon, can also adversely affect skeletal mineralization (Abrams *et al.*, 1974). Available evidence indicates that the primary physiological role of calcitriol, or 1,25-dihydroxyvitamin D, is to maintain sufficient levels of calcium and phosphorus in serum to adequately support skeletal mineralization (Li *et al.*, 1998). The widespread use of calcium-containing compounds and vitamin D sterols to manage patients with CKD probably explains the relatively low prevalence of osteomalacia in the current dialysis population.

Nutritional vitamin D deficiency is common among patients with CKD as judged by measurements of serum 25-hydroxyvitamin D levels (Eastwood *et al.*, 1976; Thomas *et al.*, 1998). The disorder may aggravate secondary HPT among persons with mild to moderate CKD, and it can cause osteomalacia among those with overt hypocalcemia (Ghazali *et al.*, 1999). Losses of vitamin D-binding protein (DBP) in the urine may further compromise vitamin D nutrition among patients with proteinuria owing to diabetes, amyloidosis, or various types of glomerulonephritis (Malluche *et al.*, 1979). Long-term treatment with phenytoin and/or phenobarbital have been implicated as causes of osteomalacia in persons with normal renal function, and a higher incidence of symptomatic bone disease was reported among dialysis patients receiving these agents (Pierides *et al.*, 1976a).

Nutritional vitamin D deficiency is often overlooked or not considered as a cause of osteomalacia among patients with CKD because of the widespread use of polar metabolites of vitamin D such as calcitriol or paricalcitol to treat secondary HPT, in particular, in those receiving dialysis. Such compounds do not, however, satisfy the nutritional requirements for vitamin D, and they may not support the localized needs for calcitriol in bone and other tissues (Holick, 2006, 2007; Vieth *et al.*, 2007). These agents are not substrates for the 1α-hydroxlase enzymes in extrarenal tissues that regulate calcitriol synthesis locally to fulfill autocrine and paracrine functions. The pleiotropic actions of vitamin D on cell-mediated immunity, inflammation, and other processes in tissues that express the 1α-hydroxlase enzyme are probably mediated through this mechanism (Holick, 2006, 2007). Localized tissue-specific effects of 1,25-dihydroxyvitamin D on cell proliferation and differentiation may also account for the lower incidence of certain cancers, such as breast, prostate, and colon among others, in populations where the prevalence of nutritional vitamin D deficiency is less common than those where it is higher (Holick, 2006, 2007; Vieth *et al.*, 2007).

In the past, bone aluminum toxicity was a frequent cause not only of adynamic bone but also of osteomalacia among patients treated with dialysis (Coburn *et al.*, 1986). Unlike other forms of osteomalacia, hypercalcemia is seen often when aluminum is the cause. The mechanisms responsible for the development of osteomalacia owing to bone aluminum accumulation include adverse effects of aluminum on the recruitment of fully differentiated osteoblasts, inhibition of differentiated osteoblastic functions, and disturbances in the physical-chemical processes of crystal deposition and crystal growth by aluminum ions during skeletal mineralization (Blumenthal and Posner, 1984; Goodman *et al.*, 1984; Posner *et al.*, 1986; Sedman *et al.*, 1987).

Risk factors for aluminum-related osteomalacia included previous parathyroidectomy, a history of renal transplantation and graft failure, bilateral nephrectomy, and diabetes mellitus (Coburn *et al.*, 1986). The ingestion of citrate can markedly enhance intestinal aluminum absorption, and such compounds should not be given to patients with CKD who are taking medications that contain aluminum (Froment *et al.*, 1989; Molitoris *et al.*, 1989). Elevated plasma PTH levels owing to persistent secondary HPT appear to offset the adverse effects of aluminum on bone, which may explain the higher prevalence of aluminum-related bone disease among diabetic patients and those with a history of parathyroidectomy (de Vernejoul *et al.*, 1985; Felsenfeld *et al.*, 1982).

Mixed Lesion of Renal Osteodystrophy

Apart from uncomplicated osteomalacia from any cause, mixed lesions of renal osteodystrophy occur when a mineralization defect develops in individuals with preexisting skeletal manifestations of hyperparathyroidism. Persistent hypocalcemia and/or hypophosphatemia, either alone or arising from nutritional vitamin D deficiency, is responsible in some patients (Sherrard *et al.*, 1974). Impaired skeletal mineralization oiwng to bone aluminum toxicity may occur in others.

Historically, mixed lesions of renal osteodystrophy developed as bone aluminum deposition worsened among patients with previously uncomplicated secondary HPT hyperparathyroidism owing to CKD (Sherrard, 1986). In contrast, osseous changes of hyperparathyroidism could emerge as secondary HPT progressed among patients with established osteomalacia from any cause. Under these circumstances, the histological features of hyperparathyroidism became superimposed on those of osteomalacia, resulting in a mixed histological picture. This occurred in some patients with aluminum-related osteomalacia who responded favorably to treatment with the chelating agent deferoxamine (Sherrard, 1986). Mixed renal osteodystrophy can thus represent a transitional state between the high-turnover skeletal lesions of mild hyperparathyroidism or osteitis fibrosa cystica and the low-turnover lesions of osteomalacia or adynamic bone (Goodman and Leite Duarte, 1991).

Amyloid Deposition in Bone

Localized deposits of amyloid within bone develop in some patients with CKD who are treated with dialysis for 7 to 10 years or longer (Bardin *et al.*, 1985; Cary, 1985). Such deposits are composed of a unique fibril of amyloid derived from β_2-microglobulin (β_2M), a normal plasma constituent (Gejyo *et al.*, 1985; Morita *et al.*, 1985). These occur typically as cystic lesions at the ends of long bones near tendon insertions either in isolation or as multiple cysts (Kleinman and Coburn, 1989). Radiographs are useful for identifying such lesions in the femoral head, femoral neck, and proximal humerus. Pathological fractures can occur at these sites, particularly at the hip. Amyloid cysts are found not infrequently in metacarpal and carpal bones.

Amyloid deposition also occurs in the soft tissues surrounding joints, particularly the shoulder, and it is an important cause of a painful scapulo-humeral arthritis (Kleinman and Coburn, 1989). Extensive deposits of amyloid within or adjacent to cervical vertebrae can lead to destructive spondyloarthropathy, and displacement fractures of the cervical spine can result in spinal cord compression and paralysis (Bai *et al.*, 2004; Kessler *et al.*, 1990). Amyloid deposition is a common cause of medial nerve compression and the carpal tunnel syndrome among long-term hemodialysis patients (Bardin *et al.*, 1985; Bazzi *et al.*, 1995; Kleinman and Coburn, 1989; Koch, 1992).

Symptoms of bone and joint pain and complaints of joint stiffness owing to the localized accumulation of β_2M are difficult to distinguish from those arising from other types of renal bone disease. Key clinical features of dialysis-related amyloidosis include generalized arthritis, erosive arthritis, and joint effusions (Kleinman and Coburn, 1989). These are not manifestations of a generalized metabolic bone disorder but represent the consequences of local amyloid deposition in bone and/or periarticular tissues. Clinical manifestations of dialysis-related amyloidosis appear rarely among patients who have been treated with dialysis for less than five years. The disorder is more common among those undergoing renal replacement therapy for extended periods or who begin dialysis after the age of 50 years (van Ypersele de Strihou *et al.*, 1991).

The fraction of patients afflicted with amyloidosis increases progressively as a function of the number of years of treatment with dialysis (Bazzi et al., 1995; Zingraff and Drüeke, 1991). Clinical management is often unsatisfactory, but interventions are often needed to control pain arising from bone and joint involvement. The carpal tunnel syndrome improves after surgical intervention, but it can reoccur. Successful renal transplantation may provide symptomatic improvement, but there is little evidence that amyloid deposits in bone or in soft tissue resolve after kidney transplantation (Jadoul *et al.*, 1989).

THERAPEUTIC OPTIONS

Hyperparathyroidism: High-Turnover Renal Bone Disease

Effective strategies for managing secondary HPT among patients with CKD address the key pathogenic factors that account for the development and progression of the disorder. These include alterations in calcium and phosphorus metabolism and in vitamin D metabolism and nutrition (Goodman, 2001). Interventions that address each of these disturbances are an integral component of the general clinical management of any patient with CKD whether or not dialysis is required. They are often however, insufficient to fully control secondary HPT among patients with CKD, and more definitive measures may be required (Goodman, 2001).

Treatment with vitamin D sterols and/or with calcimimetic agents is necessary to effectively modify parathyroid gland function, to control plasma PTH levels, and to manage the bone disease of secondary HPT in many patients with CKD (Goodman, 2001). The approach differs substantially, however, between patients with mild to moderate CKD, who have significant residual renal function, and those with little or no residual renal function, who require dialysis. Modest calcium supplementation is often necessary and beneficial among patients with stage 3 or stage 4 CKD, but the use of large doses of calcium may lead to calcium retention and aggravate vascular calcification in those undergoing dialysis (Goodman *et al.*, 2000b). Vitamin D sterols and calcimimetic agents can be used either alone or together to control plasma PTH levels among dialysis patients with secondary HPT (Block *et al.*, 2004b; Goodman, 2002). In contrast, vitamin D sterols represent the only definitive pharmacological intervention for the disorder among patients with less advanced CKD (Coburn and Elangovan, 1998). Calcimimetic compounds are not approved for use in such patients.

Secondary HPT in CKD, Stages 1 to 4

Diet and nutrition

Among patients with CKD who do not require treatment with dialysis, inadequate dietary calcium intake, marginal vitamin D nutrition, and overt vitamin D deficiency can adversely affect calcium metabolism and provide an ongoing stimulus for PTH synthesis and secretion by the parathyroid glands (Coburn *et al.*, 1973a). In more advanced cases of CKD, phosphorus retention and hyperphosphatemia may develop, which can further aggravate secondary HPT (Slatopolsky and Delmez, 1994). Specific interventions are required to address these issues.

Dietary calcium intake is suboptimal in many patients with CKD, particularly in those who adhere to phosphorus-restricted diets that contain few dairy products (Slatopolsky and Delmez, 1994). Such diets are used commonly among patients with stage 3 or stage 4 CKD, but they typically provide only 500 to 600 mg of elemental calcium per day. Hypocalcemia thus occurs in some patients, whereas hypocalciuria is observed consistently among patients with stage 3 or stage 4 CKD who are not receiving oral calcium supplements or vitamin D sterols, results documented repeatedly in recent clinical trials (Charytan *et al.*, 2005; Coburn *et al.*, 2004; Coyne *et al.*, 2006).

Under circumstances where calcium intake is inadequate or intestinal calcium absorption is compromised, increases in PTH secretion owing to secondary HPT diminish calcium excretion in the urine and mobilize calcium from bone (Goodman, 2005b, 2005c). These adaptive responses serve to attenuate reductions in serum calcium concentration. They can be offset by providing additional amounts of calcium in the diet or by giving oral calcium supplements that enhance intestinal calcium transport, reduce PTH secretion, and lower plasma PTH levels (Rudnicki *et al.*, 1993). Dietary calcium supplementation is often required among patients with CKD to maintain a daily calcium intake of 1000 to 1200 mg as recommended currently.

The use of calcium-containing, phosphate-binding agents such as calcium carbonate or calcium acetate can support calcium nutrition among patients with CKD, stages 2 to 4, but these compounds are often not prescribed because serum phosphorus levels remain normal in most cases. If they are given, some of the additional calcium will be absorbed from the intestine predominantly by passive, vitamin D-independent mechanisms (Sheikh *et al.*, 1989). This serves to correct hypocalcemia and lower plasma PTH levels. Calcium excretion in the urine should be monitored regularly and the doses of calcium-based compounds adjusted appropriately to avoid hypercalciuria.

Apart from the need to avoid dietary calcium deprivation, current guidelines suggest that the total intake of elemental calcium both from dietary and medicinal sources not exceed 2000 mg/day among patients with CKD (Eknoyan *et al.*, 2003). The recommendation is based largely on concerns about the use of very large oral does of calcium-containing compounds as phosphate-binding agents among patients undergoing dialysis and the reported association between this therapeutic strategy and the occurrence of soft-tissue and/or vascular calcification (Blacher *et al.*, 2001; Goodman *et al.*, 2000b; Guérin *et al.*, 2000). The risks of calcium retention and/or calcium loading are much lower, however, among patients with less advanced CKD where both urine output and the capacity to excrete calcium in the urine are preserved. Concerns about calcium retention among patients with mild to moderate CKD should not preclude the judicious use of oral calcium supplements to maintain calcium nutrition and to prevent or attenuate the development of secondary HPT.

Dietary phosphorus restriction and phosphate-binding agents are the first interventions recommended traditionally to control secondary HPT owing to CKD even when serum phosphorus concentrations remain within the normal range as summarized in current treatment guidelines (Eknoyan *et al.*, 2003). To be effective, phosphate-binding compounds should be given with meals to sequester phosphorus within the intestinal lumen and thus diminish its absorption. The effect of calcium-containing compounds to lower plasma PTH levels when given to control serum phosphorus levels in this context is caused in part by improvements in calcium nutrition and by increases in net intestinal calcium absorption as already discussed (Bleyer *et al.*, 1999; Emmett, 2004; Pflanz *et al.*, 1994). Calcium-free, phosphate-binding agents may also promote intestinal calcium transport by diminishing the amounts of phosphorus available to form insoluble complexes with calcium in the lumen of the gut, thus leaving more free calcium ions to be absorbed across the intestinal epithelium (Chertow *et al.*, 1999a). Decreases in intestinal phosphorus absorption during treatment with phosphate-binding agents alter phosphorus metabolism systemically and promote renal calcitriol synthesis by enhancing renal 1α-hydroxylase activity, thereby increasing vitamin D-dependent intestinal calcium transport (Portale *et al.*, 1984, 1986, 1989). Reductions in the serum levels of FGF23 may also enhance renal calcitriol production (Llach and Massry, 1985; Moallem *et al.*, 1998; Portale *et al.*, 1984). As such, phosphate-binding agents lower plasma PTH levels primarily by indirect mechanisms.

As noted previously, inadequate vitamin D nutrition and overt vitamin D deficiency are common among patients with CKD (Coburn *et al.*, 2004). Vitamin D nutrition is assessed by measuring the serum level of 25-hydroxyvitamin D, the predominant circulating metabolite of vitamin D. Values less than 30 ng/mL are considered to be inadequate and provide biochemical evidence of vitamin D deficiency. Repletion with generic vitamin D is recommended to maintain serum 25-hydroxyvitamin D levels above this biochemical threshold. Current guidelines suggest that this be done among patients with CKD, stages 1 to 4, but, for reasons that are unclear, the recommendation has not been extended to patients undergoing dialysis (Eknoyan *et al.*, 2003).

Either ergocalciferol, vitamin D_2, or cholecalciferol, vitamin D_3, can be used to restore vitamin D nutrition among patients with CKD. Larger doses are recommended for patients with overt vitamin D deficiency and very low serum 25-hydroxyvitamin D levels compared with those with vitamin D insufficiency and lesser reductions in serum 25-hydroxyvitamin D (Eknoyan *et al.*, 2003). Specific information about the dosing of ergocalciferol is provided in certain practice guidelines because this compound is more widely available for use clinically (Eknoyan *et al.*, 2003). The guidance is based, however, on results obtained in persons with normal renal function, not in those with CKD.

Some authorities suggest that serum 25-hydroxyvitamin D levels much greater than 30 ng/mL are required to fully satisfy the nutritional requirements for vitamin D (Dawson-Hughes *et al.*, 2005; Norman *et al.*, 2007; Vieth *et al.*, 2007). The issue is the focus of ongoing discussions and further research. The biochemical definition of adequate vitamin D nutrition may thus be revised in the future not only for persons with normal renal function but also for those with CKD.

Plasma PTH levels decrease modestly after vitamin D nutrition is restored following treatment with ergocalciferol in some patients with secondary HPT owing to CKD. Greater reductions are seen in stage 3 than in stage 4 CKD, but plasma PTH levels remain elevated in many patients (Zisman *et al.*, 2007). Treatment with ergocalciferol is thus alone insufficient to control secondary HPT in a substantial proportion of patients with stage 3 or stage 4 CKD, but this is not the primary reason for providing either ergocalciferol or cholecalciferol to such individuals. Rather, the goal is to maintain adequate vitamin D nutrition among patients known to be at risk for vitamin D deficiency. Studies to compare the efficacy of ergocalciferol to that of cholecalciferol for correcting vitamin D deficiency or for lowering plasma PTH levels among patients with CKD have not been reported.

Pharmacological interventions

Vitamin D sterols Apart from dietary and nutritional considerations, treatment with vitamin D sterols is required to adequately control plasma PTH levels in many patients with CKD. The primary therapeutic objective is to reduce plasma PTH levels and to manage the bone disease of secondary HPT. Unfortunately, bone biopsy and bone histology are not readily available to most clinicians, and less invasive biochemical tests are typically used to guide clinical management.

Plasma PTH levels have served traditionally as the most reliable biochemical index of the severity of secondary HPT and its skeletal consequences. The results are used widely for clinical diagnosis and for monitoring disease progression (Eknoyan *et al.*, 2003; Goodman *et al.*, 2002b, 2003b). Available guidelines recommend that treatment for secondary HPT be initiated either when plasma PTH levels exceed 70 pg/mL for patients with stage 3 CKD or when values exceed 110 pg/mL for those with stage 4 CKD (Eknoyan *et al.*, 2003). Values above these thresholds are usually associated with histological evidence of hyperparathyroidism in bone (Hamdy *et al.*, 1995).

A number of PTH assays are available commercially (Boudou *et al.*, 2005). The results obtained with many of them differ, sometimes substantially, from those provided by the method used in published reports that serve as the basis for current practice guidelines (Souberbielle *et al.*, 2006). Such differences must be recognized and their potential impact on the interpretation of PTH values should be considered when results obtained with any particular assay are used for diagnostic purposes. Most immunometric assays detect not only full-length PTH, which is comprised of 84 amino acids, but also one or more amino-terminally truncated peptide fragments of PTH (Goodman, 2003b; Goodman *et al.*, 2003b). Variations in the extent of cross-reactivity with these peptide fragments largely explain disparities among results using different PTH assays (Souberbielle *et al.*, 2006).

Several polar metabolites of vitamin D can be used to treat secondary HPT among patients with CKD. Calcitriol became available for this purpose in the late 1970s (Brickman *et al.*, 1972). It was the first vitamin D analogue shown to be consistently effective for correcting hypocalcemia and for reducing plasma PTH levels among patients with secondary HPT owing to CKD, including those receiving dialysis (Brickman *et al.*, 1974a, 1974b).

Treatment with daily oral doses of calcitriol reduces plasma PTH levels by raising serum calcium concentrations, which activates the calcium-sensing receptor (CaSR) in parathyroid tissue and inhibits PTH secretion (Brickman *et al.*, 1974a, 1974b; Llach *et al.*, 1977). Calcitriol also downregulates pre-pro-PTH gene transcription and diminishes PTH mRNA expression (Cantley *et al.*, 1985; Russell *et al.*, 1984; Silver *et al.*, 1985). Successful treatment with oral doses of calcitriol diminishes the histological severity of secondary HPT in bone and may attenuate bone loss from the appendicular skeleton among patients with CKD (Bianchi *et al.*, 1994; Coen *et al.*, 1986). Increases in bone mass, as measured by dual energy x-ray absorptiometry (DXA), have also been reported (Przedlacki *et al.*, 1995; Ruedin *et al.*, 1994). Similar results have been achieved during treatment with daily oral doses of alfacalcidol, or 1α-hydroxyvitamin D_3, which undergoes 25-hydroxylation in the liver to form calcitriol (Hamdy *et al.*, 1995; Kanis *et al.*, 1977; Pierides *et al.*, 1976b; Rix *et al.*, 1999). Alfacalcidol is used widely in Europe, but it is not available for use clinically in the United States.

Because calcitriol and alfacalcidol enhance intestinal calcium absorption, both compounds are useful for correcting hypocalcemia among patients with CKD. Increases in serum calcium concentration are responsible, at least in part, for the effect of these agents to lower plasma PTH levels. Treatment is started with small doses initially, usually 0.125 to 0.25 μg/day of calcitriol or 0.25 to 0.5 μg/day of alfacalcidol. Doses are subsequently adjusted upward, as needed, either to correct hypocalcemia or to control plasma PTH levels if serum calcium and phosphorus levels do not become elevated (Coburn and Elangovan, 1998).

Serum calcium concentrations should be monitored regularly during treatment with either calcitriol or alfacalcidol because the development of hypercalcemia and/or hypercalciuria can adversely affect renal function, cause nephrolithiasis, or lead to nephrocalcinosis (Coburn and Elangovan, 1998; Goodman and Coburn, 1992; Sanchez *et al.*, 1999). These events are uncommon in adults with stage 3 or stage 4 CKD when the daily doses of calcitriol and alfacalcidol do not exceed 0.5 μg and 0.9 μg, respectively (Bianchi *et al.*, 1994; Goodman and Coburn, 1992; Nordal and Dahl, 1988). Caution is warranted, however, when higher doses are used.

Treatment with daily oral doses of 0.125 μg of calcitriol was more effective than placebo in preventing increases in plasma PTH levels during 12 months of follow-up among patients with CKD, stages 3 and 4 (Ritz *et al.*, 1995). Similarly, plasma PTH levels remained controlled for 24 months and the histological changes of hyperparathyroidism in bone improved after 12 months of treatment with daily oral doses of alfacalcidol among adult patients with creatinine clearance values between 15 and 50 mL/min (Hamdy *et al.*, 1995).

Two other analogues of vitamin D are now available for treating secondary HPT among patients with CKD who do not require dialysis (Coburn *et al.*, 2004; Coyne *et al.*, 2006). Doxercalciferol and paricalcitol are both vitamin D_2 derivatives, and they are available currently as oral preparations in the United States. Doxercalciferol, or 1α-hydroxyvitamin D_2, is the vitamin D_2 equivalent of alfacalcidol. In practical terms, it is a prohormone that is converted into the biologically more potent metabolite 1,25-dihydroxyvitamin D_2 after undergoing 25-hydroxylation in the liver. A unique structural modification to the A ring of the secosteroid characterizes paricalcitol, which is 19-nor-1,25-dihydroxyvitamin D_2.

Clinical studies in humans and work in experimental animals suggest that vitamin D_2 analogues are less potent than their vitamin D_3 counterparts in promoting intestinal calcium and phosphorus absorption and in raising serum calcium and phosphorus concentrations (Finch *et al.*, 1999; Gallagher *et al.*, 1994; Takahashi *et al.*, 1997; Weber *et al.*, 2001). They may thus offer a more favorable safety profile than calcitriol and other metabolites of vitamin D_3 when used to treat secondary HPT among patients with mild to moderate CKD in whom episodes of hypercalcemia and/or hyperphosphatemia can adversely affect kidney function. Unfortunately, only limited information is available from controlled clinical trials to support this contention (Sprague *et al.*, 2003), and no controlled clinical trials have been done in patients with stage 3 or stage 4 CKD.

Both doxercalciferol and paricalcitol effectively lower plasma PTH levels when used to treat secondary HPT among patients with CKD, stages 3 and 4 (Fig. 7 and Fig. 8) (Coburn *et al.*, 2004; Coyne *et al.*, 2006). They do so without substantially raising serum calcium or phosphorus concentrations, probably by diminishing pre-pro-PTH gene transcription. In clinical trials lasting 24 weeks, the frequency of episodes of hypercalcemia and/or hyperphosphatemia was no greater among patients treated with daily oral doses of either doxercalciferol or paricalcitol compared with placebo (Coburn *et al.*, 2004; Coyne *et al.*, 2006). Calcium excretion in the urine increased modestly during treatment with both agents, but overt hypercalciuria did not occur. Kidney function did not change from baseline values during treatment with either compound (Coburn *et al.*, 2004; Coyne *et al.*, 2006). Doxercalciferol and paricalcitol are thus generally safe and effective for lowering plasma PTH levels among patients with secondary HPT owing to CKD, stages 3 and 4.

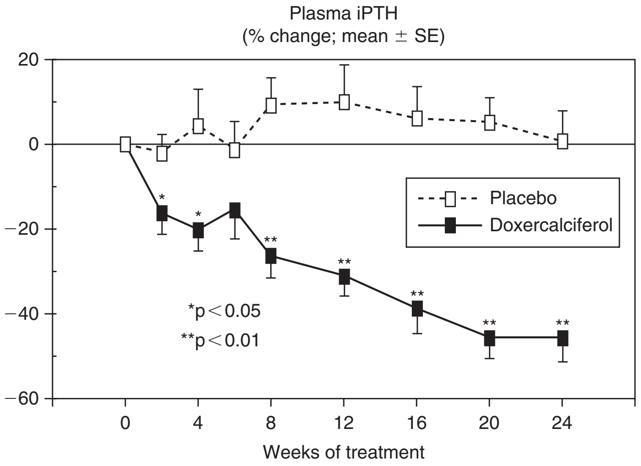

FIGURE 7 The percentage change in plasma PTH levels during 24 weeks of treatment with daily oral doses of either doxercalciferol (solid symbols) or placebo (open symbols) among patients with stage 3 or stage 4 CKD. (From reference (Coburn *et al.*, 2004)).

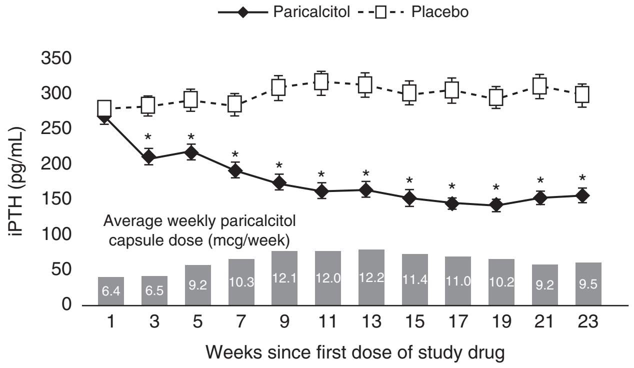

FIGURE 8 Plasma PTH levels during 24 weeks of treatment with daily oral doses of either paricalcitol (solid symbols) or placebo (open symbols) among patients with stage 3 or stage 4 CKD. The results are combined data from three separate randomized, placebo-controlled clinical trials. Paricalcitol was given orally three times per week in two of the studies, whereas daily oral doses were used in the third. Bars depict the average weekly dose of paricalcitol. (From reference (Coyne *et al.*, 2006)).

Long-term safety and efficacy data have yet to be reported with either agent among patients with CKD. The role of either compound in modifying the progression of secondary HPT has not been determined. Similarly, studies to evaluate the impact of treatment with either doxercalciferol or paricalcitol on bone histology or on bone mass have not been done among patients with mild to moderate CKD.

Current guidelines suggest that plasma PTH levels be maintained between 35 and 70 pg/mL among patients with stage 3 CKD and between 70 and 110 pg/mL among those with stage 4 CKD (Eknoyan *et al.*, 2003). Periodic adjustments to the doses of calcitriol or alfacalcidol may be required to achieve these biochemical objectives, but the issue has been evaluated critically in clinical trials. Virtually all studies to date have used prespecified percentage reductions in plasma PTH levels or a plasma PTH level below a predetermined value to assess therapeutic efficacy.

None have been done using plasma PTH values that fall within a specified therapeutic target range to judge biochemical outcomes or to assess drug efficacy.

To limit the risks of soft-tissue and vascular calcification, the dose of any vitamin D sterol should be reduced or treatment withheld temporarily if hypercalcemia or hyperphosphatemia develop (Eknoyan *et al.*, 2003). Treatment should also be withdrawn when plasma PTH levels fall below the lower limit of the therapeutic target range because adynamic renal osteodystrophy can occur when values remain low for sustained periods (Goodman *et al.*, 1994). If plasma PTH levels rise substantially after vitamin D therapy is withdrawn, treatment should be restarted by using smaller doses.

Secondary HPT in CKD, stage 5

Diet and nutrition

As discussed previously for patients with less advanced CKD, measures to support adequate calcium and vitamin D nutrition are also required in many with stage 5 CKD who are treated with dialysis. Nearly all have hyperphosphatemia and most receive phosphorus-restricted diets that contain only limited amounts of elemental calcium. As such, dietary calcium intake is usually inadequate as judged by conventional criteria. Intestinal calcium absorption is suboptimal because endogenous renal calcitriol production is impaired and serum calcitriol levels are reduced, often substantially.

The defect in intestinal calcium transport among patients with stage 5 CKD can be offset partially either by raising the dietary calcium content or by prescribing oral calcium supplements (Indridason and Quarles, 2000). A portion of the additional calcium provided will be absorbed by vitamin D-independent mechanisms (Sheikh *et al.*, 1988, 1989). Unfortunately, the amounts of calcium required to maintain adequate calcium nutrition among patients undergoing dialysis have not been established, and current guidance is based on limited information.

Because the capacity to excrete calcium in the urine is reduced markedly among patients with advanced CKD, it is recommended that the daily intake of elemental calcium not exceed 1500 to 2000 mg/day among those treated with dialysis (Eknoyan *et al.*, 2003). This includes both dietary and medicinal sources. The objective is to limit the risk of calcium retention and to avoid circumstances where total body calcium balance remains positive for extended periods. The use of large oral doses of calcium as a phosphate-binding agent has been associated with soft-tissue and vascular calcification among patients receiving dialysis, and arterial calcification may progress more rapidly among hemodialysis patients managed with calcium-containing compounds compared with those receiving calcium-free compounds (Chertow *et al.*, 2002; Goodman *et al.*, 2000b; Guérin *et al.*, 2000; Spiegel *et al.*, 2007).

When calcium-containing compounds are used exclusively as part of a phosphate-binding strategy, the doses of elemental calcium required to control hyperphosphatemia often far exceed 1500 to 2000 mg/day (Goodman *et al.*, 2000b; Slatopolsky *et al.*, 1986). Such amounts can raise serum calcium concentrations among patients undergoing dialysis regularly, and they can cause episodes of hypercalcemia even in those who are not receiving treatment with vitamin D sterols (Bleyer *et al.*, 1999; Fournier *et al.*, 1986; Moriniere *et al.*, 1989). Calcium-free agents such as sevelamer and lanthanum carbonate are thus often required to manage hyperphosphatemia when the total daily intake of calcium is constrained as recommended in current practice guidelines. Although aluminum-containing, phosphate-binding agents represent another alternative, they should generally be avoided to limit the risks of aluminum retention and toxicity (Coburn *et al.*, 1986).

Sevelamer hydrochloride, or hydrogel of cross-linked poly-allylamine hydrochloride, is an ion exchange polymer that binds phosphorus in the intestinal lumen and diminishes its absorption (Chertow *et al.*, 1997). Sevelamer does not contain calcium or aluminum. It is as effective as calcium acetate for controlling serum phosphorus levels among patients undergoing dialysis, but episodes of hypercalcemia occur less often among those given sevelamer (Chertow *et al.*, 1999b). Daily doses averaging 5 to 6 g are needed to maintain serum phosphorus levels in the range of 5.8 to 6.0 mg/dL, or 1.8 to 2.0 mM, among patients undergoing thrice-weekly hemodialysis, but larger doses are required in many patients (Chertow *et al.*, 1999a). Symptoms arising from the upper gastrointestinal tract such as nausea and vomiting are not uncommon, and some patients complain of bloating or abdominal fullness. The relationship between these side effects and the doses of sevelamer remain uncertain, but they may limit the total amount of sevelamer that can be given to control serum phosphorus levels.

Serum total cholesterol and LDL cholesterol levels decrease by 20% to 30% during treatment with sevelamer, whereas HDL cholesterol levels rise, changes probably attributable to the binding of cholesterol and bile acids within the intestinal lumen (Chertow *et al.*, 1997, 1999a; Wilkes *et al.*, 1998). Serum carbon dioxide levels also decline in some patients, reflecting modest decreases in plasma bicarbonate and mild academia (De Santo *et al.*, 2006). Bicarbonate levels before hemodialysis may thus be lower among patients treated with sevelamer compared with those receiving calcium carbonate. The release of protons from the resin in exchange for inorganic phosphate accounts for this biochemical change. A new formulation of sevelamer that contains carbonate rather than chloride as the exchange anion has been developed to circumvent this problem (Delmez *et al.*, 2007).

Lanthanum carbonate represents another calcium-free, phosphate-binding agent (Hutchison, 1999). Its capacity to bind phosphorus is equivalent to that of aluminum hydroxide, which has long been considered to be among the most potent phosphate-binding agents. The phosphate-binding capacity of lanthanum carbonate exceeds that of calcium acetate, calcium carbonate, and sevelamer (Hutchison, 1999). Unlike calcium-based compounds, the binding of phosphorus by lanthanum is unaffected by pH (Joy and Finn, 2003). Clinical trials using amounts as high as 3000 mg/day, given in divided doses with meals, confirm its efficacy for lowering serum phosphorus levels among patients undergoing dialysis (Al Baaj *et al.*, 2005; Finn and Joy, 2005; Hutchison *et al.*, 2005, 2006). Gastrointestinal symptoms are not uncommon, however, among patients treated with lanthanum carbonate.

A very small fraction of lanthanum is absorbed from the gastrointestinal tract. It can thus be detected in serum and in several tissues, including liver and bone. The implications of these findings remain uncertain. Histomorphometric assessments of bone biopsy samples obtained after several years of treatment with lanthanum carbonate among patients undergoing hemodialysis reveal no adverse effects on skeletal mineralization or on bone remodeling (D'Haese *et al.*, 2003). Safety and efficacy have been documented among patients treated for as long as three years (Hutchison *et al.*, 2006), but additional studies are required to determine whether lanthanum accumulates in bone or other tissues with long-term therapy (D'Haese *et al.*, 2003).

Pharmacological interventions

Use of oral vitamin D sterols For many years, treatment with small daily oral doses of either calcitriol or alfacalcidol was the only definitive therapeutic intervention for secondary HPT among patients undergoing dialysis regularly (Coburn and Elangovan, 1998; Salusky *et al.*, 1987). The doses employed typically ranged from 0.125 to 1.0 μg/day for calcitriol and were approximately twofold higher for alfacalcidol. Both were effective in lowering plasma PTH levels among patients receiving either hemodialysis or peritoneal dialysis (Quarles *et al.*, 1988; Salusky *et al.*, 1987; Salusky and Goodman, 1996). They also raised serum calcium concentrations and were useful for correcting hypocalcemia, a common consequence of CKD during the 1970s and 1980s when aluminum-containing rather than calcium-containing compounds were used widely as phosphate-binding agents.

Episodes of hypercalcemia and/or hyperphosphatemia are frequent dose-limiting side effects of treatment with oral doses of calcitriol or alfacalcidol. Hypercalcemia is not uncommon in adult patients when the dose of calcitriol exceeds 0.50 μg/day. Hypercalcemia develops more often among patients receiving calcium-containing, phosphate-binding compounds, in those with adynamic renal osteodystrophy, and after plasma PTH and serum alkaline phosphatase levels have decreased and approach normal values during treatment (Coburn *et al.*, 1977; Goodman *et al.*, 2003a).

Intermittent oral doses of calcitriol, given twice or thrice weekly, have also been used to manage secondary HPT both in patients receiving hemodialysis and in those undergoing peritoneal dialysis (Kwan *et al.*, 1992; Martin *et al.*, 1992; Moe *et al.*, 1998). Although, larger cumulative weekly doses of calcitriol can be achieved by using an intermittent dosing strategy, there is little evidence that this approach is more effective than daily oral calcitriol therapy for controlling secondary HPT (Bacchini *et al.*, 1997; Caravaca *et al.*, 1995; Liou *et al.*, 1994; Monier-Faugere and Malluche, 1994; Muramoto *et al.*, 1991).

Use of parenteral vitamin D sterols Treatment with intermittent intravenous doses of vitamin D sterols is the most widespread approach for treating secondary HPT among persons undergoing hemodialysis, at least in the United States. The use of oral doses of calcitriol or other vitamin D sterols is now uncommon. In contrast, oral vitamin D therapy continues to be used widely in Europe and elsewhere.

Calcitriol was the first vitamin D sterol to be given intravenously during thrice-weekly hemodialysis sessions to control plasma PTH levels among patients with secondary HPT (Slatopolsky *et al.*, 1984). Potential advantages of the intravenous route of administration included assured patient compliance and the ability to deliver larger cumulative weekly doses of calcitriol in an effort to lower plasma PTH levels. It was also suggested that the high serum levels of calcitriol achieved after bolus intravenous injections were more effective in suppressing pre-pro-PTH gene transcription and in reducing plasma PTH levels, but evidence to support this contention is limited (Fischer and Harris, 1993; Slatopolsky *et al.*, 1984).

In short-term studies of adult hemodialysis patients with mild to moderate secondary HPT, plasma PTH levels decreased promptly within two weeks during treatment with thrice-weekly intravenous doses of calcitriol (Fig. 9)

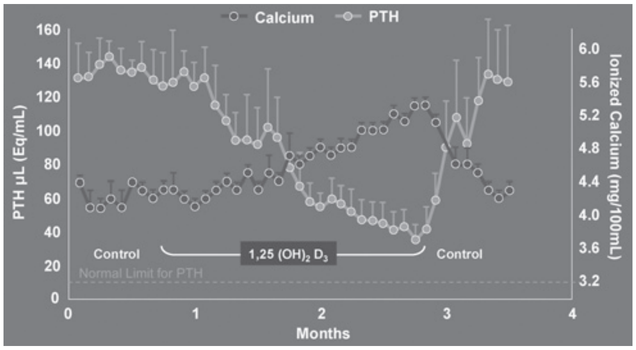

FIGURE 9 Plasma PTH and blood ionized calcium concentrations during two weeks of treatment with thrice-weekly intravenous doses of calcitriol. Plasma PTH levels were measured using a single-antibody mid-region immunoassay for PTH. (From reference (Slatopolsky *et al.*, 1984)).

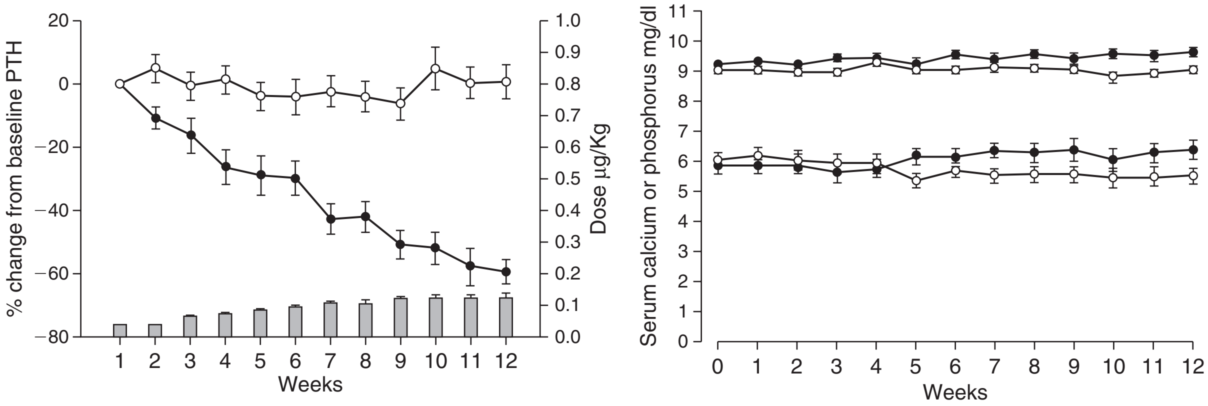

FIGURE 10 The percentage reduction in plasma PTH levels from pre-treatment values during 12 weeks of treatment with thrice-weekly intravenous doses of paricalcitol among hemodialysis patients with secondary hyperparathyroidism (left panel). Bars indicate the average weekly dose of paricalcitol in µg per kilogram of body weight. Serum calcium and phosphorus concentrations at each study visit are also presented (right panel). (From reference (Martin *et al.*, 1998)).

(Slatopolsky *et al.*, 1984). In a separate study of patients undergoing hemodialysis regularly, plasma PTH levels fell by 52% after 12 months and by 71% after 24 months of treatment using doses of calcitriol that ranged from 0.5 µg to 2.25 µg three times weekly (Sprague and Moe, 1992). Greater reductions in plasma PTH levels can be achieved with higher thrice-weekly doses, but values may fall below the target range currently recommended (Dressler *et al.*, 1995). Patients with marked parathyroid gland enlargement are less likely to respond favorably to treatment (Malberti *et al.*, 1996).

Little information has been provided about the efficacy of intravenous calcitriol therapy for controlling plasma PTH levels and for managing secondary HPT beyond two years of follow-up. Data on other important clinical outcomes is also unavailable. These include the impact of treatment on bone mass, on the need for surgical parathyroidectomy, and on skeletal fracture rates (Foley *et al.*, 2005).

As with oral calcitriol therapy, the doses of calcitriol that can be given intravenously to manage secondary HPT are limited by increases in serum calcium and phosphorus concentrations (Slatopolsky *et al.*, 1984; Sprague and Moe, 1992). Such changes can aggravate vascular and soft-tissue calcification. Recurrent episodes of hypercalcemia occur more often among patients ingesting large doses of calcium as a phosphate-binding agent, an approach that remains quite common. Because of these concerns, treatment with intravenous doses of new vitamin D sterols such as paricalcitol and doxercalciferol has largely replaced intravenous calcitriol therapy in the management of secondary HPT among patients undergoing hemodialysis. Other compounds such as 22-oxacalcitriol, or maxacalcitol, have been developed, but they are not available for use clinically in the United States (Brown, 1998).

In a placebo-controlled clinical trial, treatment for 12 weeks with thrice-weekly intravenous doses of paricalcitol lowered plasma PTH levels by an average of 60% from baseline values among patients with moderately severe secondary HPT (Fig. 10) (Martin *et al.*, 1998). Initial doses of 2 to 3 µg were titrated upward to a mean dose of approximately 8 µg thrice weekly to reduce plasma PTH levels. Serum calcium and phosphorus concentrations rose modestly compared with placebo. Episodes of hypercalcemia were more frequent in subjects treated with paricalcitol but often occurred after plasma PTH levels had decreased substantially (Martin *et al.*, 1998). Sustained reductions in plasma PTH levels were achieved among patients treated with thrice-weekly intravenous doses of paricalcitol for 12 months, but greater increases in serum calcium and phosphorus levels were observed during long-term follow-up (Lindberg *et al.*, 2001). The frequency of persistent elevations in serum calcium and/or phosphorus concentrations was modestly lower among hemodialysis patients managed with paricalcitol compared with calcitriol in one prospective clinical trial (Sprague *et al.*, 2003).

In short-term studies, thrice-weekly doses of doxercalciferol, given orally or intravenously, also effectively lowered plasma PTH levels among hemodialysis patients with moderately severe secondary HPT. Values decreased by approximately 60% from pretreatment levels after 16 weeks using oral doses ranging from 2.5 to 10 µg three times weekly (Fig. 11) (Frazao *et al.*, 2000). Similar reductions in plasma PTH levels were observed over the same interval of follow-up during treatment with thrice-weekly intravenous doses of doxercalciferol ranging from 1 to 4 µg (Fig. 12) (Tan *et al.*, 1997). Serum calcium concentrations rose modestly during treatment with oral or intravenous doses, but the number of episodes of hypercalcemia and hyperphosphatemia was greater among patients given doxercalciferol compared with placebo regardless of the route of drug administration. Hypercalcemia and hyperphosphatemia occur less frequently, however, among subjects

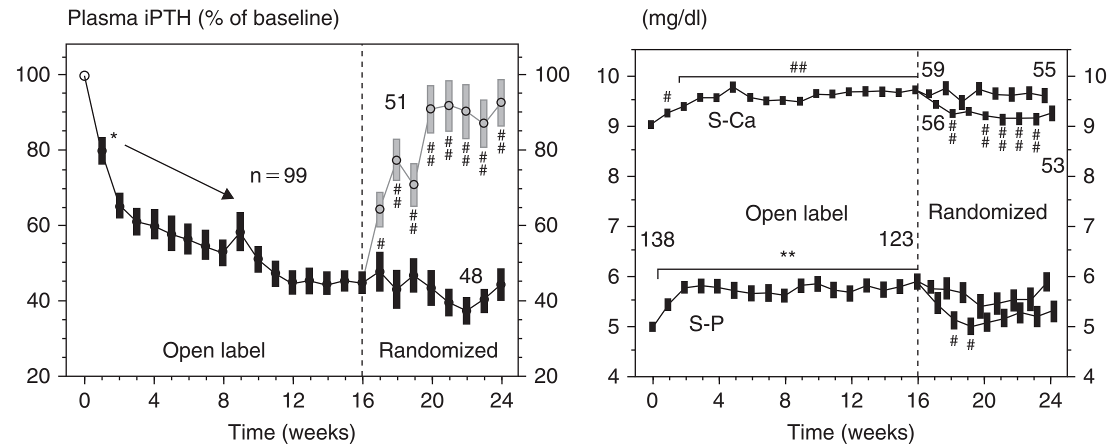

FIGURE 11 The percentage change in plasma PTH levels from pre-treatment values during treatment with thrice-weekly oral doses of doxercalciferol among hemodialysis patients with secondary hyperparathyroidism (left panel). All patients were initially given doxercalciferol for 16 weeks. Subjects were then assigned randomly to receive either doxercalciferol or placebo for another eight weeks. Mean serum calcium and phosphorus concentrations during the study are also shown (right panel). (From reference (Frazao *et al.*, 2000)).

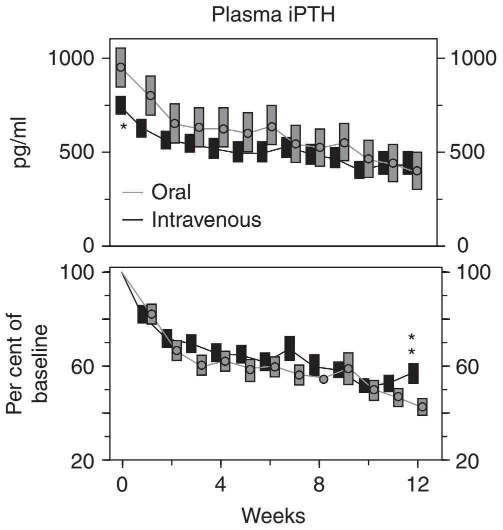

FIGURE 12 Plasma PTH levels (top panel) and the percentage change in plasma PTH (bottom panel) during treatment with thrice-weekly doses of doxercalciferol given orally or intravenously among hemodialysis patients with secondary hyperparathyroidism. Oral doses of doxercalciferol ranged from 2.5 μg to10.0 μg whereas intravenous doses ranged from 1.0 μg to 4.0 μg with each hemodialysis session. (From reference Maung *et al.*, 2001)).

managed with intravenous doses than with oral doses of doxercalciferol (Maung *et al.*, 2001). Episodes of hypercalcemia or hyperphosphatemia usually resolve within three to seven days after treatment is withdrawn (Tan *et al.*, 1997). The use of intravenous rather than oral doses of doxercalciferol may thus provide a better alternative for managing secondary HPT when serum calcium and phosphorus levels are not controlled optimally or when episodes of hypercalcemia and/or hyperphosphatemia have occurred previously (Frazao *et al.*, 2000).

Additional studies are needed to determine whether the frequency of episodes of hypercalcemia and hyperphosphatemia is less during treatment with doxercalciferol than with calcitriol or other vitamin D sterols. Clinical trials to compare the safety and efficacy of paricalcitol with that of doxercalciferol have not been done. The value of paricalcitol and doxercalciferol for achieving and maintaining biochemical control of secondary HPT after several years of follow-up has not been assessed. Similarly, information about the impact of treatment with either paricalcitol or doxercalciferol on bone mass and on skeletal fracture rates among patients undergoing dialysis has not been reported.

The effect of vitamin D sterols to downregulate pre-pro-PTH gene transcription and to lower plasma PTH levels is limited in duration, and ongoing treatment is required to sustain these responses (Goodman, 2001). Plasma PTH levels thus often rise substantially within weeks after treatment is withdrawn among patients who have responded previously to vitamin D therapy (Frazao *et al.*, 2000). As such, the impact of periodic adjustments to the doses of vitamin D sterols must be considered when evaluating interval changes in plasma PTH levels during clinical management.

Plasma PTH levels should be monitored regularly during treatment with intermittent doses of vitamin D sterols, whether given orally or intravenously. Doses should be reduced when plasma PTH levels approach values that are four to five times the upper limit of normal to diminish the risk of inducing adynamic bone, which can develop after intermittent calcitriol therapy (Goodman *et al.*, 1994; Salusky and Goodman, 1996, 2001). Whether treatment with paricalcitol or doxercalciferol produces similar reductions in bone formation and turnover is not known. Data on bone histology after the treatment of secondary HPT with these vitamin D sterols have not been reported among patients managed with dialysis.

Calcimimetics The calcimimetic agent cinacalcet hydrochloride first became available in 2004 for use clinically to manage secondary HPT among patients undergoing dialysis. Cinacalcet acts as an allosteric activator of the calcium-sensing receptor (CaSR), lowering the threshold for receptor activation by extracellular calcium ions and thereby inhibiting PTH secretion (Hammerland *et al.*, 1998). It thus reduces plasma PTH levels by a mechanism distinct from that of the vitamin D sterols and provides a second pharmacological intervention for secondary HPT among persons with CKD (Goodman, 2003a, 2005a).

Cinacalcet is available only as tablets for oral administration. A formulation for intravenous use among patients receiving thrice-weekly hemodialysis is not available owing largely to the hydrophobic nature of the compound. Nevertheless, cinacalcet is absorbed rapidly after oral administration (Harris *et al.*, 2004). Peak plasma levels are achieved within 60 to 90 minutes and depend largely on the doses given (Antonsen *et al.*, 1998; Harris *et al.*, 2004; Silverberg *et al.*, 1997). Plasma PTH levels decrease abruptly after single oral doses reaching a nadir after two to four hours, but values rise subsequently toward predose levels during the remainder of the day (Goodman *et al.*, 2002a; Harris *et al.*, 2004). The immediate effects of cinacalcet to inhibit PTH secretion and to lower plasma PTH do not differ substantially between patients with mild or advanced secondary HPT, and they do not diminish with ongoing therapy (Moe *et al.*, 2005b).

Treatment with cinacalcet is initiated by using single daily oral doses of 30 mg. Doses are titrated sequentially upward at two- to four-week intervals to 60 mg, 90 mg, 120 mg, or to the maximum daily dose of 180 mg to control plasma PTH levels (Block *et al.*, 2004b). As expected, serum calcium concentrations decrease as plasma PTH levels decline. Serum calcium levels should thus be monitored regularly during cinacalcet therapy. The largest reductions occur during the first week, and serum calcium levels should be measured one week after treatment is started to assess this biochemical change. Smaller decrements in serum calcium are seen with subsequent dosage adjustments. Symptomatic hypocalcemia has not been reported among dialysis patients treated by using a dose titration scheme (Block *et al.*, 2004b).

Serum phosphorus levels also often decline during treatment with cinacalcet (Goodman *et al.*, 2002a). Reductions in the mobilization of phosphorus to form bone are largely responsible. Elevated serum calcium and phosphorus values are common among hemodialysis patients who are frequently treated with calcium-containing compounds and/or vitamin D sterols. These biochemical disturbances have been associated in observational studies with adverse outcomes including cardiovascular events and mortality (Block *et al.*, 2004a). Additional work is required, however, to determine whether improvements in the control of serum calcium and phosphorus levels during the treatment of secondary HPT with cinacalcet modifies important clinical outcomes. Unlike the vitamin D sterols, neither hypercalcemia nor hyperphosphatemia preclude the use of cinacalcet for lowering plasma PTH levels in patients with overt secondary HPT.

Among patients with inadequately controlled secondary HPT despite previous treatment with vitamin D sterols and phosphate-binding agents, plasma PTH levels declined progressively during 26 weeks of treatment with cinacalcet (see Fig. 12) (Block *et al.*, 2004b). The proportion of patients who achieved a plasma PTH level less than 250 pg/mL rose progressively over time, and 41% of subjects reached this therapeutic endpoint (Fig. 13) (Block *et al.*, 2004b). Serum calcium and phosphorus levels decreased modestly despite the continued use of vitamin D sterols in two-thirds of patients.

Treatment with cinacalcet is similarly effective for controlling plasma PTH levels when used together with smaller doses of vitamin D sterols or without concurrent vitamin D therapy. Serum calcium and phosphorus concentrations can be maintained more often within the ranges recommended by current practice guidelines among patients treated with cinacalcet than with conventional therapies (Moe *et al.*, 2005a), but prospective clinical trials to address this issue have yet to be reported.

The efficacy of cinacalcet does not differ according to disease severity as judged by baseline PTH values (see Fig. 13) (Block *et al.*, 2004b; Martin *et al.*, 2005). Cinacalcet thus appears to be similarly effective for lowering plasma PTH levels among patients with mild, moderate, or severe secondary HPT (Fig. 14). Although CaSR expression is diminished in hyperplastic parathyroid tissue, such changes do not substantially modify the biochemical response to treatment (Chen and Goodman, 2004).

The use of daily oral doses of cinacalcet induces an oscillating hormone concentration profile during the day (Lindberg *et al.*, 2003; Seidel *et al.*, 1993). These short-term variations must be considered when interpreting PTH results during clinical management (Goodman, 2005a). Values will be affected by the length of the interval between oral drug administration and the time at which blood samples are collected (Goodman, 2005a). Plasma PTH levels obtained 24 hours after preceding doses of cinacalcet have been used in all published clinical trials to assess therapeutic efficacy and to guide dosage adjustments, and a similar approach is recommended in clinical practice (Block *et al.*, 2004b; Goodman, 2005a; Lindberg *et al.*, 2003; Quarles *et al.*, 2003). Measurements done 12 to 24 hours after daily doses of cinacalcet thus provide biochemical information similar to that reported in clinical trials to inform therapeutic decisions (Block *et al.*, 2004b; Goodman, 2005a; Lindberg *et al.*, 2003; Quarles *et al.*, 2003).

In all published clinical trials, single daily oral doses of cinacalcet have been used to manage secondary HPT among patients undergoing dialysis. Information about the safety and efficacy of cinacalcet using alternative dosing strategies in such patients is not available.

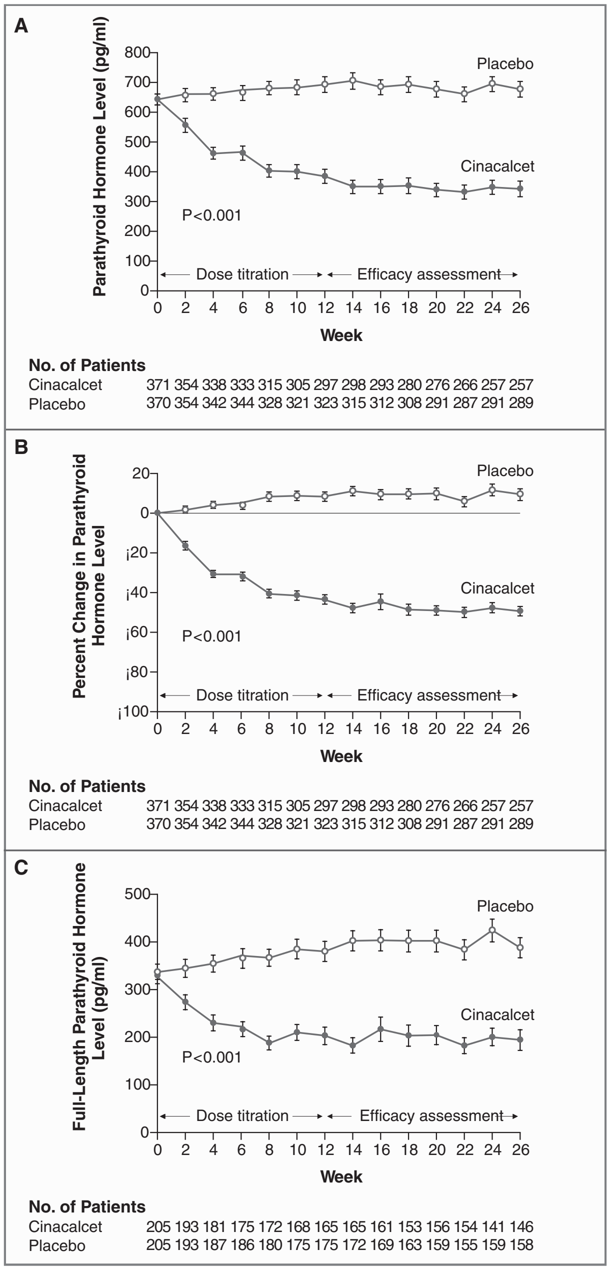

FIGURE 13 The percentage of patients treated for 26 weeks with cinacalcet or placebo who achieved a mean plasma PTH level ≤250 pg/mL or a decrease in plasma PTH equal to or greater than 30% (top panel). The proportion who achieved a plasma PTH level ≤250 pg/mL increased progressively during the study (middle panel), and the fraction of cinacalcet-treated patients that experienced a 30% or greater decrease in plasma PTH levels from pre-treatment values did not differ according to disease severity as judged by baseline, or pre-treatment, plasma PTH determination (bottom panel). (From reference (Block *et al.*, 2004b)).

Parathyroidectomy Some patients undergoing dialysis and others with less advanced CKD fail to respond adequately to treatment with vitamin D sterols or cinacalcet, and plasma PTH levels cannot be controlled adequately. Such patients generally have advanced secondary HPT that requires surgery to control the disorder. Plasma PTH levels are markedly elevated, and attempts to lower them during treatment with vitamin D sterols are frequently constrained by recurrent episodes of hypercalcemia and/or hyperphosphatemia that limit the doses that can be given safely.

For patients with hypercalcemia and a history of exposure to of aluminum-containing medications, the possibility of bone aluminum toxicity should be considered and the diagnosis excluded by using bone biopsy and bone histomorphometry, if necessary, before proceeding with surgery. Aluminum-related bone disease can worsen and overt osteomalacia can develop during the weeks or months immediately after parathyroidectomy among patients with bone aluminum toxicity (Coburn and Slatopolsky, 1990). Additional causes of hypercalcemia, such as sarcoidosis, other granulomatous disorders, malignancy, and treatment with large doses of calcium or vitamin D analogues, should be considered and excluded.

Apart from hypercalcemia, hyperphosphatemia that persists or reoccurs frequently during treatment with vitamin D sterols or despite ongoing management with dietary phosphorus restriction and phosphate-binding represents another reason to consider parathyroidectomy. Additional indications include persistent bone pain, skeletal fractures, intractable pruritus that does not respond to intensive dialysis or to other medical interventions, evidence of progressive extraskeletal calcification, and calciphylaxis among patients with markedly elevated plasma PTH levels (Llach, 1990).

Either subtotal or total parathyroidectomy is done most often among patients treated with dialysis (Kaye *et al.*, 1989; Kaye, 1989). Hyperparathyroidism reoccurs in 15% to 30% of those who have had a previous successful subtotal parathyroidectomy, a finding likely owing to ongoing hyperplasia in the residual parathyroid tissue. For patients who are candidates for renal transplantation, subtotal parathyroidectomy is preferred because the remaining tissue serves to maintain calcium homeostasis after renal function is restored.

An alternative surgical approach is total parathyroidectomy followed by the implantation of parathyroid gland fragments into subcutaneous tissues in the forearm or elsewhere to preserve some glandular function postoperatively. Caution is warranted, however, regarding this approach, and several limitations must be acknowledged. The implanted tissue can enlarge and develop autonomous secretory behavior. As such, hyperparathyroidism may recur. The implanted fragments can also spread locally into surrounding tissues, rendering surgical resection difficult.

Adynamic Bone

The appropriate management of patients with adynamic renal osteodystrophy depends largely on whether the cause can be identified definitively. Among patients receiving large doses of calcium-containing, phosphate-binding agents, the amounts utilized should be reduced to avoid ongoing parathyroid gland suppression. Treatment with vitamin D sterols should be withdrawn when plasma PTH levels have been lowered excessively, in particular, when accompanied by recurrent episodes of hypercalcemia. If bone aluminum deposition is the underlying cause, eliminating the source of ongoing aluminum loading may be sufficient to reverse the adynamic lesion. Bone formation increases substantially in many patients after treatment with aluminum-containing compounds is withdrawn (Hercz *et al.*, 1988).

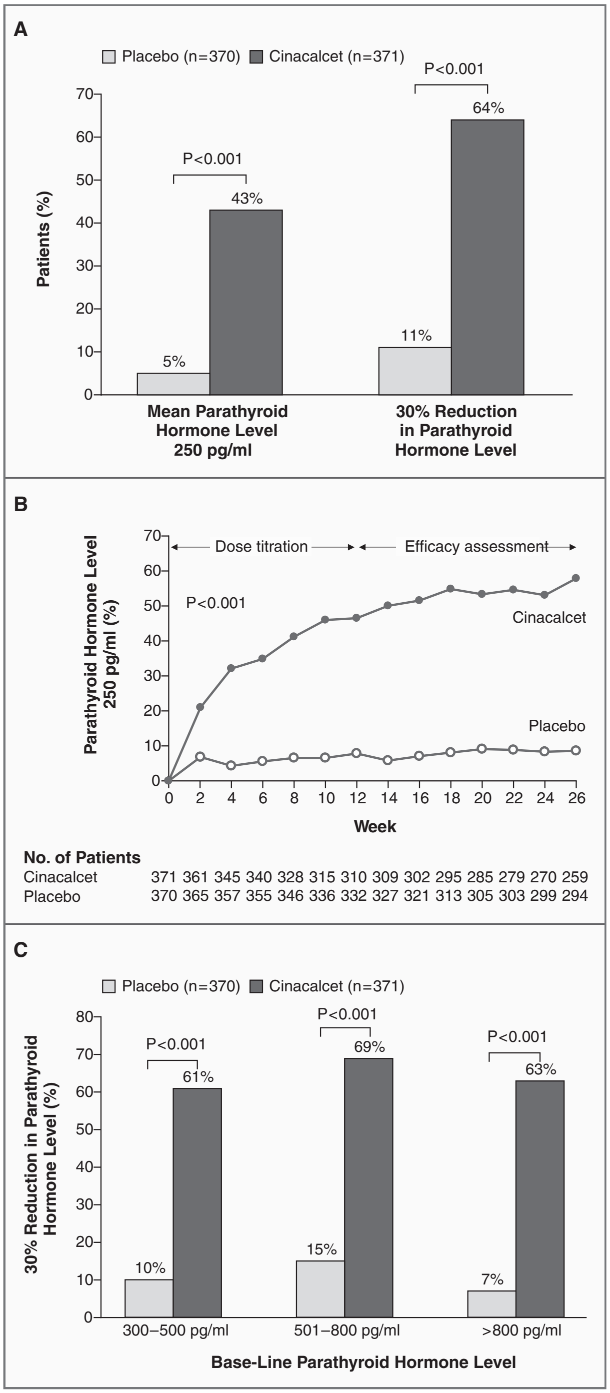

FIGURE 14 The percentage of patients treated for 26 weeks with cinacalcet or placebo who achieved a mean plasma PTH level ≤250 pg/m or a decrease in plasma PTH equal to or greater than 30% from pretreatment values (top panel). The proportion who reached a plasma PTH level ≤250 pg/m increased progressively during treatment with cinacalcet (middle panel), and the proportion who had a 30% or greater decrease in plasma PTH levels from pretreatment values did not differ according to disease severity as judged by baseline, or pre-treatment, plasma PTH determination (bottom panel). (From reference (Block *et al.*, 2004b)).

Although relative hypoparathyroidism is thought to be a factor in the pathogenesis of adynamic renal osteodystrophy, the use of exogenous doses of PTH or teriparatide has not been evaluated as a therapeutic strategy. Little is known about the impact of treatments for other causes of adynamic bone such as diabetes or postmenopausal osteoporosis among patients receiving dialysis. Nevertheless, the use of bisphosphonates is not recommended. These compounds are normally eliminated from the body by excretion in the urine. The renal clearance of bisphosphonates is markedly diminished among patients with advanced CKD, and these agents are not removed effectively by dialysis. As such, extensive sequestration in bone may occur with potentially adverse consequences.

Osteomalacia

The treatment of osteomalacia should be directed toward the specific cause identified. Overt vitamin D deficiency or marginal vitamin D nutrition should be corrected by the administration of either ergocalciferol or cholecalciferol over several months. The efficacy of treatment is assessed by follow-up measurements of serum 25-hydroxyvitamin D levels. Values should be maintained above 30 ng/mL. Ongoing therapy may be required to achieve this objective, but the matter has not been studied in detail among patients undergoing dialysis regularly. Other factors that adversely affect skeletal mineralization, such as hypocalcemia or hypophosphatemia that cannot be explained by vitamin D deficiency, must also be identified and eliminated.

Among patients with bone aluminum toxicity, the source of aluminum exposure should be identified and suitable corrective measures undertaken to avoid further aluminum loading. Oral medications and dialysis solutions that contain aluminum are implicated most often. Therapeutic interventions that preclude further aluminum exposure are often sufficient to effectively manage aluminum-related osteomalacia in subjects who do not have severe bone pain and/or recurrent skeletal fractures. Others, however, are markedly incapacitated, and more definitive interventions are required.

Deferoxamine (DFO) effectively mobilizes aluminum from bone and other tissues and enhances its removal during hemodialysis and peritoneal dialysis procedures (Malluche *et al.*, 1984; Ott *et al.*, 1986). Doses can be given either intravenously or subcutaneously, but low-dose subcutaneous therapy is recommended (Hercz *et al.*, 1986; Milliner *et al.*, 1986). Bone pain and other musculoskeletal symptoms attributable to bone aluminum toxicity generally improve after four to ten months of treatment in severely affected cases (Coburn *et al.*, 1986; Ott *et al.*, 1986).

Serum calcium levels decrease modestly and episodes of hypercalcemia become less frequent or resolve altogether during the successful treatment of aluminum-related osteomalacia with DFO. Serum alkaline phosphatase levels often rise, suggesting improvements in skeletal mineralization and osteoblastic activity (Coburn *et al.*, 1986). Plasma PTH levels also increase modestly, and this biochemical change may contribute to increases in bone formation and remodeling as bone aluminum levels decline (Ott *et al.*, 1986). The amount of surface-stainable aluminum in bone decreases in most patients who are treated with DFO, but those who have undergone previous parathyroidectomy respond less well or not at all (Ott *et al.*, 1986).

Serious and often lethal infections with *Rhizipus* and *Yersinia* can develop during DFO therapy among patients undergoing dialysis (Boelaert *et al.*, 1985; Gallant *et al.*, 1986; Hoen *et al.*, 1988; Segal *et al.*, 1988; Windus *et al.*, 1987). The chelation of plasma iron by DFO enhances its delivery into the cytoplasm of certain organisms thus increasing their pathogenic potential (Abe *et al.*, 1990; Robins-Browne and Prpic, 1985; Van Cutsem and Boelaert, 1989). As such, DFO must be used judiciously in the management of aluminum-related bone disease, and treatment should be confined only to those severely affected. In asymptomatic patients with aluminum deposition in bone, bone histology and bone formation improves solely by withdrawing aluminum-containing medications and replacing them with calcium-containing compounds to control serum phosphorus levels (Hercz *et al.*, 1988).

Evidence of bone toxicity owing to aluminum deposition should be documented fully by using bone biopsy and bone histomorphometry before DFO therapy is initiated. Doses should not exceed 0.5 to 1.0 g/week, and plasma aluminum levels should be measured regularly. Subcutaneous rather than intravenous DFO therapy is recommended. Subcutaneous doses do not produce the high serum levels of ferrioxamine that occur after intravenous doses, a factor thought to enhance iron uptake by pathogenic organisms, thereby increasing the risk of opportunistic infection.

Mixed Renal Osteodystrophy

A definitive diagnosis of mixed renal osteodystrophy requires bone biopsy and histomorphometric assessments, procedures not done commonly in clinical practice. Certain laboratory findings such as persistent hypocalcemia, low serum 25-hydroxvitamin D levels, or elevated serum aluminum levels suggest the presence of an underlying defect in skeletal mineralization, but they are by no means conclusive. Similarly, histological changes of hyperparathyroidism in bone would be expected if plasma PTH were persistently elevated, but such findings do little to distinguish patients with mixed lesions of renal osteodystrophy from those with uncomplicated secondary HPT. If confirmed by bone biopsy, therapeutic measures to control secondary HPT and to eliminate causes of inadequate skeletal mineralization are required among patients with mixed renal osteodystrophy.

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Chapter 69

Osteogenesis Imperfecta

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INTRODUCTION

Osteogenesis imperfecta (OI) is a highly variable heritable disease of bone characterized by recurring bone fractures. It is the most common single gene defect causing bone disease and its typical clinical presentation and the natural history have been well chronicled in many review articles and book chapters. The disease has received attention from basic scientists because it has become the paradigm for mutations affecting a well-defined extracellular matrix protein. The results of these biochemical and molecular studies have provided the foundation for understanding the broad spectrum of heritable diseases of connective tissue, and OI will continue to be the disease that provides the breakthroughs for this class of heritable diseases. The purpose of this chapter is to provide an update to the well-described clinical classifications and point out recent advances in the diagnosis and therapy of the disease. Most of the chapter will review the consequences of mutations of type 1 collagen on the structure of the collagen fibers, the biology of the osteoblast, and the pathophysiology of the resulting bone disease. It will emphasize how this information influences our understanding of osteoporosis and other heritable diseases of connective tissue. In addition, outstanding problems requiring further research attention, particularly relating to cell and somatic gene therapy, will be presented.

CLINICAL CLASSIFICATIONS AND VARIANTS OF OI

The most widely used clinical classification for OI continues to be the one developed by Sillence that is based on disease severity (Sillence, 1988). For purposes of correlating clinical phenotype with the underlying genetic and pathophysiological basis of the disease, grouping the classes into deforming and nondeforming bone disease can be helpful even though this too is an arbitrary distinction. Three degrees of deforming OI – types II, III, and IV – are associated with decreasing severity of growth retardation and limb deformity and all result from a mutation affecting the structure of the type 1 collagen molecule (Table. I). The consequences of this mutation on the structure of the extracellular matrix and the biology of bone cell are very similar, varying only in their degree of severity. In contrast, most cases of nondeforming OI, type I, are the result of mutations affecting the production of an otherwise normal type I collagen molecule.

Deforming OI

The criteria for identifying individuals as OI type II, III, or IV are somewhat arbitrary. Although this classification helps to stratify patients according to their disease severity, the typing does not necessarily predict the natural history of the disease or the type of underlying genetic mutation. Furthermore, with the implementation of bisphosphonate in children, disease severity and natural history are being greatly improved and eventually may force a revaluation of this classification system.

Most infants who succumb to OI in the perinatal period are considered OI type II. The hallmarks of type II include shortened and deformed limbs in the presence of normal birth weight and length. The cranium is unusually soft and molded and may be fractured at birth. Intracranial bleeding may have occurred. The sclerae are deep blue. The limbs are deformed and short, raising the consideration of hypophosphatasia, achondrogenesis, and thanatophoric dwarfism in the diagnosis. Multiple fractures are seen on x-ray and the extremities appear broad and crumpled. The critical problem is neonatal pulmonary insufficiency that may lead to death in the first postnatal week (Shapiro, 1989). Faulty thoracic musculoskeletal development also limits respiratory function in the majority of cases. However, prolonged survival has been documented in occasional type II cases.

Infants born with fractures and deformity who survive the perinatal period are grouped as OI type III. The natural history of type III includes the presence of multiple

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TABLE I An All-Inclusive Clinical and Molecular Classification of Osteogenesis Imperfecta

Molecular Classification	Clinical Classification	Clinical Severity	Molecular Mechanism
Dominant Negative OI	Type II	Perinatal lethal	Glycine substitutions preferentially located in C terminal helical domain of either collagen chain
	Type III	Progressive Deforming	Glycine substitutions preferentially located in midhelical domain of either collagen chain
	Type IV	Moderately Deforming	Glycine substitutions preferentially located in midhelical domain of the a2 collagen chain
	Type V	Moderately Deforming	Non-type I collagen gene mutation
	Type VI	Moderately Deforming	Non-type I collagen gene mutation
	Type VII	Perinatal lethal	Mutation in the 3-prolyl hydrolase protein complex
	Type I	Nondeforming	Glycine substitutions preferentially located in N terminal helical domain of either collagen chain
	Sp1-associated osteoporosis	Osteoporosis-associated risk factor	Excessive alpha 1 chain production may lead to accumulation of alpha 1(I) trimer molecules with the bone matrix
	Noncollagen mutations	Mutation of proteins that interact with the collagen microfibril	Murine knockout studies suggest that noncollagenous proteins of bone may contribute to formation of a stable bone matrix. The osteoporosis of Marfan's may be an example
Haploid Insufficiency	Type I	Classical mild OI	Complete nonfunctional Col1A1 allele usually due to premature stop codon
	Osteoporosis	Associated risk factor for osteoporosis	Partial inactivation of a Col1A1 allele either due to a mutation affecting processing of the transcript or the activity of the promoter

fractures, significant molding of the calvarium, and deformity of the extremities at birth. Fractures may continue to occur in infancy and childhood precluding a normal pattern of ambulation. Deformity of the thoracic cage (pectus carinatum, pectus excavatum) may be present in early childhood and may advance as the scoliosis and vertebral compression increase. Type III OI subjects develop scoliosis at an early age. Vertebral compression, most commonly of the central or "codfish" type, begins shortly after birth and progresses relentlessly prior to puberty (Engelbert, 2003). The volume of the thoracic cage decreases with age and eventually cardiorespiratory insufficiency, including right-sided cardiac failure, may occur (Vitale, 2007). As adults, these individuals are very short, frequently less than 4 feet in height. Skeletal involvement is associated with a severe defect in remodeling so that the appendicular bones are usually very narrow although in some cases, a "broad bone" x-ray appearance is seen. In either event, the epiphyseal zone is markedly dysplastic, which effectively limits endochondral and therefore somatic bone growth. Because of severe limb deformity and a marked degree of osteopenia at an early age, type III subjects are wheelchair bound. Because of molding of the base of the skull, type III subjects are at risk for the development of basilar invagination that may cause brainstem compression with both respiratory and neurologic complications and sudden death (Janus, 2003; Kovero, 2006). Charnas reported communicating hydrocephalus in 17 out of 76 subjects with OI (Charnas, 1993). Seizure disorders were observed in 5 out of 76 cases. Brainstem compression requiring surgical decompression or reinforcement of the craniocervical junction is extremely complex, requiring mechanical support, trans-oral clivectomy, and decompression of the posterior fossa where respiratory center function is compromised (Bhangoo and Crockard, 1999; Sawin and Menezes, 1997).

Infants who appear normal at birth but develop deformity upon ambulation generally fall into the OI type IV group, although this form can merge into features seen in Moderate short stature is common, but ambulation is generally possible with the aid of rods and braces. Radiologically, the extent of skeletal dysplasia in type IV disease is more severe than found in type I cases, but less than in type III OI. There is more severe osteoporosis with significant cortical and trabecular bone loss. Cystic changes may appear in the long bones. Vertebral compression fractures occur frequently. In addition to more severe scoliosis, there is moderate deformity of the pelvis and lower extremities that limits ambulation without support. Dentinogenesis imperfecta (DI) is most frequent in OI types III and IV, and overall, affects about 15% of OI patients among the different phenotypes (Lund, Jensen *et al.*, 1998; Petersen and Wetzel, 1998; Rios, 2005). It appears as a bluish-brown translucent discoloration. Deciduous teeth tend to be more severely affected than permanent teeth. These teeth are soft because of a defect in underlying dentin (type I collagen) so that the enamel layer is lost, which leads to extreme dental erosion even in children. Expert dental care is essential in affected children. Hearing loss, present in all OI phenotypes (Garretsen, 1997) has its onset in children (Kuurila *et al.*, 2000) or during second or third decade (Kuurila, 2002). Conductive, mixed, and sensorineural hearing loss have been documented in OI (Shapiro *et al.*, 1982). The lesions are a combination of bony trauma (i.e., fractures of the stapes crura and footplate) and damage to vestibular structures in the inner ear (Kuurila, 2003). These lesions are not identical to those of otosclerosis.

Until recently there were no examples of OI resulting from mutations in genes other than type 1 collagen. As a result of forming an integrated research, diagnostic, and treatment team with the opportunity to examine the broad spectrum of patients with deforming OI, Glorieux has identified at least three additional OI-like diseases (OI type V–VII) that are not a result of a mutation in the type I collagen genes. Type V OI has radiologic features of type IV OI with subtle differences including calcification of the interosseous membrane and a metaphyseal band adjacent to the growth plate. Hyperplastic callus formation is frequently found in this form of OI (Ramirez, 2003 ; Cheung, 2007) and can be confused with osteosarcoma (Vieira, 2006). The disease is inherited as an autosomal dominant and no mutations with the type I collagen genes have been found (Glorieux *et al.*, 2000). OI type VI is similar in severity to type IV OI but has high levels of serum alkaline phosphatase and a mineralization defect but no radiological features of rickets (Glorieux, 2002). In both cases, linkage studies have been possible and have excluded type 1 collagen genes. The importance of these new clinical phenotypes is that they demonstrate that molecules other than type 1 collagen are essential for formation of a stable bone matrix and will eventually lead to a discovery of the role that these gene products play in bone biology.

Murine models have also led to the discovery of genes that modify type I collagen as another mechanism for severe OI. Type VII OI, a severe recessively inherited form resembling type II OI that does not map to collagen loci (Ward, 2002), has been shown to be a mutation in either prolyl 3-hydroxylase (Cabral, 2007) or cartilage-associated protein (CTRAP), which is a binding partner of the 3-hydroxylase complex (Morello, 2006). In either case, there is defective hydroxylation of the single prolyl 3-hydroxylase site within the alpha 1(I) chain with excessive lysyl hydroxylation suggesting that the 3-hydroxy site is essential for collagen assembly. Another murine model resembling OI is the fro/fro mouse, which has been shown to be a defect in sphingomyelin phosphodiesterase 3 (Smpd3) (Aubin, 2005). Although a human form of OI has yet to be mapped to this locus, the mouse points an aspect of lipid metabolism important for bone formation that is likely to have a clinical homologue.

Another unifying feature of most of the deforming forms of OI is the strong dominant negative properties of the mutation. In those cases where affected individuals choose to have children, the disease is passed on in an autosomal dominant manner. However, in most cases the disease results from a random and isolated mutation in the germ cell of a parent. Usually this event is a one-time occurrence and the likelihood of another affected individual is extremely small. However, in 5 to 10 percent of cases one of the parents sustained a somatic mutation during his or her embryonic development such that a proportion of their somatic and germ cells have an OI mutation (Cole and Dalgleish, 1995 ; Lund *et al.*, 1997). The mosaic parent does not have features of OI despite having a significant number of mutant cells (Cabral, 2004)and most of their children have normal bones. However, if the child is conceived with a germ cell carrying the OI mutation, then full-blown OI results. As a result of this possibility for recurrence, families with one child with OI who wish to have more children are advised to undergo prenatal testing (Byers, 2006) or sensitive ultrasound analysis with the possibility that a subsequent child might be affected (Parilla, 2003; Ruano, 2004). With the recognition of type VII OI as a recessive disorder with a defined molecular defect, it will be important to identify mutations other noncollagen genes that modify type I collagen to distinguish germinal mosaicism from a true recessive disorder.

MILD NONDEFORMING OI

Although this class of OI patients is relative common, it is not unusual to be unappreciated by the family or their physician. In cases with blue sclera (Sillence *et al.*,1993), mild short stature, ligamentous laxity, mild scoliosis, and a strong family history of recurring fractures and osteoporosis, the diagnosis is not difficult. Radiographs are not diagnostic and fractures usually heal without deformity. In many cases, there is not a high degree of morbidity and once puberty is attained, fractures are rare. Perhaps the most important reason for identifying these individuals is to initiate anti-osteoporosis therapy as soon as menopausal symptoms have developed because these individuals are particularly susceptible to accelerated bone loss when sex steroids are lost (Bischoff *et al.*, 1999). Other patients who fit into this category can have symptoms that overlap with mild Ehlers-Danlos syndrome (EDS). The overlap appears to be related to where the mutation is placed within the collagen molecule, making this type of mutation of interest to the structural biologist (Feshchenko *et al.,* 1998; Raff *et al.,* 2000) (Cabral, 2005 ; Makareeva, 2006). More recently, mutations affecting processing of the C-terminal propeptide of the COL1A1 gene (Symoens, 2004) and even point mutation in the Y position of the collagen helix (Cabral, 2007) have been shown to have an OI/EDS overlap syndrome. The importance to the clinician is to recognize that the two genetic phenotypes can coexist so that measures to strengthen the skeleton are initiated when appropriate.

Probably the most difficult situation that arises within this class of patients is child abuse. Although OI is frequently invoked as a potential explanation for the infants and young child with multiple fractures, it is rare that any of the clinical features that are characteristic of OI are present (Steiner *et al.,* 1996; Byers, 2006) and instead there usually is no family history of recurring fractures, short stature, or abnormal radiographs. Because OI can result from a new mutation within the type I collagen genes and potentially other extracellular matrix genes, lack of a family history does not exclude the diagnosis. Genetic testing based on protein electrophoresis may lack the sensitivity for mild forms of OI, necessitating DNA sequencing of the collagen genes (Cabral, 2006). Recently the term *temporary brittle bone disease* has been invoked to explain certain forms of multiple fractures in a setting without an obvious perpetrator (Paterson *et al.,* 1993) but the concept has not gained acceptance (Ablin and Sane, 1997; Chapman and Hall, 1997). A recent report finds that infants and children with this diagnosis have reduced bone mass by radiographic absorptiometry or computed tomography (Miller and Hangartner, 1999) and a mechanism of prenatal bone unloading has been proposed (Miller, 2003). Progress is being made in developing standards for DEXA evaluation of infants (Koo, 2004, 2006) so there is hope that a more objective measure of bone (radiological and rapid DNA sequencing) may replace the diagnostic decisions that are based on emotion and opinion frequently in an antagonistic environment. This topic is probably best addressed in literature prepared by the Osteogenesis Imperfecta Foundation on their website (http://www.oif.org).

The relationship of mutations within the type I collagen genes and osteoporosis has been difficult to establish. In families where OI mutations and osteoporosis have been identified, careful clinical scrutiny usually identifies features of the disease (Shapiro *et al.,* 1992; Spotila *et al.,* 1991). Nonglycine substitutions in the helical domain have been found in a few instances, but the significance of these findings is difficult to interpret (Spotila *et al.,* 1994). Quantitative trait linkage (QTL) studies of populations of individuals with osteoporosis have failed to demonstrate linkage to either type I collagen gene (Niu *et al.,* 1999; Cardon *et al.,* 2000). However, an Sp1 polymorphism was shown a statistical association to individuals with osteoporosis (Grant *et al.,* 1996) and this linkage has been examined by numerous investigators with varying outcomes (Mann, 2003). The most recent and comprehensive study does demonstrate an effect on bone mass and increase in vertebral fracture but the effect is small requiring a large study to achieve statistical significance (Ralston, 2006).

NEEDED ADVANCES IN CLINICAL DIAGNOSIS AND MANAGEMENT

In most cases, the diagnosis of OI can be made on clinical grounds alone. Unfortunately radiographs are generally insensitive to the diminished bone mass associated with type 1 OI. DEXA has been shown to be effective in detecting the diminished bone mass associated with type 1 OI in adults although its application to children and particularly infants has not been adequately developed. Such standards would be particularly helpful in evaluating individuals with suspected abuse. pQCT might be more sensitive in young children to discriminate individuals with normal or diminished bone mass (Fredericks *et al.,* 1990; Keen *et al.,* 1999). Other clinical tests that may be helpful in the diagnosis OI are serum level of procollagen propeptide fragments reflecting the rate of type I collagen formation (Kauppila *et al.,* 1998; Lund, Jensen *et al*., 1998) and the generation of collagen-derived cross-links reflecting removal (Bank *et al.,* 2000). Here again standards need to be developed for age and sex, degree of sexual maturation, or menopause to make these tests of bone turnover useful in OI.

In many instances, it is advantageous to know the underlying molecular abnormalities for purposes of genetic counseling and in some cases for predicting natural history. Frequently molecular testing is also used to help in the evaluation of children with recurring fractures. The OIF website has a comprehensive listing of the molecular diagnostic services available (http://www.oif.org/site/PageServer?pagename=Testing). The analysis can begin as a screening test on fibroblasts derived from an affected individual for biochemical abnormalities of the collagen molecules followed by confirmation at the genetic level by DNA sequencing or it can go directly to DNA sequencing of the collagen loci. Both approaches have an excellent track record for finding mutations although not all the patients with OI yield an identified mutation. With increasing power and reduced cost of DNA sequencing, this is likely to become the method of choice. For clinicians not familiar with interpretation of genetic testing, it is suggested that services that directly communicate with the requestor regarding test results is preferable to those who refer the requestor to a genetic counselor.

Given the complexity in the clinical management of OI, a multidisciplinary clinical team approach to treatment is of greatest value for both the patient and the field. Not only are there significant orthopedic and medical issues, but problems of daily living are pervasive. Proper handling during infancy, mainstreaming within schools, driving an automobile, attending college, scoliosis and pulmonary insufficiency, neurological symptoms, pregnancy and genetic risk, and acceleration of bone disease after menopause are complex problems that are difficult for an individual clinician to manage and require an experienced and broad-based treatment team. Many of these issues are covered on the OIF website (http://www.oif.org) and in their literature. Because OI is a relatively rare and highly variable disorder, no single clinician or clinic can perform phenotype/genotype or clinical intervention studies with the power that multi-site studies achieve in areas such as pediatric oncology. The OIF is working to develop this concept as linked clinical research centers where patients are recruited for common protocols. The first step in the process is the establishment of a patient registry with the intent to identify patients who have an interest in participating in clinical research protocols http://www.osteogenesisimperfecta.org/oir/. The progress of this exciting initiative can be viewed at http://www.oif.org/site/PageServer?pagename=RegistryLCRCs.

ANIMAL MODELS OF OI

Naturally arising forms of OI have been described in cows (Agerholm *et al.,* 1994) and dogs (Campbell, 2001; Seeliger, 2003). Although these animal models have value for evaluating orthopedic strategies, the maintenance of large animal models is extremely expensive so they are not useful for the initial development of new medical or genetic strategies. However, once an intervention has the possibility of a clinical trial then a larger animal model will probably be a requirement for FDA approval.

To date, the mouse has contributed the most to our understanding of the disease and is the initial platform for evaluating new therapies. Lethal OI has been produced experimentally by insertion of a collagen transgene containing a glycine point mutation (Stacey *et al.,* 1988) or an internal deletion within the helical domain of the molecule (Khillan *et al.,* 1991). Whereas transgene insertions are relatively easy to produce, there is significant variability in the clinical severity even within the same pedigree (Pereira *et al.,* 1994), reducing their value as a experimental model. OI mutations within the endogenous collagen gene yield a more reproducible phenotype. The oim/oim mouse arose from a spontaneous mutation within the Col1A2 gene. Because the mutation produces a nonfunctional alpha-2(I) chain, the disease results from an accumulation of alpha 1(I) trimer molecules (Chipman *et al.,* 1993). It has been widely used even though this is a recessive form of OI that is quite unlike the more common dominantly inherited forms of OI. In addition, a patient with a lack of Col1A2 chain production resulting in a mild form of EDS not OI (Malfait, 2006) was recently identified indicating that the phenotypic consequences of COL1A2 deficiency is more complex than is currently appreciated. A cysteine (G349C) substitution has been knocked into the Col1A1 gene to produce a moderately severe form of OI (Brit IV mouse) that has a stable phenotype and is very representative of the common forms of OI (Forlino *et al.,* 1999; Kozloff, 2004; Kuznetsova, 2004). The only murine model of type I OI is the heterozygous Mov 13 mouse in which one of the two Col1A1 genes is nonfunctional due to a retroviral insertion within the first intron. The heterozygous mouse was initially thought to have normal bone and was used for generating a double knockout of the Col1A1 gene, which has an embryonic lethal phenotype (Lohler *et al.,* 1984). However, more sensitive testing did demonstrate diminished bone mass and underproduction of type 1 collagen (Bonadio *et al.,* 1990). Unfortunately, the mice succumb to leukemia in their early adulthood limiting their value to basic bone research. A viable model for OI type 1 needs to be produced by genetic knock in.

The fro/fro mouse has a bone fragility phenotype that is not associated with a defect in collagen (Sillence, Ritchie *et al.,* 1993). Linkage studies followed by direct gene analysis uncovered the unanticipated locus to be sphingomyelin phosphodiesterase 3 (Smpd3) (Aubin, 2005). The biochemical and physiological consequences of this gene mutation await elucidation.

MOLECULAR BASIS OF OI

A comprehensive listing of the mutations within type I collagen genes resulting in OI is now maintained in the OI mutation database at http://www.oiprogram.nichd.nih.gov/consortium.html/ (Marini, 2007). They can be broadly correlated with clinical severity with the deforming forms of OI being associated with mutations that interrupt the helical stability of the collagen molecule or its interaction with noncollagenous protein of the collagen fibril whereas most forms of type I OI are associated with underproduction of an otherwise normal type I collagen.

Deforming OI

Essentially all of these mutations act in a dominant negative manner, i.e., it is the presence of the abnormal gene product that causes the disease. Because of this mechanism, the disease either develops as a sporadic new mutation or is inherited in an autosomal dominant manner. Those cases in which recurrence is observed represent germinal mosaicism in one parent and not recessive inheritance. The only known exception to this statement is a null mutation within the Col1A2 gene (see later discussion).

The three-dimensional structure of the collagen fibril can be altered by a substitution for glycine in the collagen (gly-x-y) triplet, inframe deletion, an inframe insertion, or by exon skipping. Depending on the helical location of a mutation, these produce a variety of clinical pictures that range from lethal (OI type II) to severely deforming (OI type III) to mildly deforming (OI type IV). Glycine substitution in the helical domain of the collagen alpha 1(I) chain is the most common defect. Glycine, the smallest amino acid, is a critical component that must fit in a sterically restricted space where the three chains of the triple helix come together. Substitutions usually occur in the first base of the genetic code for glycine (GGN) and do not alter the length of the chain but disrupt helical stability. The eight potential amino acid substitutions are cysteine, alanine, arginine, aspartic acid, cysteine, glutamic acid, serine, valine, and tryptophan. How the substitution affects the conformation of the collagen helix is still not well understood and current biochemical analysis does not always predict clinical severity. Because the helix assembles from the C-terminal propeptide, a mutation in the C terminal helical and propeptide region results in greater instability and more severe disease while mutations located in the midhelical domain tend to be less severe. However, mutations within the midhelical domain can have a severe phenotype suggesting that subdomains within the helix are critical for function beyond just contributing to an intact helical structure such as ligand-binding domains for various noncollagenous components of the collagen matrix (Marini, 2007). Mutations located at the N-terminal domain of either chain can be extremely mild and fall into the category of type I OI.

Because the exons that encode the helical domain maintain the reading frame, mutations in the consensus donor or acceptor site can lead to exon skipping, producing a shortened helix that has the same effect on helical stability as a glycine substitution (Byers *et al.,* 1983). Much less common are mutations that delete a portion of the gene and along with it a number of inframe exons (Mundlos *et al.,* 1996) or mutations that insert a segment of intron that remains inframe with the entire transcript. In the latter case, a nonhelical segment is inserted within the helical domain disrupting the structure of the collagen helix (Wang *et al.*, 1996).

The one exception to the statement that severe disease results from a dominant negative mutation in either type I collagen gene is a null mutation of the Col1A2 gene. Formation of the heterotrimeric collagen molecule requires that the alpha 2(I) chain account for 50% of the available chains at the time the procollagen molecule is assembled. When this requirement is not met, either because of underproduction of the alpha 2(I) chain or overproduction of the alpha 1(I) chain, then homotrimeric molecules are formed. Severity of disease depends on the balance between homotrimeric and heterotrimeric molecules within the bone matrix. This may explain why there is a spectrum of disease severity from OI type III when both Col1A2 alleles are affected, to measurable osteopenia and fragility in the heterozygous state (McBride *et al.,* 1998; Saban *et al.,* 1996), to an association with osteoporosis due to the sp1 polymorphic alteration in the Col1A1 gene. This variation in disease severity acts in a recessive manner or as quantitative trait in which gene dosage contributes to the severity of bone disease.

NONDEFORMING OI

The most common mutation causing type I OI reduces the output of otherwise normal type I collagen. Because of the 2:1 requirement for formation of heterotrimeric collagen, the level of Col1A1 production directly influences the accumulation of normal type I collagen molecules. Reduced output from a single Col1A1 allele reduces the production of heterotrimeric collagen and the unincorporated alpha 2(I) chains are degraded intracellularly (Gotkin, 2004). The genetic mechanism for a clinical phenotype resulting from complete inactivation of one allele is referred to as haploid insufficiency. In fact, a spectrum of disease severity related to gene dosage is observed in the Mov 13 mouse. The homozygous Mov 13 is an embryonic lethal that does not reach the stage of skeletal development (Lohler *et al.,* 1984), whereas the heterozygous Mov 13 is a model for OI type I (Bonadio *et al.,* 1990). Similarly, the severity of osteopenia in type I OI is probably related to the degree that one of the Col1A1 alleles underperforms. It would not be surprising to find that a more subtle underproduction from a Col1A1 allele could be a contributing quantitative trait to the development of osteoporosis.

Mutations introducing a premature stop codon are the most frequent cause for a null Col1A1 allele (Redford-Badwal *et al.,* 1996; Slayton *et al.,* 2000; Willing *et al.,* 1994). Premature stop codons arising in all but the terminal exon of a gene usually lead to rapid destruction of the transcript rather than producing a truncated protein. The process is called nonsense mediated RNA decay and it appears to mediated by a nuclear protein complex that is able to recognize a premature stop codon and to target an RNA with this feature for rapid destruction (Medghalchi *et al.,* 2001). This appears to be an important mechanism for preventing a truncated protein from expression thus saving the cell from proteins with unintended function (Isken, 2007). Mutations of these surveillance genes are incompatible with development (Medghalchi *et al.,* 2001) in mice and can result in mental retardation in humans (Tarpey, 2007). Demonstrating that a truncated alpha 1(I) chain is produced from a Col1A1 transcript *in vitro* serves as the basis for uncovering the presence and location of the stop codon (Bateman *et al.,* 1999). Otherwise, finding the mutation by a molecular approach is laborious and can be missed.

A second mechanism for producing a null Col1A1 allele is retention of an intron within the mature transcript. Intron retention instead of exon skipping can result when a mutation of a splice donor site is located in a small intron such that the combination of the intron and the flanking upstream and downstream exon is regarded as an acceptable exon (Stover *et al.*, 1993). However, the presence of the mutant donor site retains the transcript within the splicing apparatus of the nucleus (S35 domain) and it is eventually destroyed (Johnson *et al.*, 2000). Although this is an uncommon cause of a null allele, it has provided insight into the normal pathway for splicing a complex transcript such as collagen and demonstrated that splicing of otherwise normal but small collagen introns is relatively slow. Because approximately 25% of the Col1A1 introns are small and thus are candidates to be affected in this mechanism, it would not be surprising to find polymorphic changes within the gene that contribute to the rate of splicing which in turn could be associated with mild osteoporosis.

Finally, a nonfunctional collagen gene can result from synthesis of a procollagen chain, which is unable to incorporate within the triple helical molecule. Frameshift mutations within the terminal exon of either collagen gene have been identified that lead to synthesis of full-sized procollagen chain, which is rapidly degraded intracellularly when it fails to incorporate into the collagen molecule (Willing *et al.*, 1993). In the case of the Col1A1 product, mild OI is observed, but when it occurs in the Col1A2 product, homotrimeric molecules are formed causing a more severe bone phenotype (see earlier discussion).

CONSEQUENCES OF OI-PRODUCING MUTATIONS

Formation on the Extracellular Matrix and Osteoid Mineralization

Many approaches have been employed to characterize the triple helical structure and intramolecular organization of collagen fibrils in normal and OI subjects. X-ray diffraction analysis of collagen-like peptides show the destabilizing effect of a glycine point mutation (Beck *et al.*, 2000) and studies using NMR and circular dichroism lead to a similar conclusion (Baum and Brodsky, 1999; Liu *et al.*, 1998; Melacini *et al.*, 2000). At the intramolecular level, x-ray diffraction has shown small fibers with less well-defined lateral growth and more fiber disorganization in tissue obtained from OI subjects (McBride *et al.*, 1997). At the biochemical level, mutations that interrupt the helix decrease the thermal stability of procollagen molecules and render the molecule more susceptible to proteolytic attack by tissue proteases (Bachinger *et al.*, 1993). This may explain the observation that mutant collagen molecules are not uniformly distributed through matrix but are found on the surface of bone (Bank *et al.*, 2000). Because 25% of the collagen produced by cells containing a Col1A1 mutation are normal and 50% of the collagen produced by cells containing a Col1A2 mutation are normal, tissue proteases probably select against the mutant molecules (Bateman and Golub, 1994) allowing for a substrata of relatively normal collagen fibers to accumulate.

Transmission and scanning EM have shown that the periodicity of OI fibrils is normal but the fibrils are disorganized and have wide variation in fiber diameter (Eyden and Tzaphlidou, 2001). Fibril formation can be studied *in vitro* from components synthesized by cultured cells (Hashizume *et al.*, 1999; Holmes *et al.*, 2001). The importance of other proteins that modify the size and organization of otherwise normal type I collagen fibrils has been revealed by EM studies. These changes can affect the mechanical properties of the collagen fibers formed *in vitro* (Christiansen *et al.*, 2000; Ottani *et al.*, 2001). For example, the co-polymerization of type V collagen within the type I collagen fibril influences the size and structure of the type I collagen fibril (Kypreos *et al.*, 2000; Mizuno *et al.*, 2001). Another modifier of the collagen fiber size is the incorporation of unprocessed type I procollagen producing another form of EDS that can overlap with features of type I OI. The EDS–OI-like symptoms appear to result from impairing cleavage of the procollagen propeptide secondary to glycine substitution disruption in the N-terminal helical domain. A similar problem might be expected with a mutation affecting cleavage of the C terminal propeptide (Holmes *et al.*, 1996). Induced mutations in certain noncollagenous proteins such as decorin (Danielson *et al.*, 1997), fibromodulin (Ezura *et al.*, 2000), and microfibrillin (Kielty *et al.*, 1998) can affect the structure or organization of type I collagen fibers indicating that physical interaction between the two components play an important role in this process. It would not be surprising that OI mutations may affect some of these binding interactions, adding to the complexity of the bone phenotype.

The interaction of the mineralizing phase of new bone formation with the matrix has been studied by high-voltage electron microscopy (Landis, 1996a, 1996b), Fourier transform infrared microspectroscopic analysis (Cassella *et al.*, 2000; Camacho, 2003), and small angle x-ray scattering (Fratzl *et al.*, 1996). These methods demonstrate that although the absolute amount and composition of hydroxyapatite within an OI bone is probably not abnormal, the crystal structure is deformed and probably contributes to the overall weakened nature of the bone (Camacho *et al.*, 1999). Thus while the primary defect is in helix formation, the ultimate determination of bone strength reflects how the helix influences the interaction of noncollagenous proteins and minerals and at this point the critical features of the molecule that control these interactive events are not fully understood.

INTRINSIC PROPERTIES OF THE OI OSTEOBLAST

The presence of intracellular mutant procollagen molecules and the physiological response to the abnormal extracellular osteoid combine to have a profound impact on the biology of the osteoblast that further influences the severity and natural history of disease. It has been appreciated for some time that the rough endoplasmic reticulum of OI fibroblasts are grossly dilated (Lamande *et al.,* 1995) and the secretion of fully formed procollagen is impaired (Fitzgerald *et al.,* 1999; Lamande and Bateman, 1999). The role that hsp47 chaperone protein has in determining the trafficking of normal and mutant molecules within these cells is likely to be an important determinant for the biology of the affected osteoblast (Kojima *et al.,* 1998). In fact, gene knockout of the hsp47 protein is an embryonic lethal in which type I procollagen is produced that is susceptible to protease digestion (Nagai *et al.,* 2000; Nagata, 2003 ; Kubota, 2004), suggesting that this chaperone protein plays an essential role in normal triple helix formation, secretion, and fibril assembly (Ishida, 2006). Specific Y position amino acids with the collagen triplets are required for hsp47 to collagen (Koide, 2006) and many of the abnormalities in collagen production may result from loss of this interaction. The central role of chaperone proteins in explaining the disease severity in the Brit IV mouse has been demonstrated (Forlino, 2007). The retention of the mutant procollagen molecule also leads to post-translational overmodification of the lysine residues in the helical domain that may further affect the quality of fibril formation.

The inherent ability of an OI fibroblast or bone cell to produce collagen and proliferate *in vitro* is impaired and probably a consequence of the retained procollagen molecules with the distended rough ER. *In vitro* studies of osteoblasts derived from OI humans (Fedarko *et al.,* 1995; Fedarko *et al.,* 1996) or OI mice (Balk *et al.,* 1997) show diminished markers of osteoblastic differentiation as well as reduced rate of cell proliferation. However, the cells can be driven into osteoblastic differentiation when given exogenous bone morphogenic protein (BMPs). The explanation for this observation is not clear but may reflect the quality or quantity of the extracellular matrix that is made by the preosteoblastic cell that is necessary for osteoblast differentiation *in vitro*. Possibly the high rate of bone turnover that is characteristic of this disease (see next section), may lead to exhaustion and/or premature senescence of stem cells capable of generating vigorous osteoblastic cells *in vitro*. If stem cell exhaustion is also present in intact bone, then an additional factor of osteoblast number will contribute to the severity of the bone disease.

PHYSIOLOGICAL DEMANDS ON THE OI OSTEOBLAST

Intact bone, probably through the osteocyte, is able to sense its mechanical environment and initiate a new round of bone formation when the load on the region exceeds its ability to carry it. This fundamental principle of bone biology is continuously called upon in OI because the matrix that is produced is never able to support the load placed on the skeleton. This situation is illustrated in the histology of OI bone that shows a state of high turnover characterized by increased numbers of osteoblasts and osteocytes (Jones *et al.,* 1999) and an increased number of osteoclasts. Dynamic labeling shows that increased number of double labeled surfaces of normal thickness (Rauch *et al.,* 2000). Biochemical markers for bone formation and resorption in growing children and adults do reflect the histological findings although the measurements are variable because of differences in growth rate and the underlying mutation (Lund, Hansen *et al.,* 1998;Braga, 2004). Some of these variables can be overcome in murine models in which there is uniformity of the genetic defect and sufficient numbers of mice of similar age and sex.

Analysis of murine models is particularly instructive in appreciating the pathophysiology of the OI mutation (Kalajzic, 2002). Because net total bone formation in OI bone is low and its intrinsic properties for matrix production in culture are impaired, the OI osteoblast or its lineage is viewed as underproductive. This view can lead to the conclusion that stimulation to increase its rate of matrix formation should be beneficial to the disease. However, the OI osteoblast lineage is under constant stimulation to proliferate to build up sufficient numbers of precursor cells to progress to full osteoblast differentiation and produce the matrix that was resorbed by the activated osteoclasts. The activated osteoblastic lineage can be demonstrated by measuring the content of col1a1 mRNA in OI bone or the activity of a type I collagen promoter transgene that is sensitive to osteoblastic activity. In both cases, a high level of transcriptional activity for type I collagen can be demonstrated relative to normal bone. The net effect is an uncoupling between the signals transmitted from the bone matrix to be bone lineage in which the bone cells do respond at the gene level but cannot deliver at the protein level. The lineage is already maximally stimulated in response to the activated osteoclastic pathways but the new matrix that is produced does not improve the mechanical properties of the bone.

Studies on the oim mouse model illustrates that it is the balance between matrix formation and resorption that determines bone strength in OI (Fig. 1). During periods of rapid linear growth, the deficit between formation and resorption is maximal because at this time bone turnover is enhanced even beyond the level that is responsive to mechanical forces. Although normal bone has the reserve within the bone lineage to increase its rate of matrix formation, the OI bone lineage is already maximally stimulated so that it is during the period of linear growth that the deficit in net bone formation is most severe. This may explain why growth retardation and fractures are so severe in the rapidly growing child. With the completion of puberty and cessation of linear growth, bone remodeling slows and a balance between formation and resorption becomes more favorable. Thus, puberty does not improve bone strength

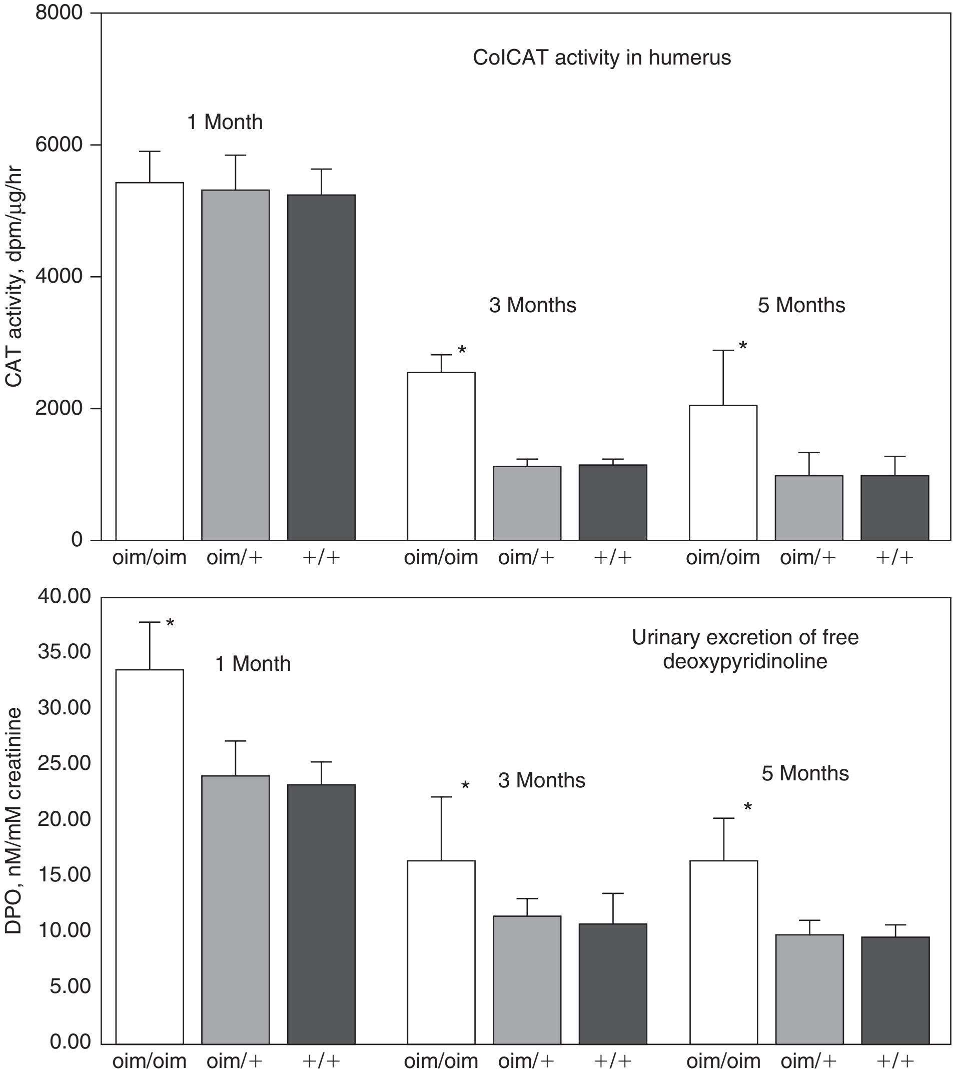

FIGURE 1 Relative balance of bone formation and resorption in OIM mice during the period of rapid somatic growth (1 month), adulthood (3 months), and older age (5 months). During somatic growth, the activity of the oim/oim osteoblast population does not exceed that of normal animals whereas bone resorption of the 1-month-old animal is greater than normal. Thus, it is during the period of rapid bone growth that the deficit between bone formation and resorption is maximal. With stabilization of the skeleton, the continuous demand for new bone formation in the oim/oim mouse become evident and during this time the deficit between bone formation and resorption is less severe.

by stimulating the lineage but instead it stabilizes the skeleton and reduces the need for bone remodeling. When menopause reinstates a state of high bone resorption, the balance between formation and resorption again becomes unfavorable and fractures can return. In effect, the pathophysiology of OI can be view as a consequence of the activated osteoclast lineage and this probably explains the success of bisphosphonate treatment in OI.

Nondeforming OI secondary to an expressed collagen mutation probably represents a mild variant of deforming OI discussed earlier. However, OI type 1 resulting from haploid insufficiency of a COL1A1 chain may have subtle differences. Analysis of the heterozygous Mov 13 mice show diminished bone volume and half-normal levels of col1a1 mRNA. This is consistent with human OI in which low levels of procollagen propeptide in blood reflect the null mutation in type I collagen-producing cells (Minisola *et al.,* 1994). Histomorphometry does show increased osteoblast cellularity and bone forming units and dynamic histomorphometry suggest a decrease in osteoid seams (McCarthy *et al.,* 1997). Excessive osteoclastic activity does not appear to be present. In both mouse and man with type I OI, significant skeletal remodeling becomes apparent upon sexual maturation so that the mechanical properties of the bone approach normal (Bonadio *et al.,* 1993; Jepsen *et al.,* 1997). Although further analysis of a murine model that is healthy into adulthood is necessary, it would appear that the deficit between bone formation and resorption in type I OI is much less than in deforming forms of OI, particularly after the adult skeleton is established.

Thus, it is during adulthood that a relatively normal bone matrix is accumulated and fractures are uncommon. It is only during growth and in menopause that this relationship is unfavorable, again underscoring the value of bisphosphonates for improving bone strength during these periods.

THERAPY OF OI

Anti-Resorptive Agents

Bisphosphonate therapy in infants or young children with OI has changed the natural history of the disease. When administrated properly, it can reduce fracture frequency, increase bone mineral content, and improve the radiographic assessment of the bone shape in growing children (Astrom and Soderhall, 1998; Glorieux *et al.,* 1998). The positive effects are most dramatic in infants from ages 1 to 3 in which the severity of the classification of the disease can be improved (Plotkin *et al.,* 2000) (Astrom, 2007). In infants and young children, remarkable improvement is seen in the quality of life, which includes motor function and muscle strength (Montpetit, 2003 ; Land, 2006). Infants begin to ambulate and children participate in more strenuous physical activities. Bone pain and diaphoresis, which is a major complaint often overlooked by physicians as well as families, is greatly diminished by the treatment. Linear growth is not impaired and in fact augmentation of final stature in the most severely affected subjects can exceed their predicted outcome (Zeitlin, 2003). Fractures heal at their expected rate. The major effects on bone mineral density (BMD) occur during the first 2 years of treatment, but continued treatment for at least 4 years does result in continued improvements in BMD and fracture rate (Rauch, 2003; Rauch, 2007). Less dramatic increases in bone density are being observed in adults with mild OI although the effect on fracture frequency has not been apparent (Shapiro, 2003; Chevrel, 2006). Bisphosphonate treatment appears to be effective in OI type V (Fleming, 2005; Zeitlin, 2006) and type VI (Land, 2007) and it can be anticipated that it should be effective in OI type VII.

Initial studies have used cyclical intravenous administration of pamidronate and currently prospective studies using oral forms of bisphosphonates are ongoing (Glorieux, 2000). Other published studies using agents that are active orally or require less frequent intravenous dosing appear to be equally effective (Sakkers, 2004; Seikaly, 2005; Gatti, 2005; DiMeglio, 2006). Other studies are currently ongoing so it is difficult to say which form is most optimal for any subset of affected individuals.

The success of bisphosphonate appears to be related to the unremitting osteoclastic activity (Rauch, 2002). The effect of the drug can be monitored by measuring the level of collagen-derived cross-links in blood or urine. Clinical symptoms of bone pain and diaphoresis also correlate with the inhibitory effect of the drug on osteoclastic activity suggesting that it is the process of high bone turnover and associated high blood flow, not unlike a pagetic lesion, that underlie these symptoms. Even though the matrix it now accumulates still contains the mutant collagen molecules, the balance between formation and degradation is improved and the accumulation of matrix improves bone strength relative to a bone without matrix. The experience in children indicates that linear growth and repair of fractures is not compromised by the use of the bisphosphonate. Murine studies show less bowing and a diminished fractured frequency, even though bone strength by direct mechanical testing cannot be demonstrated. Further work is needed using animal models to confirm that the drug is acting primarily at the level of the osteoclast and does not have other actions that contribute to clinical improvement. For example, if the bisphosphonate acts to reduce the level of bone turnover it might have the additional benefit of extending the longevity of the osteoblast lineage or allowing the resident osteoblast to achieve full differentiation and time to remodel the existing matrix from a woven to lamellar bone.

ANABOLIC AGENTS

Growth hormone, IGF1, and PTH have the potential to increase bone mass. Except for a treatment protocol with growth hormone in children with deforming OI, most of the experience with these agents has been anecdotal. Like all children who are initially started on growth hormone, OI children do experience an initial acceleration of growth rate and increase in BMD (Marini, 2003). Because the treatment duration has been limited, a bone mass increase in excess to the increase in body size has not been reported, although stable (42)Ca isotopic studies demonstrate an improvement in mineral incorporation while on treatment (Vieira *et al.,* 2000; Vieira *et al.,* 1999). Given the underlying physiological basis of OI, it would be surprising that an agent that stimulates more bone turnover as part of its anabolic action would have a long-term beneficial effect (Wright, 2000). The osteoblast lineage is already maximally stimulated and the addition of agents that enhance osteoclastic activity will only contribute to the deficit between formation and degradation. Perhaps the milder forms of OI that lack the inherent high rate of bone resorption will be more responsive to an anabolic agent (Antoniazzi *et al.,* 1996) as suggested by the murine studies of the heterozygous OIM mouse (King, 2005). Potentially the combination of growth hormone and bisphosphonate might provide a compromise that is acceptable and studies of this combination are underway. PTH is also being evaluated for OI. Although the molecular and cellular mechanism of PTH enhancement of bone formation is still controversial, its known action to enhance

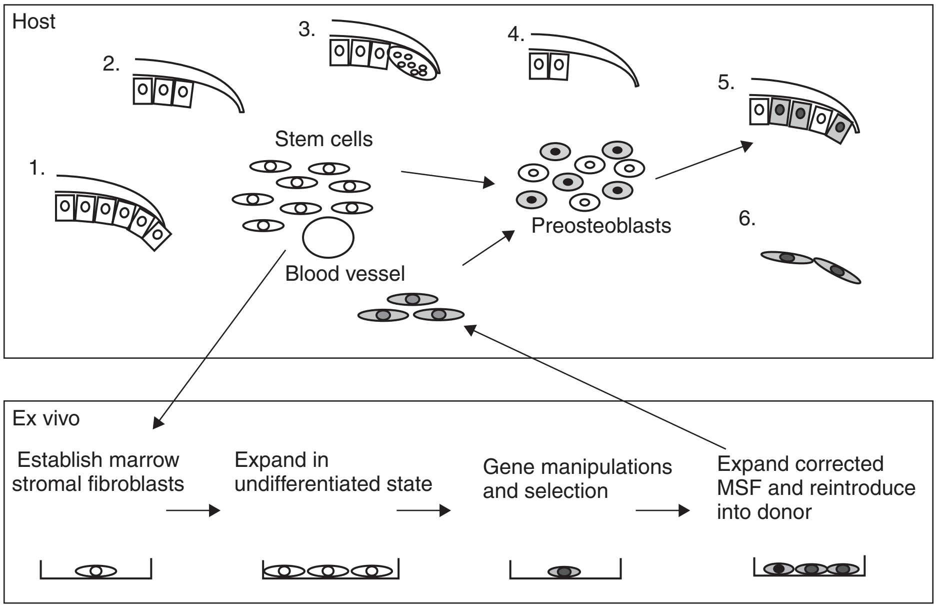
Host
1.
2.
3.
4.
5.
6.
Stem cells
Preosteoblasts
Blood vessel
Ex vivo
Establish marrow stromal fibroblasts
Expand in undifferentiated state
Gene manipulations and selection
Expand corrected MSF and reintroduce into donor

FIGURE 2 Strategy for somatic gene therapy for OI. The host bone is undergoing rapid and continuous bone turnover in which activated osteoblasts (1) start a cycle of bone remodeling; (2) Osteoclasts remove damaged bone matrix; (3) followed by a new wave of bone formation (4 and 5). Cells that contribute to the new round of bone formation are captured, probably by bone marrow aspiration, and grown in a culture dish under nondifferentiating conditions. Genetic manipulations include elimination and replacement of the mutant gene followed by expansion of the population of corrected cells. These cells are reintroduced into the host (darkened cells) and they begin to participate in the cycle of new bone formation. Over time the corrected cells outpopulate the endogenous cells and the quality of the bone matrix improves (5 and 6).

commitment of progenitor cells to bone as opposed to fat (Rickard, 2006), and its anti-apoptotic effect on mature osteoblasts (Bringhurst, 2002) should favorably promote bone formation by osteoblasts in the individual with OI.

CELL AND GENE THERAPY

Because bisphosphonates do not correct the primary cause for OI and their long-term use and its long-term effectiveness are still uncertain, steps to correct underlying genetic mutation are being evaluated in both humans and mice. The possibility that gene therapy is a feasible strategy in OI came from the analysis of individuals who are somatic mosaic for an OI mutation that constitute up to 40% of mutant cell within bone but do not have evidence of bone disease (Cabral, 2004). Those studies suggest that the deleterious effect of OI cells can be neutralized by the presence of normal cells so that if it were possible to introduce normal cells into an individual with OI, the severity of bone disease would be reduced (Figure 2). Furthermore, because bone turnover is high and the endogenous osteoblast lineage is activated in OI, introduction of normal cells into this environment would rapidly populate the bone with cells having a normal proliferative rate and making a normal matrix that would outproduce the effort from the resident OI cells. This treatment strategy requires the ability to introduce cells from the osteoblast lineage into OI subjects. Ideally, autologous bone progenitor cells could be engineered to replace the OI collagen gene activity or the progenitors could be derived from a tissue-matched normal individual. The latter choice was the rationale for non-autologous bone marrow transplant procedures in a limited number of children with severe OI (Horwitz *et al.*, 1999; Horwitz *et al.*, 2001). The long-term success of these initial studies has been difficult to assess and even the basic rationale of this approach is being questioned based on animal experimental data. Thus, there are two primary questions that need to be answered before cell therapy can be considered an alternative. First, is there a source of tissue-matched bone progenitor cells available for transplantation, and second, is it possible to deliver progenitor cells to bone that can affect an improvement in bone quality?

The immune compatible progenitor problem has been approached using a wide variety of genetic engineering techniques. The goal is to reduce the relative output of mutant collagen chain production relative to the normal allele. Numerous RNA suppression techniques such as hammerhead (Smicun, 2003; Peace, 2005) and hairpin (Lian *et al.*, 1999) ribozymes, U1snRNA (Beckley *et al.*, 2001; Liu, 2002), RNA transplicing (Mansfield *et al.*, 2000), RNase P (Kawa *et al.*, 1998) and RNAi (Millington-Ward, 2004) have achieved partial suppression in cultured cells but have not been evaluated in murine models of OI. Overexpression of the normal allele (Pochampally, 2005) is another approach that may achieve a more favorable output of bone matrix. Complete inactivation of the mutant allele has been achieved by specific integrations of a fragment of DNA containing a transcription stop sequence from an adeno-associated viral vector (Chamberlain, 2004). Because the integration event targets both the normal and mutant allele with equal affinity as well as integrating other sites randomly, a selection technique for the correctly targeted cell is necessary. Although the resulting cells now have a type I OI phenotype, it is clearly an improvement over the parental cells. Another strategy, yet to be employed in OI, are RNA/DNA oligonucleotides that utilize host gene repair mechanism to correct a point mutation (Ino, 2004; Bertoni, 2005; Tagalakis, 2005). The potential to correct, not just inactivate a mutation, is a particularly attractive aspect of this approach but the efficiency is quite variable and a strategy to identify correctly targeted cells from a large mutant background have not been developed.

Despite the genetic engineering problems, the most severe hurdle for somatic gene therapy for OI is the reintroduction into a host of cells that are capable of homing to bone and participating in new bone formation. Although there is no question regarding the ability of marrow stromal cells to differentiate into mature osteoblasts *in vitro* or in subcutaneous implant (Dennis *et al.,* 1999; Bilic-Curcic, 2005), demonstration that this is possible when administered systemically is still unconvincing. The plasticity of this cell source has been cited for adult stem cell from bone marrow or fetal cord blood (Chang, 2006) after systemic injection for numerous nonskeletal tissues. However, the interpretation of experiments have come under increasing scrutiny (Wagers, 2002, 2004) to suggest that the apparent success may be due to donor-host cell fusion (Sherwood, 2004; Quintana-Bustamante, 2006), engraftment of donor-derived myeloid cells (Balsam, 2004) or excessive optimism (Theise, 2005). Most studies in man and mouse can demonstrate a low degree (1–5%) of engraftment of bone or bone marrow stroma as assessed by a nonspecific, donor-derived transgenic or unique endogenous genetic marker (Ding *et al.,* 1999; Onyia *et al.,* 1998; Pereira *et al.,* 1998; Nilsson *et al.,* 1999; Dominici, 2004). These experiments were repeated using a differentiation-restricted transgenic visual marker gene (Kalajzic, 2002) to identify the donor cells that achieved systemic distribution and that lined the surface of bone and other tissues. In bone, many donor-derived cells had markers of an osteoclast (Wang, 2005; Boban, 2006) whereas in lung they had markers of macrophages (Boban, 2007). Bone cell differentiation from donor-derived marrow stromal cells can be demonstrated in this transplantation model, but the donor cells with osteogenic properties remain at the site of intramarrow injection and do not circulate (Wang, 2005). These data do not support the strategy that genetically modified cells can be used to repopulate bone via a systemic route.

The underlying genetic abnormality also determines the success of stromal cell transplantation. When the disease is non-cell autonomous, (i.e., the transplanted cells are engineered to secrete a deficient soluble factor), an improvement in the disease phenotype occurs irrespective of where the cells established residence. This is most obvious when the transplanted cells express a cytokine (Brouard *et al.,* 1998) or clotting factor (Chuah *et al.,* 2000). This mechanism may explain the recent success of bone fragment transplantation for infantile hypophosphatasia (Cahill, 2007; Whyte, 2003). Clinical improvement of a disease that is cell autonomous, such as OI, requires that the cells populate the affected bone, proliferate, differentiate, and participate in bone turnover. This is a standard that has not yet been met in any murine studies. The one exception is when the transplanted cells have undergone prolonged expansion *ex vivo* (Dahir *et al.,* 2000; (Oyama *et al.,* 1999; Niyibizi, 2004; Wang, 2006). For mouse, this type of treatment rapidly leads to cell immortalization, making interpretation of a transplantation experiment difficult.

As new pharmacologic or genetic therapies are developed, it will be increasingly necessary to establish a set of criteria that is used to judge its success relative to current therapies. Although bisphosphonates still need further evaluation to appreciate fully their most effective use in different age and disease classifications, they do represent a significant advance in the treatment of OI and probably have to be regarded as the threshold that a new therapy has to exceed. Particularly as it relates to somatic gene therapy for OI, to develop criteria that (a) demonstrate successful engraftment and (b) document improvements in bone health that are equivalent to or exceed medical therapy (Table II, column A). Whether the transplanted individual is mouse or man, it must be demonstrated that the transplanted cells populate the bone, expand in number over time, participate in the new bone formation, and provide a continuous source of new bone cells over the life of the transplanted subject. These studies are easier to perform in the mouse when the transplanted cells are engineered to contain visual transgenes driven by bone-specific promoters. Not only does the transgenic marker identify the source of the cell, it can reflect its level of participation in new bone formation. Moreover, this approach can be used to determine whether donor stem cells removed from the transplanted bone are still able to generate differentiated osteoblastic cells *in vitro*. If the transplanted cells are participating in new bone formation, the quality and quantity of the bone should improve over time. Radiographs should show remodeled bones with improved architecture and bone density measurements at well-defined sites will be increased in humans. QCT scanning can be used to assess bone mass and architecture in mice. Bone histomorphometry and dynamic labeling studies should confirm these clinical measures of bone health. Particularly in the mouse, it should be possible to demonstrate improved mechanical properties of the bone after the intervention. Biochemical studies can also contribute to the impression of success by demonstrating that markers of new

TABLE II Criteria for Successful Therapy of OI. Assessment of Current Trials of Bisphosphonate and Cell Transplantation in Man and Mouse.

	A. Drug Therapy		B. Cell Therapy	
Criteria	Man	Mouse	Man	Mouse
Cellular evaluation				
Cell engraftment	–	–	+	+
New bone formation	+++	+++	NR	NR
Increasing cell number; graft persistence	+++	+++	NR	NR
Bone evaluation				
BMD, QCT	+++	+++	+	NR
X-ray	++	+	NR	NR
Bone histology	+++	+++	+	+
Structural properties	NA	+++	NA	NR
Biochemical	+	++	NR	NR
Clinical criteria				
Quality of life	+++	+	NR	NR
Growth, weight gain	+++	+	++	+
Muscle strength	++	++	NR	NR
Fracture rate	+	+	+	NR

NR – *not reported*; NA – *not ascertainable*.
+ *Present*.
- *Absent*.

bone formation remain elevated while the level of markers reflecting bone degradation gradually subside.

The clinical evaluation of transplanted individuals is equally important to the assessment of success. Bone pain and diaphoresis are major symptoms that are daily facts of life for many patients with OI. Muscle weakness can be profound and in growing children greatly delay acquisition of motor milestones. Successful interruption of the pathophysiological cycle of OI appears to reduce greatly these symptoms. Bone pain diminishes, diaphoresis is greatly reduced, and muscle tone improves. Children begin to ambulate and adults find that their activities of daily living are easier to perform. Because the symptoms are so profound for individuals with OI, they need to be recorded in a prospective manner as clinical outcome measure. These types of measurements are difficult to record in the mouse although cage activity can be quantitated and probably would be informative. Certainly fracture frequency, linear growth, and weight gain are important to record although they can be influenced by many other factors unrelated to the intervention.

Even disregarding the molecular steps necessary to correct the genetic abnormalities in OI stromal cells, the success of transplantation of normal cells into a normal or OI host is relatively ineffective at this time (Table II, column B). Given the experimental difficulties of performing these studies in a controlled and quantitative manner in humans, greater emphasis must be placed on studies in experimental animals in which the problems inherent to the procedure can be identified and solved. Ultimately, these problems will be solved and this will open a new era of therapeutic opportunities for OI and other osteopenic bone diseases.

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Chapter 70

Hereditary Deficiencies in Vitamin D Action

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INTRODUCTION

Hereditary deficiencies in vitamin D action can be caused by disturbances in the synthesis of the hormonal form of the vitamin, 1,25-dihydroxyvitamin D (1,25(OH)$_2$Dcalcitriol), or defects in the interaction of calcitriol and its target tissues.

Vitamin D derived from endogenous production in the skin or absorbed from the gut is transformed into its active form by two successive steps: hydroxylation in the liver to 25-hydroxyvitamin D [25(OH)D], followed by 1α-hydroxylation in the renal proximal tubule to 1,25(OH)$_2$D. Some other cells exhibit 1α-hydroxylase activity: placental decidual cells, keratinocytes, macrophages of various origins, and some tumor cells. The role of the extrarenal production of 1,25(OH)$_2$D is unknown, and under normal conditions it does not significantly contribute to the circulating levels of the hormone. Hydroxylation at carbon 24, to produce 24, 25-dihydroxyvitamin D [24,25(OH)$_2$D] or 1,24,25-trihydroxyvitamin D, is performed in a wide range of normal tissues and is believed to be important in the removal of vitamin D metabolites. All these enzymes are mitochondrial mixed-function oxidases containing cytochrome P450 and having ferredoxin- and heme-binding domains. Cloning of cDNA for porcine 25-hydroxylase and the mouse, rat, and human 24- and 1-hydroxylase, as well as the genes for the later two enzymes, has been reported (Akeno *et al.*, 1997; Fu *et al.*, 1997a; Jehan *et al.*, 1998; Jones *et al.*, 1999; Monkawa *et al.*, 1997; Murayama *et al.*, 1999; Ohyama *et al.*, 1993; Shinki *et al.*, 1997; Takeyama *et al.*, 1997). Hereditary defects along the cascade of 1,25(OH)$_2$D synthesis lead to a deficiency in the vitamin D hormonal form.

Isolated deficiency of 25(OH)D is a very rare autosomal recessive disorder, attributed to, but not proven to be caused by deficiency in the hepatic vitamin D-25 hydroxylase activity (Gill *et al.*, 2000).

This discussion will therefore concentrate on hereditary defects in the renal 25-hydroxyvitamin D-1α-hydroxylase activity. Regarding interaction with the target tissue, as detailed elsewhere in this book, calcitriol effects are mediated via a high-affinity intracellular receptor: the vitamin D receptor (VDR). VDR acts as a ligand-modulated transcription factor that belongs to the steroid, thyroid, and retinoic acid receptors gene family (Baker *et al.*, 1988; Burmester *et al.*, 1988; Evans, 1988; Ozur *et al.*, 1991). Thus, hereditary defects in the interaction of calcitriol and its target tissues could evolve from defects in hormone binding to VDR, defects in the heterodimerization with RXR or the interaction of the receptor complexes with DNA, or defects in the transcription modulation function.

Though VDRs were demonstrated in most, if not all, tissues, leading to the increased recognition of multiple target organs and actions of the hormone, it seems that most, if not all, clinical signs and symptoms associated with deficient vitamin D actions are caused by perturbations in mineral metabolism.

1,25(OH)$_2$D is the most powerful physiological agent that stimulates active transport of calcium, and to a lesser degree phosphorus and magnesium, across the small intestine (DeLuca, 1984; Mayer *et al.*, 1984). Thus, deficiencies in vitamin D action will lead to a decrease in the net flux of mineral to the extracellular compartment, causing hypocalcemia and secondary hyperparathyroidism. Hypophosphatemia will ensue as a result of both reduced absorption of phosphorus owing to deficient calcitriol action on the gut and increased renal phosphate clearance owing to secondary hyperparathyroidism. Low concentrations of calcium and phosphorus in the extracellular

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fluid will lead to defective mineralization of organic bone matrix. Defective bone matrix mineralization of the newly formed bone and growth plate cartilage will produce the characteristic morphological and clinical signs of rickets, whereas at sites of bone remodeling, it will cause osteomalacia. Deficiencies in vitamin D action may impair the differentiation of osteoblasts and thus their functional capacity to mineralize bone matrix; this may be an additional mechanism leading to rickets and osteomalacia (Owen *et al.*, 1991; Reichel *et al.*, 1989).

Because all hereditary deficiencies in vitamin D action will lead to the same clinical, radiological, histological, and most of the biochemical aberrations, those common features will be discussed first.

CLINICAL FEATURES OF RICKETS AND OSTEOMALACIA

Children with hereditary deficiencies in vitamin D action will appear normal at birth and usually develop the characteristic features of rickets within the first 2 years of life. Defects in bone mineralization are particularly evident in regions of rapid bone growth, including, during the first year of life, the cranium, wrist, and ribs. Rickets at this time will lead to widened cranial sutures, frontal bossing, posterior flattening of the skull (craniotabes), bulging of costochondral junction (rachitic rosary), indentation of the ribs at the diaphragmatic insertion (Harrison's groove), and widening of the wrists. After the first year of life with the acquisition of erect posture and rapid linear growth, the deformities are most severe in the legs. Bow legs (genu varum) or knock-knee (genu valgum) deformities of varying severity develop as well as widening of the ends of long bones. If not treated, rickets may cause severe lasting deformities, compromise adult height, and increase susceptibility to pathological fractures.

The specific radiographic features of rickets reflect the failure of cartilage calcification and endochondral ossification and therefore are best seen in the metaphysis of rapidly growing bones. The metaphyses are widened, uneven, concave, or cupped, and because of the delay in or absence of calcification, the metaphyses could become partially or totally invisible (Fig. 1). In more severe forms or in patients untreated for prolonged periods, rarefaction and thinning of the cortex of the entire shaft, as well as bone deformities and greenstick fractures, will become evident.

The clinical features of osteomalacia are subtle and could be manifested as bone pain or low-back pain of varying severity in some cases. The first clinical presentation could be an acute fracture of the long bones, public ramii, ribs, or spine. The radiographic manifestations could be mild, e.g., generalized, nonspecific osteopenia or more specific, such as pseudofractures, commonly seen at the medial edges of long bones shaft.

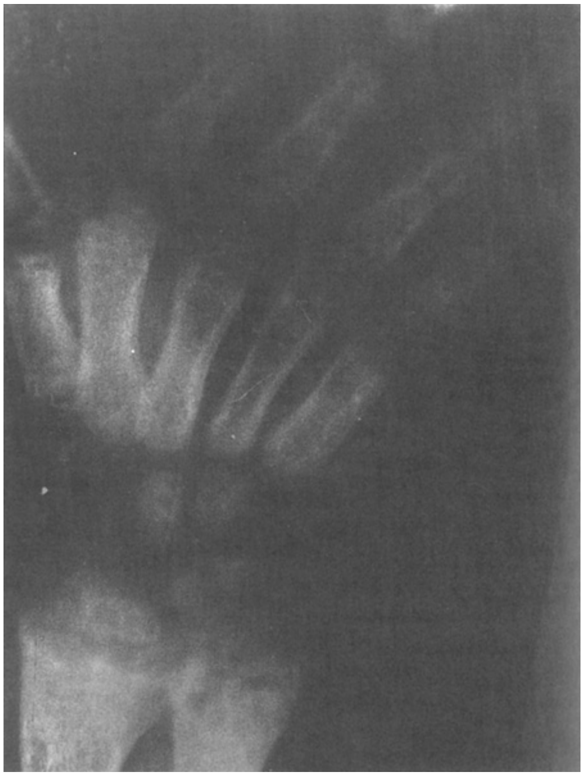


FIGURE 1 Radiographs of wrists and hands of a patient with VDDR. Note the signs of severe rickets and demineralization.

In hypocalcemic rickets and/or osteomalacia, as is the case in deficiencies in vitamin D action, radiographic features of secondary hyperparathyroidism such as subperiosteal resorption and cysts of the long bones may exist.

The characteristic histological feature of rickets and osteomalacia is deficiency or lack of mineralization of the organic matrix of bone. Because in clinical practice a bone specimen can be obtained only from the iliac crest, the histological picture is osteomalacia. Osteomalacia is defined as excess osteoid (hyperosteoidosis) and a quantitative dynamic proof of defective bone matrix mineralization obtained by analysis of time-spaced tetracycline labeling (Parfitt, 1983; Teitelbaum and Bollough, 1979).

The biochemical parameters characterizing deficiencies in vitamin D action can be divided into those associated with vitamin D status, the primary disturbance in mineral homeostasis and the respective compensatory mechanisms, and changes in bone metabolism. Changes in mineral and bone metabolism will be similar in all states of deficient vitamin D action, whereas serum levels of vitamin D metabolites will characterize each of the two classes delineated in the introduction (Table I).

As previously discussed, deficiencies in vitamin D action will lead to hypocalcemia and secondary hyperparathyroidism. Thus, the characteristic biochemical features are low to low-normal concentrations of serum calcium

TABLE I Biochemical Features of Vitamin D-Dependent Rickets (VDDR) Types I and II

	Serum concentrations						
	Calcium	Phosphorus	Alkaline phosphatase	iPTH	25OHD	$1,25(OH)_2D$	$24,25(OH)_2D$
VDRR-I	↓	↓	↑	↑	N–↑	↓	↑
VDRR-II	↓	↓	↑	↑	N–↑	↑	↓

(depending on compensatory parathyroid activity), low urinary calcium excretion, hypophosphatemia, increased serum immunoreactive parathyroid hormone (iPTH) levels, increased urinary cyclic AMP excretion, and decreased tubular reabsorption of phosphate (the last two measures reflecting the biological activity of elevated iPTH). Biochemical markers associated with increased osteoid production, such as bone-specific alkaline phosphatase and osteocalcin, will be elevated in states of rickets and osteomalacia (Cole *et al.*, 1985). However, as $1,25(OH)_2D$ stimulates osteocalcin synthesis *in vivo* and *in vitro*, serum levels of this biochemical bone marker are unreliable measures in hereditary vitamin D-deficient states.

HEREDITARY DEFICIENCY IN $1,25(OH)_2D$ PRODUCTION (VITAMIN D-DEPENDENT RICKETS TYPE I)

Prader *et al.* (1961) were the first to describe two young children who showed all the usual clinical features of vitamin D deficiency despite adequate intake of the vitamin, thus coining the name "pseudovitamin D deficiency." Complete remission depended on continuous therapy with high doses of vitamin D; thus the term "vitamin D-dependent rickets" (VDDR). However, remission of the disease could be achieved by continuous therapy with physiological (microgram) doses of 1α-hydroxylated vitamin D metabolites (Delvin *et al.*, 1981; Fraser *et al.*, 1973).

Family studies have revealed that it is an autosomal recessive disease. Linkage analysis in a subset of French-Canadian families assigned the gene responsible for the disease to chromosome 12q13 (De Brackeleer and Larochell, 1991; Labuda *et al.*, 1990). The gene encoding the 1α-hydroxylase of mouse kidney, human keratinocyte, and peripheral mononuclear cells was localized on chromosome 12*q*13.1-*q*13.3, which maps to the disease locus of VDDR-I (Fu *et al.*, 1997b; Kitanaka *et al.*, 1998; Smith *et al.*, 1999; St. Arnaud *et al.*, 1997, Wang *et al.*, 1998; Yoshida *et al.*, 1998). There are no direct measures of the renal enzyme proving defective 1α-hydroxylase activity. This is virtually impossible to obtain, because of both the difficulty in obtaining tissue and the low level of expression. There are, however, several indirect observations to support this etiology. First, circulating levels of 25-hydroxyvitamin D (25(OH)D) are normal or elevated, depending on previous vitamin D treatment. Second, serum concentrations of $1,25(OH)_2D$ are very low (see Table I). Third, although massive doses (100 to 300 times the daily recommended dose) of vitamin D or 25(OH)D are required to maintain remission of rickets, physiological replacement doses of calcitriol are sufficient to achieve the same effect. Fourth, it was reported that cells isolated from the placenta of two women with this disease had deficient activity of the decidual enzyme 25(OH)D-1α-hydroxylase (Glorieux *et al.*, 1995). It is noteworthy that human decidual cells do produce $1,25(OH)_2D$ and that this enzyme was regulated by feedback mechanisms (Delvin and Arabian, 1987; Diaz *et al.*, 2000; Weisman *et al.*, 1979). Finally, the 1α-hydroxylase gene from more than 25 families with VDDR-I and some of their first-degree healthy relatives were analyzed by direct sequencing, site-directed mutagenesis, and cDNA expression in transfected cells (Fu *et al.*, 1997; Kitanaka *et al.*, 1998–1999; Smith *et al.*, 1999; St. Arnaud *et al.*, 1997; Wang *et al.*, 1998; Yoshida *et al.*, 1998). All patients had homozygous mutations, whereas parents or other healthy siblings were heterozygous for the mutation. Most patients of French-Canadian origin had the same mutation causing a frameshift and a premature stop codon in the putative heme-binding domain. The same mutation was observed in additional families of diverse origins (Smith *et al.*, 1999). All other patients had either a base-pair deletion causing premature termination codon upstream from the putative ferredoxin and heme-binding domains, or missense mutations (Fu *et al.*, 1997b; Kitanaka *et al.*, 1999; Smith *et al.*, 1998; Wang *et al.*, 1998; Yoshida *et al.*, 1998). No 1α-hydroxylase activity was detected when the mutant enzyme was expressed in various cells. The sequence of the human 1α-hydroxylase gene from keratinocytes and peripheral blood mononuclear cells has been shown to be identical with the renal gene (Fu *et al.*, 1997b; Kitanaka *et al.*, 1999; Smith *et al.*, 1999; Wang *et al.*, 1998; Yoshida *et al.*, 1998), thus supporting the use of these accessible cells as a proxy to study the renal tubular enzymatic defect. Taken together, these observations support the notion that the etiology of this hereditary disease

TABLE II Response to Treatment of Patients with Vitamin D-Dependent Rickets (VDDR) Types I and II

	Vitamin D or 25(OH)D		1α-Hydroxylated vitamin D metabolites	
	Physiological	Pharmacological	Physiological	Pharmacological
VDDR-I	−	+	+	Toxic
VDDR-II	−	− or +	−	− or +

Note. Physiological doses are those recommended or being used as replacement therapy. Pharmacological doses are 100 times and more of the physiological doses (see details in text).

is a defect in the renal tubular 25(OH)D-1α-hydroxylase activity.

The beneficial therapeutic effect of high serum concentrations of 25(OH)D in patients treated with massive doses of vitamin D, while $1,25(OH)_2D$ levels remain low, may have several possible explanations. First, high levels of 25-(OH)D may activate the VDR whose affinity for this metabolite is approximately two orders of magnitude lower than for $1,25(OH)_2D$. Second, a metabolite of 25(OH)D may act directly on target tissues, and finally, high levels of 25(OH)D may drive the local production of $1,25(OH)_2D$ via a paracrine–autocrine pathway, assuming that the tissue enzyme is controlled differently than the renal and decidual enzyme.

The differential diagnosis of VDDR-I from other hereditary forms of hypocalcemic rickets and especially the one associated with defects in the vitamin D receptor–effector system is based on serum concentrations of calcitriol and the response to treatment with 1α-hydroxylated vitamin D metabolites (see Table I and Table II).

A similar syndrome has been described and studied in a mutant strain of pigs where the mode of inheritance as well as the clinical and biochemical features is similar to the human disease (Fox *et al.*, 1985; Harmeyer *et al.*, 1982). Piglets affected by the disease have rickets, elevated 25(OH)D with low or undetectable $1,25(OH)_2D$ serum concentrations, normal specific tissue-binding sites for tritiated $1,25(OH)_2D$, and no detectable activity of 25(OH)D-1α-hydroxylase in renal cortical homogenates. Thus, there is strong evidence that the disease state in the pig is caused solely by an inherited defect in the renal enzyme.

HEREDITARY DEFECTS IN THE VITAMIN D RECEPTOR–EFFECTOR SYSTEM (VITAMIN D-DEPENDENT RICKETS TYPE II)

Introduction

This is a rare disorder and only ~60 patients have been reported (Balsan *et al.*, 1983; Bear *et al.*, 1981; Brooks *et al.*, 1978; Castells *et al.*, 1986; Chen *et al.*, 1984; Clemens *et al.*, 1983; Cockerill *et al.*, 1997; Eil *et al.*, 1981; Feldman *et al.*, 1982; Fraher *et al.*, 1986; Fujita *et al.*, 1980; Griffin and Zerwekh, 1983; Hawa *et al.*, 1996; Hewison *et al.*, 1993; Hirst *et al.*, 1985; Hochberg *et al.*, 1984; Liberman *et al.*, 1980, 1983b; Lin and Uttley, 1993; Lin *et al.*, 1996; Malloy *et al.*, 1997, 1999, 2001, 2002, 2002a, 2004; Marx *et al.*, 1978, 1984; Mechica *et al.*, 1997; Nguyen *et al.*, 2002, 2006; Rosen *et al.*, 1979; Saijo *et al.*, 1991; Simonin *et al.*, 1992; Sockalosky *et al.*, 1980; Takeda *et al.*, 1986, 1987, 1989; Tauchiya *et al.*, 1980; Yagi *et al.*, 1993; Whitfield *et al.*, 1996; Zhu *et al.*, 1998 ; and personal communications).

Brooks *et al.* (1978) described an adult patient with hypocalcemic osteomalacia and elevated serum concentration of $1,25(OH)_2D$. Treatment with vitamin D, causing a further increase in serum calcitriol levels, cured the patient. The term vitamin D-dependent rickets type II (VDDR-II) was suggested to describe this disorder. However, reports on additional patients, about half of whom did not respond to vitamin D therapy, as well as *in vivo* and *in vitro* studies to be discussed later, seem to prove that vitamin D dependency is a misnomer. The term hereditary defects in the vitamin D receptor–effector system (the abbreviation HDVDR is suggested) or end-organ resistance to $1,25(OH)_2D$ action is therefore more appropriate to describe the etiology and pathogenesis of this syndrome. However, owing to convention and convenience, the term VDDR-II will be retained in this chapter.

Clinical and Biochemical Features

General Features

The clinical, radiological, histological, and biochemical features (except serum levels of vitamin D metabolites) are typical of hypocalcemic rickets and/or osteomalacia as previously discussed. Notable exceptions are two unrelated patients with hyperphosphatemia, despite elevated serum levels of iPTH (Liberman *et al.*, 1980; Yagi *et al.*, 1993), which can be the end result of long-standing and severe hypocalcemia that paradoxically inhibits the phosphaturic response to PTH or represents an additional hereditary renal tubular defect.

In VDDR-II there is no history of vitamin D deficiency and no clinical or biochemical response to physiological doses of vitamin D or its 1α-hydroxylated active metabolites. Serum levels of 25(OH)D are normal or elevated (depending on preceding vitamin D therapy); $1,25(OH)_2D$ concentrations are markedly elevated before or during therapy with vitamin D preparations; and 24,25-dihydroxyvitamin D ($24,25(OH)_2D$) circulating levels are inappropriately low (see Table I).

The disease manifests itself as an active metabolic bone disease in early childhood. However, late onset at adolescence and adulthood was documented in several sporadic cases including the first report by Brooks *et al.* (1978) and Fujita *et al.* (1980). These patients represented the mildest form of the disease and had a complete remission when treated with vitamin D or its active metabolites. It is unclear if the adult-onset patients belonged to the same hereditary entity, because no further studies on their VDR status have been published.

Ectodermal Anomalies

A peculiar clinical feature of VDDR-II patients, appearing in approximately half of the subjects, is total alopecia or sparse hair (Fig. 2). Alopecia usually appears during the first year of life and in one patient, at least, has been associated with additional ectodermal anomalies (Liberman *et al.*, 1980). It seems that alopecia is a marker of a more severe form of the disease as judged by the earlier onset, the severity of the clinical features, the proportion of patients who do not respond to treatment with high doses of vitamin D or its active metabolites, and the extremely elevated levels of serum $1,25(OH)_2D$ recorded during therapy (Marx *et al.*, 1984, 1986). Though some patients with alopecia could achieve clinical and biochemical remission of their bone disease, none have shown hair growth. The notion that total alopecia is probably a consequence of a defective vitamin D receptor–effector system is supported by the following: alopecia has only been associated with hereditary defects in the VDR system, i.e., with end-organ resistance to the action of the hormone, and has not been recorded with hereditary deficiency in $1,25(OH)_2D$ synthesis, i.e., low circulating levels of the hormone; alopecia is present in kindreds with different defects in the VDRs; high-affinity uptake of tritiated $1,25(OH)_2D_3$ in the nucleus of the outer root sheath of the hair follicle of rodents has been demonstrated by autoradiography (Strumpf *et al.*, 1979); and the epidermis and hair follicle contain a calcium-binding protein that is partially vitamin D-dependent (Marx *et al.*, 1984). Finally, alopecia develops in homozygote VDR knockout mice (Li *et al.*, 1997; Yoshizawa *et al.*, 1997). Taken together, it could be hypothesized that an intact VDR–effector system is important for the differentiation of the hair follicle in the fetus, which is unrelated to mineral homeostasis.

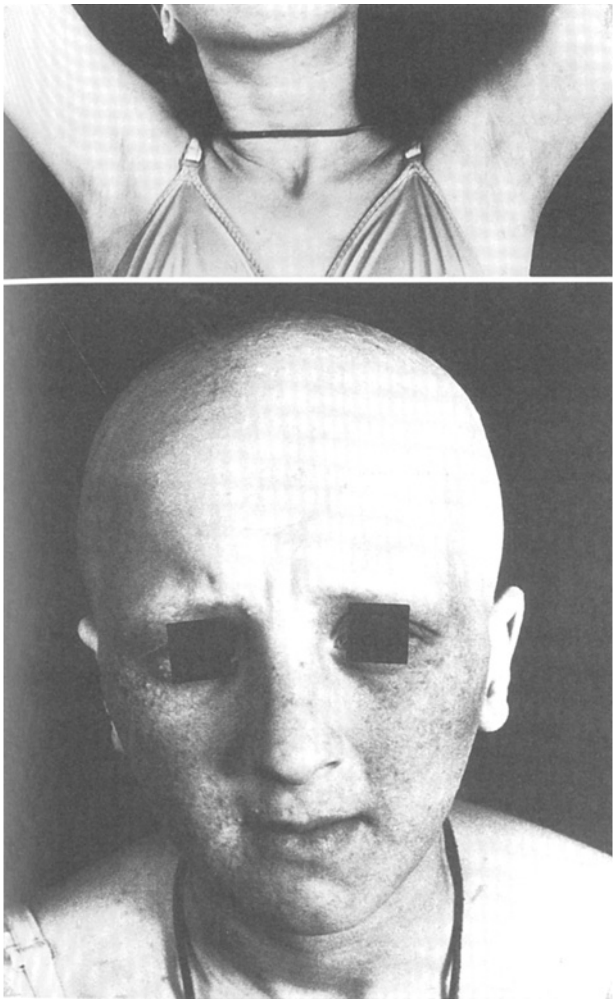


FIGURE 2 A patient with VDDR-II with total alopecia. Note: no scalp or axilla hair and no eyebrows or eyelashes.

Vitamin D Metabolism

Serum concentrations of $1,25(OH)_2D$ range from upper normal to markedly elevated values before therapy, but on vitamin D treatment may reach the highest levels found in any living system (100 times and more than the upper normal range) (Marx *et al.*, 1986). These values may represent the end results of four different mechanisms acting synergistically to stimulate strongly the renal 25(OH)D-1α-hydroxylase. Three of the mechanisms are hypocalcemia, secondary hyperparathyroidism, and hypophosphatemia. The fourth mechanism may be a failure of the negative feedback loop by which the hormone inhibits the renal enzyme activity caused by the basic defect in the VDR–effector system. This was demonstrated in two patients with VDDR-II in remission (normal serum levels of calcium, phosphorus, and PTH) in whom a load of $25(OH)D_3$ had caused a marked increase in serum $1,25(OH)_2D_3$ concentration (Balsan *et al.*, 1983; Marx *et al.*, 1984; Nguyen *et al.*, 2006). It was reported that the 1α-hydroxylase gene expression was not suppressed by 1,25(OH)$_2$D$_3$ in renal tubular cells from VDR knockout mice whereas it was suppressed in cells with normal VDR or heterozygote for the null mutation (Murayama *et al.*, 1999; Takeyama *et al.*, 1997).

1,25(OH)$_2$D is a potent inducer of the enzyme 25(OH)D-24-hydroxylase *in vivo* and *in vitro*. Serum levels of 24,25(OH)$_2$D have been very low or inappropriately low in the face of the elevated concentrations of the hormone in patients with VDDR-II (Castells *et al.*, 1986; Fraher *et al.*, 1986; Liberman *et al.*, 1980; Marx *et al.*, 1984). This probably reflects defective stimulation of 24,25(OH)$_2$D production owing to the basic deficiency in VDR activity. This assumption is supported by the observation that in mutant mice lacking the VDR, expression of 24(OH)-hydroxylase was reduced to undetectable levels and the normal induction of the enzyme by 1,25(OH)$_2$D$_3$ was not obtained (Takeyama *et al.*, 1997).

Mode of Inheritance

In approximately half of the reported kindreds, parental consanguinity and multiple siblings with the same defect suggest an autosomal recessive mode of inheritance (Marx *et al.*, 1984). Parents of patients who are expected to be obligate heterozygotes have been reported to be normal, i.e., no bone disease or alopecia and normal blood biochemistry. However, studies on cells (cultured dermal fibroblasts, Epstein–Barr transformed lymphoblasts, and mitogen-stimulated lymphocytes) obtained from parents of affected children revealed decreased bioresponses, decreased normal VDR protein and its mRNA, and a heterozygote genotype exhibiting both normal and mutant DNA alleles (Malloy *et al.*, 1989, 1990, 1999, 2002; Nguyen *et al.*, 2006; Ritchie *et al.*, 1989; Takeda *et al.*, 1990). There is a striking clustering of patients around the Mediterranean, including patients reported from Europe and America who originated from the same area. A notable exception is a cluster of some kindred from Japan (Fujita *et al.*, 1980; Tauchiya *et al.*, 1980, 1986, 1987, 1989; Yagi *et al.*, 1993).

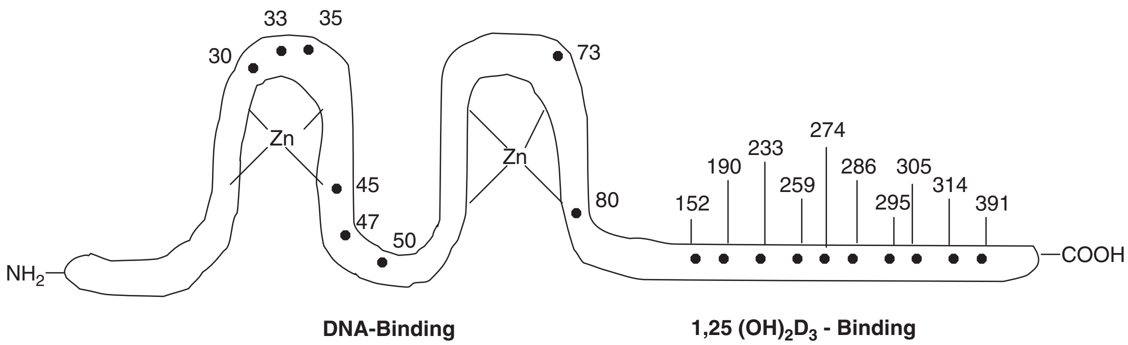
Cellular and Molecular Defects

Methods

The near ubiquity of a similar if not identical VDR–effector system among various cell types, including cells originating from tissues easily accessible for sampling, made feasible studies on the nature of the intracellular and molecular defects in patients with VDDR-II. The cells used were mainly fibroblasts derived from skin biopsies (Balsan *et al.*, 1983; Castells *et al.*, 1986; Chen *et al.*, 1984; Clemens *et al.*, 1983; Eil *et al.*, 1981; Feldman *et al.*, 1982; Fraher *et al.*, 1986; Griffin and Zerwekh, 1983; Hirst *et al.*, 1985; Hughes *et al.*, 1988; Liberman *et al.*, 1983b; Malloy *et al.*, 1989, 1990, 1994, 1999, 2002, 2004; Marx *et al.*, 1984; Nguyen *et al.*, 2002, 2006; Ritchie *et al.*, 1989; Takeda *et al.*, 1989) and peripheral blood mononuclear (PBM) cells (Koren *et al.*, 1985; Ritchie *et al.*, 1989; Takeda *et al.*, 1986, 1990; Yagi *et al.*, 1993). PBM cells contain high-affinity receptors for 1,25(OH)$_2$D$_3$ that are expressed constitutively in monocytes and are induced in mitogen-stimulated T lymphocytes and Epstein–Barr (EB)-transformed lymphoblasts. All cells have been used to assess most of the steps in 1,25(OH)$_2$D$_3$ action from cellular and subcellular uptake of the hormone to bioresponse as well as to elucidate the molecular aberrations in the VDR protein, RNA, and DNA levels.

The hormone–receptor interaction has been analyzed by several methods including the binding characteristics of [^{3}H]1,25(OH)$_2$D$_3$ to intact cells, nuclei or high-salt cellular soluble extracts, so-called cytosol (Balsan *et al.*, 1983; Castells *et al.*, 1986; Chen *et al.*, 1984; Clemens *et al.*, 1983; Eil *et al.*, 1981; Feldman *et al.*, 1982; Fraher *et al.*, 1986; Hirst *et al.*, 1985; Hochberg *et al.*, 1984; Hughes *et al.*, 1988; Koren *et al.*, 1985; Liberman *et al.*, 1983a,b; Malloy *et al.*, 1989, 1990; Marx *et al.*, 1984; Ritchie *et al.*, 1989; Sone *et al.*, 1990; Takeda *et al.*, 1985; Yagi *et al.*, 1993), measurements of VDR protein content by monoclonal antibodies with radiological immunoassay or Western blot analysis (Malloy *et al.*, 1990; Ritchie *et al.*, 1989), immunocytochemical methods in whole cells (Barsony *et al.*, 1990), and characterization of the hormone–receptor complex on continuous sucrose gradient and nonspecific DNA-cellulose columns (Balsan *et al.*, 1983; Chen *et al.*, 1984; Clemens *et al.*, 1983; Eil *et al.*, 1981; Feldman *et al.*, 1982; Hirst *et al.*, 1985; Hochberg *et al.*, 1984; Hughes *et al.*, 1988; Liberman *et al.*, 1983a,b; Malloy *et al.*, 1989; Marx *et al.*, 1984; Sone *et al.*, 1990).

The cloning and nucleotide sequencing of the human VDR gene made it feasible to study the molecular defects in patients with VDRR-II. The methods used included, among others, isolation of genomic DNA that encodes the structural portion of the human VDR, PCR amplification, screening, and sequencing of the amplified DNA fragments (Hughes *et al.*, 1988; Kristjansson *et al.*, 1993; Malloy *et al.*, 1990; Ritchie *et al.*, 1989; Sone *et al.*, 1990; Yagi *et al.*, 1993); cloning and sequencing of the VDR cDNA produced from isolated fibroblasts total RNA by reverse transcription and PCR amplification (Rut *et al.*, 1994; Saijo *et al.*, 1991; Weise *et al.*, 1993); re-creation of the mutant receptor *in vitro* by introducing the appropriate base change in normal VDR cDNA by site-directed mutagenesis that was transfected into cells that do not express endogenous VDR. Post-transcriptional action of 1,25(OH)$_2$D was tested in cells originating from patients or in cells cotransfected with VDR (either mutant or wild type fused to a promotor containing vitamin D response element,



Normal	Mutant	Normal	Mutant
30 Arg (CGA)	Stop (TGA)	152 Gln (CAG)	Stop (TAG)
33 Gly (GGC)	Asp (GAC)	190 Cys (TGT)	Trp (TGG)
35 His (CAC)	Gln (CAG)	233 Leu (GTC)	fs (GTG)
45 Lys (AAA)	Glu (GAA)	259 Gln (CAG)	Pro (CCG)
47 Phe (TTC)	Lle (ATC)	274 Arg (CGC)	Leu (CTC)
50 Arg (CGA)	Gln (CAA)	286 Trp (TGG)	Arg (CGG)
73 Arg (CGC)	Stop (TGA)	295 Tyr (TAC)	Stop (TAA)
73 Arg (CGG)	Gln (CAA)	305 His (CAC)	Gln (CAG)
80 Arg (CGG)	Gln (CAG)	314 Lle (ATC)	Ser (AGC)
		391 Arg (CGC)	Cys (TGC)

FIGURE 3 Schematic presentation of the homozygous mutation in the VDR protein in VDDR-II. The asterisks depict sites of amino acid substitutions owing to point mutations and codon changes, using the numbering system of Baker *et al.* (1988).

VDRE) (Hewison *et al.*, 1993; Hughes *et al.*, 1988; Malloy *et al.*, 1990, 1999, 2002, 2004; Nguyen *et al.*, 2002, 2006; Ritchie *et al.*, 1989; Sone *et al.*, 1990; Yagi *et al.*, 1993).

Types of Defects

Studies with the above-mentioned methods in cells originating from a variety of patients with VDDR-II revealed heterogeneity of the cellular and molecular defects in the VDR–effector system. Based on the known functional properties of the VDR, different classes of defects could be identified.

Defects in the Hormone-Binding Region (Including Heterodimerization)

Deficient Hormone Binding

There are three subgroups in this class.

(i) No Hormone Binding. This is the most common abnormality observed and is characterized by unmeasurable specific binding of [^{3}H]1,25(OH)$_2$D$_3$ to either intact cells, nuclei, or cell extracts (Balsan *et al.*, 1983; Chen *et al.*, 1984; Cockeril *et al.*, 1997; Feldman *et al.*, 1982; Fraher *et al.*, 1986; Hawa *et al.*, 1996; Koren *et al.*, 1985; Kristjansson *et al.*, 1993; Liberman *et al.*, 1983a,b; Malloy *et al.*, 1990, 2001; Marx *et al.*, 1984; Mechica *et al.*, 1997; Nguyen *et al.*, 2002; Ritchie *et al.*, 1989; Sone *et al.*, 1989). Studies in several kindreds with this defect (including an extended kindred with eight patients studied) revealed undetectable levels of VDR by immunoblots on an immunoradiometric assay in most kindreds (Kristjansson *et al.*, 1993; Malloy *et al.*, 1990; Nguyen *et al.*, 2002; Ritchie *et al.*, 1989; Weise *et al.*, 1993). DNA from these affected subjects exhibited a single base mutation in each kindred resulting in (1) conversion of a normal amino acid codon into a premature termination codon in the coding sequence of the VDR protein and (2) a frameshift in translation resulting in a premature stop codon. The truncated VDRs produced lacked hormone binding or both hormone- and DNA-binding domains (Fig. 3) (Cockeril *et al.*, 1997; Hawa *et al.*, 1996; Mechica *et al.*, 1997; Weise *et al.*, 1993). The recreated mutant VDR cDNA was expressed in mammalian cells, and the resulting mutant VDR was demonstrated to be the truncated protein that exhibited no specific hormone binding. Steady-state VDR mRNA levels were decreased or undetectable in patients' cells from one kindred (Malloy *et al.*, 1990; Ritchie *et al.*, 1989). An immunoradiometric assay for VDR did not detect the 148- and 291-amino-acid-long mutant VDR, though this portion of the receptor included both epitopes required for recognition (Weise *et al.*, 1993). These observations may indicate an unstable transcript and/or increased turnover. In cells cultured from parents of some patients, expected to be obligate heterozygotes, binding of [^{3}H]1,25(OH)$_2$D$_3$, VDR protein, and mRNA content of cells ranged from the lower limit of normal to about half the normal level.

In one patient representing a kindred with no hormone binding, a missense mutation resulted in the substitution of the hydrophobic basic arginine-274 by the hydrophilic nonpolar leucine in the hormone-binding region (Kristjansson *et al.*, 1993) (see Fig. 3). In this patient, normal transcription in a transfection assay could be elicited by 1000-fold higher concentrations of calcitriol than needed for the wild-type receptor. However, no *in vivo* or *in vitro* stimulation of 25(OH)D-24-hydroxylase could be obtained by high concentrations of $1,25(OH)_2D_3$.

Two siblings without alopecia and no response to any dose of $1,25(OH)_2D$ *in vivo* and *in vitro* had a missense mutation that caused a substitution of tryptophan by arginine at amino acid 286 of the VDR (see Fig. 3) (Nguyen *et al.*, 2002). This substitution in a normal size VDR abolished completely the binding of $1,25(OH)_2D$ to its receptor. The tryptophan in this position is critical for the positioning of calcitriol in the VDR as was unveiled by the three-dimensional arrangement of the VDR and its ligand based on its crystal structure (Rochel *et al.*, 2000).

(ii) Defective Hormone Binding Capacity. In a patient representing one kindred, the number of binding sites in nuclei and high-salt soluble cell extracts was 10% of control, with an apparent normal affinity (Balsan *et al.*, 1983; Liberman *et al.*, 1983a,b; Sone *et al.*, 1989).

Recently, a boy with total alopecia, severe rickets, and growth retardation was found to have two heterozygote different molecular defects in the ligand-binding domain (Nguyen *et al.*, 2006). The patient VDR had a low hormone-binding capacity, 10% to 30% of controls, with normal affinity and a marked deficient stimulation of $25(OH)D_3$-24-hydroxylase. The recreated mutations, each one tested separately *in vitro*, also showed deficient heterodimerization as well as different transactivation of two gene promotors. This patient, similar to another one described more than 20 years ago, could be completely cured by very high doses of 25(OH) vitamin D, 250 μg/day initially, followed by 100 μg/day and then 75 μg/day as a maintenance dose continuing for years, plus modest calcium supplementation. In both patients, it could be shown that during remission (normocalcemia, normophosphatemia, normal iPTH), $1,25(OH)_2D$ production is driven by the substrate, i.e., 25(OH) vitamin D concentrations.

(iii) Defective Hormone Binding Affinity. Binding affinity of tritiated calcitriol was reduced 20- to 30-fold, with normal capacity, in high-salt soluble dermal fibroblast extracts from one kindred (Castells *et al.*, 1986). An additional patient, representing a different kindred, had a modest decrease of VDR affinity when measured at 0°C (Malloy *et al.*, 1997).

No studies on the molecular defect were performed in patients with the last two defects.

Deficient Nuclear Uptake

The following features characterize the hormone–receptor–nuclear interaction in this defect: normal or near-normal binding capacity and affinity of $[^3H]1,25(OH)2D3$ to high-salt soluble cell extracts with low to unmeasurable hormone uptake into nuclei of intact cells (Eil *et al.*, 1981; Hewison *et al.*, 1993; Liberman *et al.*, 1983b; Takeda *et al.*, 1989; Whitfield *et al.*, 1996). These features were demonstrated in skin-derived fibroblasts in all kindreds, in cells cultured from a bone biopsy of one patient (Liberman *et al.*, 1983b), and in EB-transformed lymphoblasts of one patient (Hewison *et al.*, 1993). Occupied VDR obtained from high-salt fibroblast extracts of two kindreds demonstrated normal binding to nonspecific DNA cellulose (Liberman *et al.*, 1986). Immunocytological studies in fibroblasts of a patient with this defect showed that, immediately after 1,25(OH)2D3 treatment, VDR accumulated along the nuclear membrane with no nuclear translocation (Barsony *et al.*, 1990). Patients with this defect included a kindred with normal hair and several kindreds with total alopecia. Finally, almost all patients responded with a complete clinical remission to high doses of vitamin D and its active 1α-hydroxylated metabolites.

Attempts to characterize the molecular defect were carried in six kindreds. In three of them, no mutation in the coding region of the VDR gene was observed (Hewison *et al.*, 1993; personal communication). Studies in two kindreds revealed a normal molecular mass and quantitative expression of the VDR as judged by immunoblotting (Whitfield *et al.*, 1996). Complete sequencing of the VDR-coding region revealed a different single-nucleotide mutation in each kindred: ATC to AGC for isoleucine-314 to serine in one kindred, CGC to TCG altering arginine-391 to cysteine in the second kindred, and CAG to CCG altering glutamine-259 to proline in the third kindred (see Fig. 3) (Cockeril *et al.*, 1997; Whitfield *et al.*, 1996), This region is considered to play a role in heterodimerization of VDR with RXR, and thus it has been suggested that these patients' receptors have defects that compromise RXR heterodimerization, which is essential for nuclear localization and probably for recognition of the vitamin D-responsive element as well. The fact that no mutation in the VDR-coding region was observed in three additional kindreds with the same phenotypical defect may suggest that the genetic defect affects another component of the receptor–effector system that is essential for the VDR function as a nuclear transcription factor. It has been shown that coactivation complexes are essential for the ligand-induced transactivation of VDR (Freedman, 1999). It is worthwhile to note that in one kindred originally described with this defect (Takeda *et al.*, 1989) a missense mutation at position 140 in exon 3, encoding the DNA-binding domain, was observed (Saijo *et al.*, 1991).

Deficient coactivators of the calcitriol–VDR complex. A patient with VDDR-II without alopecia was described (Malloy *et al.*, 1999). Sequencing of the VDR-DNA revealed a missense mutation in the ligand-binding domain that caused a substitution of glutamic acid to lysine at amino acid 120 (see Fig. 3). This receptor exhibits many normal properties including calcitriol binding, dimerization, and binding to vitamin D response elements in the DNA, but a marked impairment in binding coactivators that are essential for the transactivation of the hormone–receptor complex and the initiation of the physiological response.

Defects in DNA-Binding Region

Deficient Binding to DNA. Cell preparations derived from patients with this defect demonstrate normal or near-normal binding capacity and affinity for $[^3H]1,25(OH)_2D_3$ to nuclei of intact cells and to high-salt soluble cell extracts, as well as the normal molecular size VDR of 48 to 50 kDa, as analyzed by immunoblot. Hormone–receptor complexes, however, have decreased affinity to nonspecific DNA (peak elution from DNA-cellulose columns at $0.1\,M$ KCl compared with $0.2\,M$ in normals) (Hirst *et al.*, 1985; Hughes *et al.*, 1988; Liberman *et al.*, 1986; Saijo *et al.*, 1991; Yagi *et al.*, 1993). A single-nucleotide missense mutation within exon 2 or 3, encoding the DNA-binding domain of the VDR, was demonstrated in genomic DNA isolated from dermal fibroblasts and/or EB-transformed lymphoblasts from 10 unrelated kindreds (Hughes *et al.*, 1988; Lin *et al.*, 1996; Malloy *et al.*, 1989, 1994; Mechica *et al.*, 1997; Rut *et al.*, 1994; Sone *et al.*, 1990; Saijo *et al.*, 1991; Yagi *et al.*, 1993). Eight different single-nucleotide mutations were found in the ten kindreds (see Fig. 3). Two apparently unrelated kindreds share the same mutation (Saijo *et al.*, 1991; Sone *et al.*, 1990).

All point mutations caused a single substitution of an amino acid that resides in the region of the two zinc fingers of the VDR protein that are essential for the functional interaction of the hormone–receptor complex with DNA. Interestingly, all these altered amino acids are highly conserved in the steroid receptor superfamily and seem to concentrate in three regions: the tip of the first zinc fingers (three mutations), the "knuckle" region, just C-terminal of the first zinc fingers (three mutations), and the C-terminal side of the second zinc fingers (two mutations). All of these single-amino-acid residue substitutions are associated with charge changes and thus affect hydrogen bonding. These aberrations, as well as changes in hydrophobicity, will have a deleterious effect on the interaction of the hormone–receptor complex with DNA.

Each of the mutants was recreated by introducing the appropriate base change into the normal VDR cDNA and then transfecting the mutated cDNA into mammalian cells. The functional properties of the resultant VDR expression product were indistinguishable from mutant DNA, i.e., normal hormone binding and deficient binding to nuclear extracts and nonspecific DNA (Hughes *et al.*, 1988; Saijo *et al.*, 1991; Sone *et al.*, 1990). Studies on cells obtained from parents of some of these patients revealed, as expected, a heterozygous state, i.e., expression of both normal and defective forms of VDR as well as normal and mutant gene sequences (Hughes *et al.*, 1988; Malloy *et al.*, 1989), but without any clinical or biochemical abnormalities.

In vitro Post-transcriptional and Transcriptional Effect of $1,25(OH)_2D_3$

In vitro bioeffects of the hormone on various cells in patients with VDDR-II have been assayed mainly by two procedures: induction of 25(OH)D-24-hydroxylase and inhibition of mitogen-stimulated PBM cells.

$1,25(OH)_2D_3$ induces 25(OH)D-24-hydroxylase activity in skin-derived fibroblasts (Balsan *et al.*, 1983; Castells *et al.*, 1986; Chen *et al.*, 1984; Clemens *et al.*, 1983; Feldman *et al.*, 1982; Fraher *et al.*, 1986; Gamblin *et al.*, 1985; Griffin *et al.*, 1983; Hewison *et al.*, 1993; Hirst *et al.*, 1985; Hughes *et al.*, 1988; Liberman *et al.*, 1983b; Malloy *et al.*, 1990; Ritchie *et al.*, 1989; Rut *et al.*, 1994; Sone *et al.*, 1990; Yagi *et al.*, 1993), mitogen-stimulated lymphocytes (Takeda *et al.*, 1990), and cells originating from bone (Balsan *et al.*, 1986) in a dose-dependent manner. In cells from normal subjects, maximal and half-maximal induction of the enzyme was achieved by 10^{-8} and 10^{-9} M concentrations of $1,25(OH)_2D_3$, respectively. Dermal fibroblast or PBM cells from VDDR-II patients with no calcemic response to maximal doses of vitamin D or its metabolites *in vivo* did not show any 25(OH)D-24-hydroxylase response to very high concentrations of $1,25(OH)_2D_3$ *in vitro*, whereas dermal fibroblasts from patients with a calcemic response to high doses of vitamin D or its metabolites *in vivo* showed inducible 24-hydroxylase with supraphysiological concentrations of $1,25(OH)_2D_3$, i.e., a shift to the right, *in vitro*. Physiological concentrations of $1,25(OH)_2D_3$ partially inhibit mitogen-induced DNA synthesis in peripheral lymphocytes with a half-maximal inhibition achieved at $10^{-10}\,M$ hormone (Koren *et al.*, 1985; Takeda *et al.*, 1986). Mitogen-stimulated lymphocytes from several kindreds with defects characterized as no hormone binding or deficient binding to DNA, with no calcemic response to high doses of vitamin D and its metabolites *in vivo*, showed no inhibition of lymphocyte proliferation *in vitro*, with concentrations of up to $10^{-6}\,M$ $1,25(OH)_2D_3$ (Fig. 4). Additional methods for measuring bioeffects of $1,25(OH)_2D_3$ on various cells *in vitro* were carried out only in few patients and included inhibition of dermal fibroblast proliferation (Clemens *et al.*, 1983), induction of osteocalcin synthesis in cells derived from bone (Balsan *et al.*, 1986), a mitogenic effect on dermal fibroblasts (Barsony *et al.*, 1989), and stimulation of cGMP production in cultured skin fibroblasts (Barsony and Marx, 1988). It is noteworthy that in all assays mentioned, and without exception, each patient's cells showed severely deficient responses.

With the elucidation of the molecular defects in VDDR-II, the transactivation abilities of naturally occurring mutant or recreated mutant VDRs were evaluated in a transcriptional

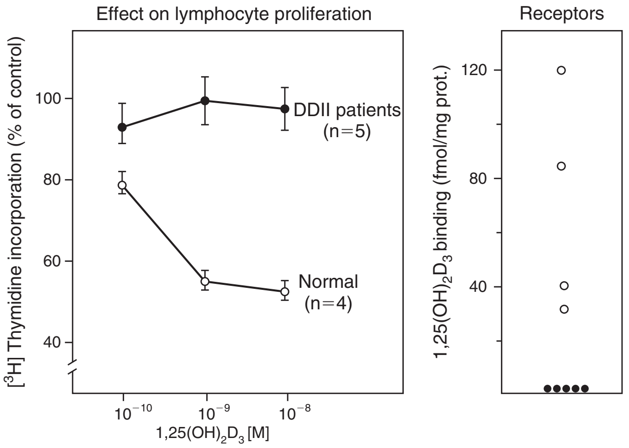


FIGURE 4 Effect of increasing concentrations of $1,25(OH)_2D_3$ on mitogen-induced lymphocyte proliferation from patients with VDDR-II (DD-II) and normal controls. The numbers of specific $[^3H]1,25(OH)_2D_3$-binding sites are depicted (*right*) in the same cell populations. VDRR-II patients tested here are with the defect characterized as no hormone binding.

activation assay. The human osteocalcin gene promoter fused to chloramphenicol acetyltransferase (CAT) gene reporter plasmid was transfected into the patients or into normal fibroblasts (Hewison *et al.*, 1993; Hughes *et al.*, 1988; Malloy *et al.*, 1990; Ritchie *et al.*, 1989; Saijo *et al.*, 1991; Sone *et al.*, 1989; Yagi *et al.*, 1993). Treating normal transfected cells with $1,25(OH)_2D_3$, caused a concentration-dependent induction of transcription, measured by increased CAT activity. No induction of transcription was observed in cells originating from patients with defects characterized as no hormone binding (Kristjansson *et al.*, 1993; Malloy *et al.*, 1990; Ritchie *et al.*, 1989) or deficient binding to DNA (Hughes *et al.*, 1988; Saijo *et al.*, 1991; Sone *et al.*, 1990; Yagi *et al.*, 1993). Moreover, in a cotransfection assay, the addition of a normal human VDR cDNA expression vector to the transfected plasmid that directed synthesis of a normal VDR restored the hormone responsiveness of resistant cells. Finally, in a patient characterized as having a deficient nuclear uptake defect no mutation was identified within the coding region of the VDR gene; no induction of 25(OH)D-24-hydroxylase activity by up to 10^{-6} *M* $1,25(OH)_2D_3$ was observed in cultured skin fibroblasts, but there was normal transactivation by $1,25(OH)_2D_3$ in the transcriptional activation assay (Hewison *et al.*, 1993).

Cellular Defects and Clinical Features

Normal hair was described with most phenotypes of the cellular defects, the exception being patients with deficient hormone-binding capacity and affinity, but this could be because only very few kindreds were described per subgroup. Normal hair is usually associated with a milder form of the disease, as judged by the age of onset, severity of the clinical features, and usually the complete clinical and biochemical remission on high doses of vitamin D or its metabolites. Notable exceptions are two patients, from two separate kindreds, with normal hair that displayed resistance both *in vivo* (no clinical remission on circulating calcitriol level up to 100 times the mean normal adult values) and *in vitro* (no induction of 25(OH)D-24-hydroxylase activity in dermal fibroblasts by up to 10^{-8} *M* $1,25(OH)_2D_3$ (Fraher *et al.*, 1986; Mally *et al.*, 2002). Only approximately half of the patients with alopecia have shown satisfactory clinical and biochemical remission to high doses of vitamin D or its active 1α-hydroxylated metabolites, but the dose requirement is ~10-fold higher than in patients with normal hair (Marx *et al.*, 1986).

It seems that patient defects characterized as deficient hormone-binding affinity and deficient nuclear uptake achieve complete clinical and biochemical remission on high doses of vitamin D or its active 1α-hydroxylated metabolites. Most of the patients with other types of defects could not be cured with high doses of vitamin D or its metabolites. However, it should be emphasized that not all of the patients received treatment for a long enough period of time and with sufficiently high doses (see "Treatment").

Diagnosis

Clinical features of early-onset rickets with no history of vitamin D deficiency, total alopecia, parental consanguinity, additional siblings with the same disease, serum biochemistry of hypocalcemic rickets, elevated circulating levels of $1,25(OH)_2D$, and normal to high levels of 25(OH)D (see Table I) support the diagnosis of VDDR-II. The issue becomes more complicated when the clinical features are atypical, i.e., late onset of the disease, sporadic cases, and normal hair. Failure of a therapeutic trial with calcium and/or physiological replacement doses of vitamin D or its metabolites may support the diagnosis, but the final direct proof requires the demonstration of a cellular, molecular, and functional defect in the VDR–effector system.

Based on the clinical and biochemical features, the following additional disease states should be considered: (1) extreme calcium deficiency: a seemingly rare situation, described in a group of children from a rural community in South Africa, who consumed an exceptionally low-calcium diet of 125 mg/day (Pettifor *et al.*, 1981). All had severe bone disease with histologically proven osteomalacia, biochemical features of hypocalcemic rickets with elevated serum levels of 1,25(OH)2D, and sufficient vitamin D. Calcium repletion caused complete clinical and biochemical remission. Nutritional history and the response to calcium supplementation support this diagnosis. (2) Severe vitamin D deficiency: during initial stages of vitamin D therapy in children with severe vitamin D-deficient rickets, the biochemical picture may resemble VDDR-II, i.e., hypocalcemic rickets with elevated serum calcitriol levels. This may represent a "hungry bone syndrome," i.e., high calcium demands of the abundant osteoid tissue becoming mineralized. This is a transient condition that may be differentiated from VDDR-II by a history of vitamin D deficiency and the final therapeutic response to replacement doses of vitamin D.

Treatment

In about half of the kindreds with VDDR-II, the bioeffects of $1,25(OH)_2D_3$ were measured *in vitro* (see earlier). An invariable correlation (with one exception) was documented between the *in vitro* effect and the therapeutic response *in vivo*; i.e., patients with no calcemic response to high levels of serum calcitriol showed no effects of $1,25(OH)_2D_3$ on their cells *in vitro* (either induction of 25(OH)D-24-hydroxylase or inhibition of lymphocyte proliferation) and vice versa. If the predictive therapeutic value of the *in vitro* cellular response to $1,25(OH)_2D_3$ could be substantiated convincingly, it may eliminate the need for time-consuming and expensive therapeutic trials with massive doses of vitamin D or its active metabolites. In the meantime, it is mandatory to treat every patient with VDDR-II irrespective of the type of receptor defect.

An adequate therapeutic trial must include vitamin D at a dose that is sufficient to maintain high-serum concentrations of $1,25(OH)_2D_3$, as the patients can produce high hormone levels if supplied with enough substrate (Brooks *et al.*, 1978; Marx *et al.*, 1978). If high serum calcitriol levels are not achieved, it is advisable to treat with 1α-hydroxylated vitamin D metabolites in daily doses of up to 6 μg/kg or a total of 30 to 60 μg and calcium supplementation of up to 3 g of elemental calcium daily; therapy must be maintained for a period sufficient to mineralize the abundant osteoid (usually 3 to 5 months). Therapy may be considered a failure if no change in the clinical, radiological, or biochemical parameters occurs during continuous and frequent follow-up while serum $1,25(OH)_2D$ concentrations are maintained at ~100 times the mean normal range.

In some patients with no response to adequate therapeutic trials with vitamin D or its metabolites, a remarkable clinical and biochemical remission of their bone disease, including catch-up growth, was obtained by treatment with large amounts of calcium. This was achieved by long-term (months) intracaval infusions of up to 1000 mg of calcium daily (Balsan *et al.*, 1986; Bliziotes *et al.*, 1988; Weisman *et al.*, 1987). Another way to increase calcium input into the extracellular compartment is to increase net gut absorption, independent of vitamin D, by increasing calcium intake (Sakati *et al.*, 1986). This approach is limited by dose and patient tolerability and was actually used successfully in very few patients.

Several patients have shown unexplained fluctuations in response to therapy or in presentation of the disease. One patient, after a prolonged remission, became completely unresponsive to much higher doses of active 1α-hydroxylated vitamin D metabolites (Balsan *et al.*, 1983), and another patient seemed to show amelioration of resistance to $1,25(OH)_2D_3$ after a brief therapeutic trial with $24,25(OH)_2D_3$ (Liberman *et al.*, 1980). In several patients, spontaneous healing occurred in their teens (Hochberg *et al.*, 1984) or rickets did not recur for 14 years after cessation of therapy (Takeda *et al.*, 1989).

Animals Models

Some New World primates (marmoset and tamarins) that develop osteomalacia in captivity are known to have high nutritional requirements for vitamin D and maintain high serum levels of $1,25(OH)_2D$, thus exhibiting a form of end-organ resistance to $1,25(OH)_2D$ (Adams and Gacad, 1988; Liberman *et al.*, 1985; Shinki *et al.*, 1983; Takahashi *et al.*, 1985). Cultured dermal fibroblasts and EB-virus-transformed lymphoblasts have shown deficient hormone-binding capacity and affinity (Adams and Gacad, 1988; Liberman *et al.*, 1985). It has been observed that marmoset lymphoblasts contain a soluble protein of 50 to 60 kDa that binds $1,25(OH)_2D_3$ with a low affinity but high capacity and thus may serve as a sink that interferes with the hormone binding and its cognate receptor (Gacad and Adams, 1993). The same group described another protein present in the nuclear extract of these cells capable of inhibiting normal VDR-RXR binding to the vitamin D response element (Arbelle *et al.*, 1996).

It is of interest that these New World primates also exhibit a compensated hereditary end-organ resistance to the true steroid hormones including glucocorticoids, estrogens, and progestins (Lipsett *et al.*, 1985). This, of course, raises the interesting possibility that the defect in the hormone–receptor–effector system involves an element shared by all the members of this superfamily of ligand-modulated transcription factors.

VDR knockout mice have been created by targeted ablation of the first or second zinc finger (Li *et al.*, 1997; Yoshizawa *et al.*, 1997). Only the homozygotic mice were affected. Though phenotypically normal at birth, after weaning, they become hypocalcemic, develop secondary hyperparathyroidism, rickets, osteomalacia, and progressive alopecia. The female mice with ablation of the first zinc finger are infertile and show uterine hypoplasia and impaired folliculogenesis. Otherwise, both VDR cell mutant mice show clinical, radiological, histological, and biochemical features that are identical to the human disease VDRR-II. Supplementation with a calcium-enriched diet can prevent or treat most of the disturbances in mineral and bone metabolism in these animal models except alopecia (Delling and Demay, 1998). It is of interest that targeting expression of human VDR to keratinocytes of VDR null mice prevented alopecia (Chen *et al.*, 2001).

CONCLUDING REMARKS

Hereditary deficiencies in vitamin D action are rare disorders. The importance of studying these diseases stems from the fact that they represent a naturally occurring experimental model that helps to elucidate the function and importance of vitamin D and the VDR–effector system in humans *in vivo*.

VDRs are abundant and widely distributed among most tissues studied and multiple effects of calcitriol are observed on various cell functions *in vitro*. Yet, the clinical and biochemical features in patients with VDDR-I and -II seem to demonstrate that the only disturbances of clinical relevance are perturbations in mineral and bone metabolism. This demonstrates the pivotal role of $1,25(OH)_2D$ in transepithelial net calcium fluxes. Moreover, the fact that, in patients with extreme end-organ resistance to calcitriol, calcium infusions correct the disturbances in mineral homeostasis and cure the bone disease may support the notion that defective bone matrix mineralization in VDRR-I and -II is secondary to disturbances in mineral homeostasis. Characterization of the molecular, cellular, and functional defects of the different natural mutants of the human VDR in VDDR-II demonstrates the essentiality of the VDR as the mediator of calcitriol action and the importance and function of its different domains. Furthermore, to function biologically, the VDR must associate with additional partners, i.e., $1,25(OH)_2D$, an RXR isoform, a specifically defined DNA region, as well as coactivators and corepressor complexes. This notion has been based primarily on *in vitro*-created point mutations and *in vitro* functional assays. However, the acid test for the relevance of the structure–function relationship is the demonstration of *in vivo* effects, in general, and deficient function under pathological conditions, in particular. Thus, studies in patients with hereditary deficiencies in vitamin D action are the essential link between molecular defects and physiological relevant effects.

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Chapter 71

Tumor-Induced Osteomalacia

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INTRODUCTION

Tumor-induced osteomalacia (TIO) is a rare, paraneoplastic disease that can be difficult to distinguish from genetic forms of hypophosphatemia and severe osteomalacia. The hallmark of TIO, and its genetic phenocopies, is renal phosphate wasting and abnormal vitamin D metabolism; specifically, lack of compensatory increase in 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) in response to hypophosphatemia. Ultimately, unchecked renal phosphorus excretion and relative $1,25(OH)_2D_3$ deficiency leads to osteomalacia, a metabolic bone disease characterized by a failure of mineralization and resultant weak, painful bones. Osteomalacia itself has myriad causes, many of which display low serum phosphate levels (Parfitt, 1990). Vitamin D ($25(OH)D_3$) deficiency is the most common cause of osteomalacia in adults and rickets in children. Osteomalacia that was resistant to vitamin D therapy led to the recognition of other causes, including TIO and genetic disorders such as X-linked hypophosphatemic rickets (XLH). McCance first described TIO in 1947, when a patient with vitamin D-resistant osteomalacia was cured by surgical resection of a bone tumor (McCance, 1947). Subsequent research into the humoral factors that are responsible for TIO, and the genetic factors responsible for inherited forms of hypophosphatemic rickets, has converged to elucidate novel phosphate homeostatic pathways.

INITIAL CLINICAL EVALUATION OF SUSPECTED OSTEOMALACIA

Osteomalacia is usually suspected clinically or found on x-rays, for example, after a fracture. Patients with osteomalacia complain of diffuse bone pain, muscle weakness, fractures, and fatigue. The pain is most often in the axial skeleton and long bones of the lower extremities. The vitamin D receptor (VDR) is also present in muscle and a myopathy may be present along with the bone symptoms, especially in the proximal muscles. Studies of adults with vitamin deficiency have noted gait instability and more frequent falls among vitamin D-deficient elderly adults compared with controls (Bischoff-Ferrari *et al.*, 2005; Burleigh *et al.*, 2007; Jackson *et al.*, 2007). In TIO, the myopathy can be very severe and may be related to the phosphorus deficiency.

Physical examination often reveals bone pain, especially with palpation of the anterior tibia and sternum. In children, long bone deformity, lower extremity bowing, and chest wall deformity is observed. If TIO is suspected, an extensive examination for masses or nodules is warranted, concentrating on soft tissues adjacent to long bones, distal extremities, the oral cavity and jaw, and the groin.

Both fractures and pseudofractures are visualized on plain films. Pseudofractures are radiolucent lines, called Looser zones, around nutrient canals that have enlarged from the failure of mineralization. Pseudofractures are generally perpendicular to the cortex of the bone. Generalized osteopenia and coarse trabeculae may be appreciated on x-ray. In children, rickets is characterized by widening of the epiphyseal growth plate and bowing the long bones, particularly the lower extremities, which can be very dramatic on x-ray.

Histological examination of the bone confirms the diagnosis of osteomalacia when clinical and radiographic evaluation is nondiagnostic. In this disorder, the organic bone matrix, osteoid, continues to be deposited at sites of remodeling at a reduced rate, whereas mineralization with hydroxyapatite is significantly inhibited (Drezner, 1999; Kumar, 2000). This leads to characteristic findings on biopsy of a widened osteoid seam (Fig. 1). The degree of matrix synthesis can be assessed with two pulses of tetracycline, taken orally for three days, separated by ten days prior to biopsy. Tetracycline is incorporated into newly synthesized matrix, and thus the space between the lines created with each pulse measures the rate of active bone deposition.

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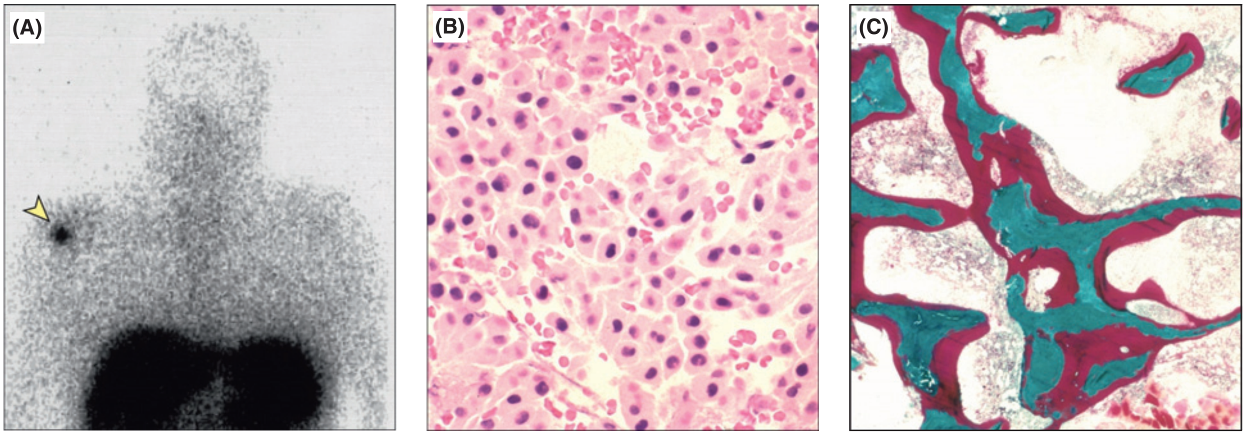

FIGURE 1 Radiographic and histological features in TIO. (**A**) Octreotide scan showing small mesenchymal tumor in the head of the humerus. (**B**) Hemiangiopericytoma with numerous pericytes and vascular channels (H&E strain). (**C**) Bone biopsy with Goldner stain. Excessive osteoid or unmineralized bone matrix composed mainly of collagen stains pink. Minerlized bone stains blue. This bone biopsy shows severe osteomalacia. (See plate section)

Rickets is characterized by the presence of similar findings at the growth plate. Although bone biopsy will confirm osteomalacia, it cannot distinguish the underlying cause.

The differential diagnosis of osteomalacia is dominated by vitamin D deficiency (Table I). With the increased use of sunscreens, decreased time spent out-of-doors, and the lack of dietary sources, vitamin D deficiency has become increasingly prevalent, especially in the elderly (Holick, 2007). If vitamin D deficiency is identified, an underlying cause of the deficiency, such as celiac sprue or other malabsorptive state, must be sought. The proliferation of gastric bypass surgery will contribute significantly to this population in the future. In rare circumstances, a patient may have primary nutritional deficiencies that lead to low serum phosphorus or calcium, for example, with alcoholism or anorexia nervosa. Genetic defects in vitamin D synthetic enzymes or in the vitamin D receptor (VDR) result in functional vitamin D deficiency. End-stage renal disease leads to a deficiency of $1{,}25(OH)_2D_3$ from loss of kidney-dependent 1α-hydroxylation; this paired with secondary hyperparathyroidism results in renal osteodystrophy, a variant of osteomalacia with a mixed picture of mineralization defect and parathyroid bone disease. Acquired and genetic renal phosphate wasting syndromes, the focus of the remainder of the chapter, are a major cause of osteomalacia. These disorders include tumor-induced osteomalacia (TIO), X-linked hypophosphatemic rickets (XLH), autosomal dominant hypophosphatemic rickets (ADHR), autosomal recessive hypophosphatemic rickets (ARHR), hereditary hypophosphatemic rickets with hypercalciuria (HHRH), and Fanconi's syndrome.

TABLE I Differential Diagnosis of Rickets and Osteomalacia

Vitamin D deficiency
Nutritional deficiency: low sunshine exposure, low dietary intake
Malabsorption: celiac disease, Crohn's disease, gastrectomy, bowel resection, pancreatitis, gastric bypass
Chronic liver disease: impaired 25-hydroxylation
Chronic renal disease: renal osteodystrophy (impaired 1α-hydroxylation and secondary hyperparathyroidism); nephrotic syndrome (renal loss of binding proteins)
Increased catabolism: anticonvulsant therapy
Vitamin D receptor defects: Vitamin D-dependent rickets type 2
Vitamin D synthetic defects: 1α-hydroxylase (CYP27B1): Vitamin D-dependent rickets type 1 25-hydroxylase (CYP27A1)
Hypophosphatemia
Nutritional deficiency: alcoholism, anorexia, starvation
Renal phosphate wasting X-linked Hypophosphatemic Rickets Autosomal Dominant Hypophosphatemic Rickets Autosomal Recessive Hypophosphatemic Rickets Hereditary Hypophophatemic Rickets with Hypercalciuria Tumor-Induced Osteomalacia Fanconi Syndrome Dent's Disease
Isolated hypocalcemia
Nutritional calcium deficiency (rare)

In general, an astute physician armed with a comprehensive history, directed physical examination, and specific laboratory tests can distinguish between the most common forms of osteomalacia. The causes of osteomalacia can generally be distinguished biochemically (Table 1) and (Fig. 2). When osteomalacia is suspected, the initial laboratory investigation should include serum levels of $25(OH)D_3$, calcium, fasting phosphorus, PTH, creatinine, alkaline phosphatase, and 24-hour urine calcium measurements. If serum phosphorus is known to be low, a $1{,}25(OH_2)D_3$ level and a 24-hour urine phosphorus collection should also be obtained [in order to

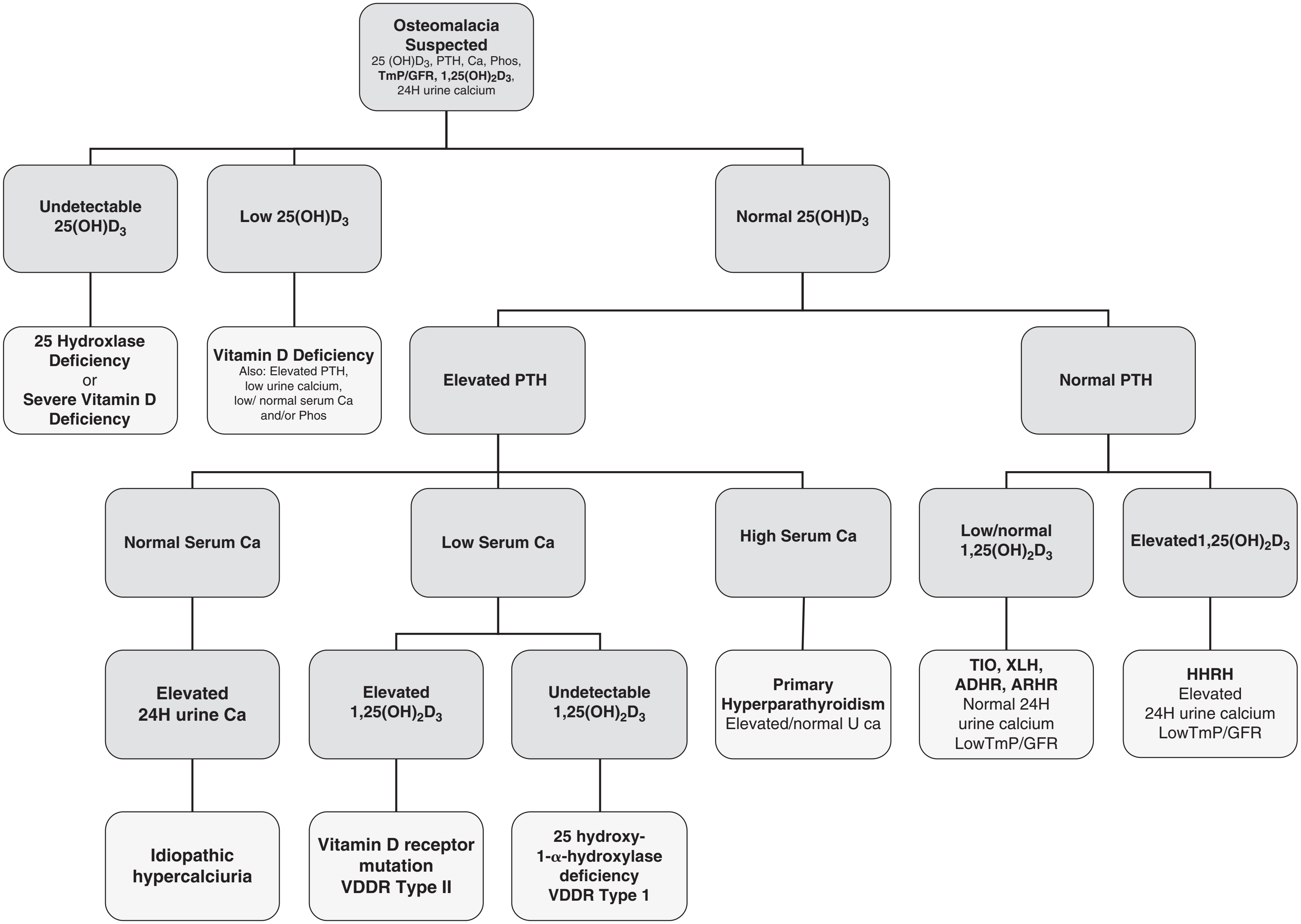
Osteomalacia Suspected
25 (OH)D_3, PTH, Ca, Phos,
TmP/GFR, 1,25(OH)$_2$$D_3$,
24H urine calcium
Undetectable 25(OH)D_3
Low 25(OH)D_3
Normal 25(OH)D_3
25 Hydroxlase Deficiency
or
Severe Vitamin D Deficiency
Vitamin D Deficiency
Also: Elevated PTH,
low urine calcium,
low/ normal serum Ca
and/or Phos
Elevated PTH
Normal PTH
Normal Serum Ca
Low Serum Ca
High Serum Ca
Low/normal 1,25(OH)$_2$$D_3$
Elevated1,25(OH)$_2$$D_3$
Elevated 24H urine Ca
Elevated 1,25(OH)$_2$$D_3$
Undetectable 1,25(OH)$_2$$D_3$
Primary Hyperparathyroidism
Elevated/normal U ca
TIO, XLH, ADHR, ARHR
Normal 24H urine calcium
LowTmP/GFR
HHRH
Elevated 24H urine calcium
LowTmP/GFR
Idiopathic hypercalciuria
Vitamin D receptor mutation
VDDR Type II
25 hydroxy-1-α-hydroxylase deficiency
VDDR Type 1

FIGURE 2 Evaluation of suspected osteomalacia.

calculate the tubular reabsorption of phosphate, transmembrane potential (TmP)/glomerular filtration rate (GFR)].

Vitamin D deficiency leads to poor absorption of minerals from the gastrointestinal tract, requiring compensatory changes to mobilize calcium and phosphorus from bone, which is mediated by PTH. In the presence of normal renal function, vitamin D deficiency is characterized by low-normal serum calcium and phosphorus as well as elevated serum PTH and alkaline phosphatase. Low urine calcium is characteristic of vitamin D deficiency because the filtered load of calcium is diminished and excess PTH results in avid reabsorption of calcium in the proximal renal tubule. Nutritional deficiencies of calcium and phosphate from severe dietary restriction and alcoholism can present with similar biochemical findings and are often exacerbating factors in the right host.

Mutations in the 25-hydroxy-1α-hydroxylase enzyme and the VDR (vitamin D-resistant rickets type I and II, respectively) are manifest with similar calcium, phosphorus, and PTH changes associated with vitamin D deficiency; however, 25(OH)D_3 levels are normal. As implied by the name, neither disorder is responsive to vitamin D supplementation. In these cases, measuring the serum 1,25$(OH)_2D_3$ is helpful, because it will be low in the absence of 1α-hydroxylase activity and elevated in the face of impaired VDR signaling.

Marked hypophosphatemia and renal phosphate wasting characterize TIO and the related genetic disorders. The serum phosphate levels should be measured fasting and the degree of hypophosphatemia can be profound. Serum calcium, 25(OH)D_3, and PTH levels are normal; however, 1,25$(OH)_2D_3$ level is generally lower than expected for the degree of hypophosphatemia, although it may be within the normal range. This failure to induce 1,25$(OH)_2D_3$ synthesis in response to hypophosphatemia contributes to the pathophysiology of these disorders. PTH is not generally elevated, with a few exceptions reported (Cheng *et al.*, 1989; Shenker and Grekin, 1984). Increased bone turnover typically elevates alkaline phosphatase. Elevated urine phosphate excretion uniquely identifies this group of disorders. Renal loss of phosphate is measured by calculating the maximal tubular resorption of phosphate normalized to the GFR, the TmP/GFR, according to a nomogram with a 24-hour urine collection for phosphorus and creatinine (Walton and Bijvoet, 1975), and can be estimated with spot urine collections (Walton and Bijvoet, 1977). With the increased renal losses in these disorders, the TmP/GFR is reduced, and can be less than 50% of normal in TIO. Urine glucose and amino acids levels should also be measured as a more generalized defect in tubular function can be seem with some tumors (Leehey *et al.*, 1985) and with Faconi's syndrome.

Although, in general, osteomalacia secondary to vitamin D deficiency can be distinguished on the basis of elevated serum PTH levels and lower serum calcium levels (in more severe cases), some patients have small changes that are indeterminate. Because urine phosphorus excretion can be elevated in vitamin D deficiency owing to secondary hyperparathyroidism, in some cases 25(OH)D_3 must be replaced and the patient reassessed for evidence of continued hypophosphatemia and hyperphosphaturia before further investigation is warranted.

DIFFERENTIAL DIAGNOSIS OF RENAL PHOSPHATE WASTING

Hypophosphatemia secondary to renal phosphate wasting has a wide differential diagnosis (Table II). These disorders can result from primary renal defects, overproduction of the phosphaturic hormone, FGF23, from normal or dysplastic bone and ectopic production of FGF23 or other phosphaturic proteins from tumors.

Fanconi's syndrome is the result of a generalized proximal tubular defect, which can result from genetic causes such as Wilson's disease and galactosemia, or acquired damage from heavy metals, connective tissue disorders, hematological malignancies (Clarke *et al.*, 1995) or medications (Colson and De Broe, 2005; Earle *et al.*, 2004; Izzedine *et al.*, 2003). Dent disease is an X-linked renal tubulopathy caused by mutations in the chloride channel, CLCN5, and is characterized by nephrolithiasis, renal failure, and Fanconi's syndrome with variable degrees of rickets and osteomalacia. The renal phosphate wasting observed in Dent's disease results from reduced membrane expression of NaPiIIc, a sodium-phosphate cotransporter (Piwon *et al.*, 2000). In addition to renal phosphate wasting, a more generalized proximal tubular defect in membrane trafficking leads to urinary losses of glucose, amino acids, and bicarbonate.

Hereditary hypophosphatemic rickets with hypercalciuria (HHRH) is a rare genetic form of hypophosphatemic rickets characterized by hypophosphatemia, renal phosphate wasting, and preserved responsiveness of 1,25$(OH_2)D_3$ to hypophosphatemia (Tieder *et al.*, 1985). This appropriate increase in 1,25$(OH_2)D_3$ leads to increased calcium absorption from the gastrointestinal tract and thus to hypercalciuria and nephrolithiasis. The genetic defect in HHRH is loss-of-function mutations in gene that endcodes NaPiIIc, one of three subtypes of the type II sodium phosphate cotransporters (Bergwitz *et al.*, 2006; Ichikawa *et al.*, 2006; Lorenz-Depiereux *et al.*, 2006b). HHRH is clinically similar to TIO with bone pain, osteomalacia, and muscle weakness as prominent features, yet the distinction is easily made with biochemical testing. Both syndromes are characterized by hypophosphatemia owing to decreased renal phosphorus reabsorption; however, patients with HHRH exhibit elevated levels of calcitriol and hypercalciuria that distinguish it from TIO, XLH, and ADHR.

TABLE II Characteristics of Renal Phosphate Wasting Syndromes

Acquired Disease	Defect	Biochemical features	Pathogenesis
TIO	Mesenchymal tumor	• Renal phosphate wasting Low Phos, low TmP/GFR • Inappriopriately low $1,25(OH)_2D$ Low or low-normal • Absence of hypercalcemia or hyperparathyroidism Normal calcium, PRH, normal urine calcium	Ectopic, unregulated production of FGF23 and other phosphatonins sFRP-4, MEPE, FGF7
Fanconi's	Renal proximal tubular defect that alters absorption of Pi, glucose, amino acids, bicarbonate	Similar to TIO with additional features of glocosuria, amino aciduria, and bicarbonate wasting	Proximal renal tubular damage owing to multiple myeloma, lymphoma, amyloidosis, light-chain disease, nephrotic syndrome, drugs, heavy metals, and heritable metabolic and renal disorders
Fibrous dysplasia	GNAS Gain of function	Biochemically indistinguishable from TIO	Increased FGF23 production from fibrous dysplastic bone; correlated with extent of bone disease
XLH	PHEX gene Loss of function	Biochemically indistinguishable from TIO	Increased FGF23 synthesis from bone
ADHR	FGF23 gene Gain of function	Biochemically indistinguishable from TIO	Increase circulating intact FGF23 owing to mutations that render it resistant to cleavage
ARHR	DMP1 gene Loss of function	Biochemically indistinguishable from TIO	Loss of DMP1 causes impaired osteocyte differentiation and increased production of FGF23
HHRN	SLC34A3 (NaPiIIc) Loss of function	• Renal phosphate wasting Low Phos, low TmP/GFR • Appropriately elevated $1,25(OH)_2D$ Elevated urine calcium and high-normal serum calcium • PRH is normal or suppressed	Defective renal sodium-dependent phosphate transport owing to loss-of-function mutations in NaPiIIc results in renal phosphate wasting without defect in $1,25(OH)_2D$ synthesis. Hypophosphatemia stimulated $1,25(OH)_2D$ production with high-normal serum calcium and elevated urine calcium.
OGD	FHFR1 Gain of function	Biochemically indistinguishable from TIO	Increased production of FGF23 by dyplastic bone

Heterozygous, dominant-negative mutations in the renal NaPiIIa gene (*NPT-2*) were identified in two patients with hypophosphatemia secondary to renal phosphate wasting and osteopenia or nephrolithiasis. The prominent symptoms of bone pain and muscle weakness seen in TIO are absent in those with *NPT-2* mutations. Furthermore, the presence of hypercalciuria and elevated calcitriol make these patients easily distinguishable from patients with TIO (Prie *et al.*, 2002). Therefore, either through mutations of the sodium-phosphate transporters themselves as in HHRH, through damage to the proximal renal tubule as in Fanconi's syndrome, or through aberrant regulation via FGF23, decreased expression or function of the renal sodium-phosphate cotransporters likely represent the common pathway in renal phosphate wasting observed in these syndromes (Shimada *et al.*, 2004b; Sitara *et al.*, 2004; Tenenhouse and Sabbagh, 2002).

FGF23, a circulating fibroblast growth factor produced by osteocytes and osteoblasts, has two currently known physiological functions: first, FGF23 promotes internalization of NaPiIIa and NaPiIIc from the brush-border membrane and thus reduces reabsorption of urinary phosphorus resulting in hypophosphatemia; second, it diminishes mRNA expression of the 25-hydroxy-1α-hydroxylase enzyme that converts vitamin D to its active form, 1,25$(OH_2)D_3$, and disrupts the compensatory increase in 1,25$(OH_2)D_3$ triggered by hypophosphatemia. In several disorders that result in expansion of abnormal bone, FGF23 is overproduced and results in renal phosphate wasting and hypophosphatemia. Fibrous dysplasia, bone lesions that replace medullary bone with fibrous tissue, are associated with disorders such as McCune–Albright Syndrome, a somatic gene defect that leads to hormone-independent activation of G protein (Gsα) coupled signaling, and a rare form of dwarfism called osteoglophonic dysplasia, owing to activating mutations in FGF receptor 1 (FGFR1) (White *et al.*, 2005). In patients with a high burden of these nonossifying lesions, phosphate wasting and lower-than-expected 1,25$(OH)_2D_3$ levels have been observed. FGF23 produced by the abnormal bone is likely responsible, because the degree of fibrous dysplasia is correlated with FGF levels as well as the degree of phosphate wasting (Riminucci *et al.*, 2003; White *et al.*, 2005).

FGF23 excess is central to the pathophysiology of TIO and three genetic disorders of renal phosphate wasting, XLH, ADHR, and ARHR. Biochemically, these disorders are indistinguishable from TIO and one another; it is only through careful assessment of the family history, inheritance pattern, and physical examination that clues to the specific disorders can be garnered. Family history and a thorough chart review to determine the onset of hypophosphatemia help to make the diagnosis, because a prior normal phosphate level decreases the likelihood of a genetic disease, whereas similarly affected family members suggest a genetic cause. Physical findings of rickets, such as lower extremity deformity, suggest the disorder has been present since childhood. A word of caution is in order because the genetic forms of hypophosphatemic rickets have highly variable penetrance and sometimes individuals may not manifest the disorder until adulthood. Furthermore, many times the affected individual represents a *de novo* mutation and there is not family history of hypophophatemic rickets. In general, however, age of onset can be helpful in distinguishing TIO from inherited forms of hypophosphatemic rickets. Most cases of TIO are diagnosed in adults in the sixth decade of life. Another important historical feature is the rate of onset, because rapid onset of symptoms is more characteristic of an acquired disorder such as TIO. However, symptoms of TIO can be present for many years before a diagnosis is made. In fact, the average time to diagnosis is 2.5 years from the onset of symptoms. At the onset of TIO, the symptoms are often vague and ill-defined but over time they crescendo into a severe syndrome of pain, fractures, and myopathy that are typically more extreme than those observed in patients with inherited forms of hypophosphatemic rickets. In cases where it is difficult to distinguish between TIO and inherited forms of hypophosphatemic rickets, genetic testing is available. Single-gene defects have been identified for XLH [PHEX (HYP Consortium, 1995)], ADHR [FGF23 (ADHR Consortium, 2000)], and ARHR [DMP1 (Lorenz-Depiereux *et al.*, 2006a)], and gene tests are commercially available (Table II).

TUMOR IDENTIFICATION

Most tumors responsible for TIO are of mesenchymal origin, and are found in bone or soft tissue (Folpe *et al.*, 2004; Jan de Beur *et al.*, 2002) (see Fig. 1). The most common sites are in the long bones and extremities, but nasopharynx, sinuses, and groin are other locations, which deserve careful scrutiny. They are generally benign and slow growing, but even quite small lesions are capable of causing profound biochemical changes. A helpful classification system divides these tumors into four categories (Weidner and Santa Cruz, 1987), the most common of which is the phosphaturic mesenchymal tumor: mixed connective tissue type (PMTMCT). The others are osteoblastoma-like tumors and ossifying and nonossifying fibrous-like tumors. PMTMCT accounts for up to 80% of the tumors (Weidner and Santa Cruz, 1987). This tumor contains a variety of cells, including spindle cells, which comprise a primitive stroma, and osteoclast-like cells, with prominent blood vessels and a cartilaginous matrix.

Because these mesenchymal tumors are typically small, localization can prove challenging. Plain films and radionuclide bone scanning are generally consistent with osteomalacia, but rarely localize the causative tumor. Whole-body CT or MRI scanning has a low yield but is often the first step. Based on the presence of somatostatin receptors in mesenchymal tumors (Reubi *et al.*, 1996), ^{111}Indium pentreotide, a radio-labeled somatostatin analogue (octreotide scanning), has been used successfully in many patients to localize tumors (Jan de Beur *et al.*, 2002; Seufert *et al.*, 2001) (see Fig. 1). Because a nonfunctional mesenchymal tumor may be identified on such a scan, definitive biochemical evidence for TIO is required before embarking on imaging studies (Jan de Beur and Levine, 2002). Other modalities that have been used successfully to locate tumors in individual patients include MRI (Avila *et al.*, 1996) and 18F fluorodeoxyglucose PET scan (Dupond *et al.*, 2005a, 2005b; Roarke and Nguyen, 2007; Vandergheynst *et al.*, 2006). One group has reported using venous sampling of FGF23 preoperatively to confirm the causal nature of the tumor identified with standard imaging modalities (Takeuchi *et al.*, 2004).

Various malignant neoplasms have also been associated with TIO. These include carcinoma of the prostate (Hosking *et al.*, 1975; Nakahama *et al.*, 1995), breast, lung (Robin *et al.*, 1994;, van Heyningen *et al.*, 1994), as well as multiple myeloma (Maldonado *et al.*, 1975; Pope and Belchetz, 1993), sarcomas (Cheng *et al.*, 1989; Linovitz *et al.*, 1976), malignant fibrous histiocytoma, and malignant neurinoma (Hauge, 1956). Although these tumors are endodermal and epidermal in origin, many produce fibrous tissue proliferation and osteoblastic lesions, which may be the source of the paraneoplastic syndrome. Interestingly, epidermal nevi and neurofibromatosis can be associated with a pattern of phosphate wasting and aberrant synthesis of $1,25(OH_2)D_3$, perhaps also through changes in the differentiation and behavior of the surrounding stroma (Aschinberg *et al.*, 1977; Goldblum and Headington, 1993; Konishi *et al.*, 1991; Saville *et al.*, 1955).

PATHOPHYSIOLOGY

Tumors causing TIO do so through the secretion of factors that decrease the renal resorption of phosphorus and inhibit the conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$. The presence of humoral mediators, later coined "phophatonin," was suggested initially by the rapid reversal of the syndrome by surgical resection of the associated tumor (McCance, 1947). Subsequent studies demonstrated that transplantation of tumor cells into animals could recapitulate the syndrome (Aschinberg *et al.*, 1977; Miyauchi *et al.*, 1988; Nitzan *et al.*, 1989). Furthermore, media derived from the cultured tumor inhibited phosphate transport in a renal cell line (Cai *et al.*, 1994), but the phosphatonin was not able to be isolated biochemically.

A genetic approach employing differential gene expression profiling of the TIO tumors identified several candidates for "phosphatonin," among them FGF23 (De Beur *et al.*, 2002; Rowe, 2000; Shimada *et al.*, 2001). Contemporaneously, mutations in FGF23 were identified in families with ADHR, an inherited form of hypophosphatemic rickets biochemically similar to TIO (ADHR Consortium, 2000). In ADHR, mutations at arginine residues 176 or 179 that reside in a subtilisin-like proprotein convertase recognition site render FGF23 resistant to cleavage and degradation, thus prolonging its biological activity (Bai *et al.*, 2003; Shimada *et al.*, 2002; White *et al.*, 2001a).

That FGF23 excess is a powerful mediator of TIO, and perhaps the predominant one, is supported by abundant evidence. When injected into mice, FGF23 reduces serum phosphate and increases fractional excretion of phosphorus (Shimada *et al.*, 2001, 2002). Mice chronically exposed to FGF23-transfected Chinese hamster ovary (CHO) cell xenografts become hypophosphatemic with increased renal phosphate clearance, show reduced bone mineralization, and have reduced expression of renal 25-hydroxyvitaminD-1α-hydroxylase with decreased circulating levels of calcitriol (Shimada *et al.*, 2001). The biochemical and skeletal abnormalities of transgenic mice that overexpress FGF23 mimic human TIO (Larsson *et al.*, 2004; Shimada *et al.*, 2004b). This suggests that FGF23 is an upstream regulator of phosphate homeostasis, able to control both renal and $1,25(OH)_2D_3$ activity to decrease phosphorus levels. In humans, FGF23 is highly expressed in many tumors associated with TIO by both mRNA and immunohistochemical evaluation (Bowe *et al.*, 2001; De Beur *et al.*, 2002; Shimada *et al.*, 2001; White *et al.*, 2001b). FGF23 is measurable in serum and is elevated in most patients with TIO (Jonsson *et al.*, 2003; Yamazaki *et al.*, 2002). Furthermore, circulating FGF23 plummets after successful surgical removal of the causative tumor.

FGF23 excess results in renal phosphate wasting in several disorders associated with dysplastic bone and fibrous cells. In patients with fibrous dysplasia, FGF23 levels correlate with the degree of phosphate wasting and the response of the dysplasia to bisphosphonate therapy was correlated with a reduction in the FGF23 levels (Riminucci *et al.*, 2003). In a study of osteoglophonic dysplasia (OD), where the underlying defect is an activating mutation of the FGFR1, one patient had normal phosphate homeostasis, normal FGF23 levels, and a low burden of cystic bone lesions. In contrast, another patient with OD and a large burden of nonossifying bone lesions exhibited phosphate wasting and an elevated serum FGF23 (White *et al.*, 2005). Thus, overproduction of FGF23 by dysplastic bone or fibrous cells appears to be a critical step in a common pathway for many of the acquired forms of renal phosphate wasting.

Genetic disorders that cause FGF23 deficiency present with an opposite phenotype to TIO. Mice lacking *Ffg23* develop hyperphosphatemia and elevated $1,25(OH)_2D_3$ (Shimada *et al.*, 2004a) and appear similar to humans with familial tumoral calcinosis. Familial tumoral calcinosis (FTC) is a human disorder associated with hyperphosphatemia, elevated $1,25(OH)_2D_3$, hypercalcemia, and ectopic calcification. Initially, loss-of-function mutations in the gene that encodes GALNT3, a protein involved in O-linked glycosylation, were identified in several families with tumoral calcinosis (Campagnoli *et al.*, 2006; Ichikawa *et al.*, 2005; Specktor *et al.*, 2006; Topaz *et al.*, 2004). This was puzzling as inactivating mutations in *FGF23* were hypothesized to be the molecular defect in FTC. Subsequently, families with loss-of-function mutations in *FGF23* itself were recognized (Araya *et al.*, 2005; Benet-Pages *et al.*, 2005; Chefetz *et al.*, 2005; Larsson *et al.*, 2005). Further investigation revealed that GALNT3 glycosylates threonine 178 in FGF23. This residue resides in the region that is cleaved to inactivate FGF23 (and the site of activating mutations in ADHR). Glycosylation protects FGF23 from degradation and defective or absent glycosylation renders FGF23 vulnerable to cleavage and inactivation (Kato *et al.*, 2006). Similarly, a mutation in the gene encoding KLOTHO, a matrix molecule that acts as a cofactor/coreceptor for FGF23 and enhances binding to FGFR1c (Kurosu *et al.*, 2006; Urakawa *et al.*, 2006), was recently identified in a patient with tumor calcinosis (Ichikawa *et al.*, 2007). Therefore, loss of function of FGF23, either directly through inappropriate glycosylation or loss of its signaling cofactor, results in tumoral calcinosis with its disordered phosphate and vitamin D homeostasis. Again, the centrality of FGF23 in disorders of hypo- and hyperphosphatemia make a compelling argument for FGF23 as an important physiological regulator of phosphate and vitamin D homeostasis.

Although FGF23 appears to be the predominant mediator of TIO, several other proteins are highly expressed in tumors associated with TIO. Matrix extracellular phosphoglycoprotein (MEPE) (De Beur *et al.*, 2002; Rowe *et al.*, 2000, 2004; Shimada *et al.*, 2001) is a matrix protein that contains several functional domains involved in bone mineralization, which may act opposite to each other depending on the cleavage products generated (reviewed in White *et al.*, 2006). One important domain, ASARM (acidic serine-aspartate-rich MEPE-associated motif), is released by cathepsisn C-mediated cleaveage (Guo *et al.*, 2002). Loss of this peptide in the presence of mutant enzyme is thought to cause ectopic calcification (Toomes *et al.*, 1999) and pharmacological administration can impair mineralization (Rowe *et al.*, 2004). Thus cleavage of MEPE to ASARM appears to act to inhibit matrix formation. However, in healthy humans, levels of MEPE appear to be positively correlated with bone density (Jain *et al.*, 2004). This effect may be because of a second domain of MEPE, called AC-100, which has been shown to increase bone formation (Hayashibara *et al.*, 2004).

Whether MEPE plays direct a role in the production of TIO is not clear. MEPE can inhibit phosphate resorption *in vitro* and *in vivo*; however, in these animals low serum phosphorus was associated with an appropriate increase in $1,25(OH)_2D_3$ levels (Rowe *et al.*, 2004). Furthermore, CHO cells overexpressing MEPE did not cause TIO when implanted in nude mice (Shimada *et al.*, 2001) and MEPE null mice have normal phosphate and vitamin D status (Gowen *et al.*, 2003). Thus, increased MEPE expression in TIO may be an appropriate response of bone to low serum phosphorus, inhibiting mineralization in the absence of substrate. PHEX, the enzyme that is defective in XLH, may interact with MEPE, preventing cleavage and subsequent release of the ASARM fragment (Guo *et al.*, 2002). PHEX may, therefore, promote bone mineralization by keeping both FGF23 and MEPE suppressed. However, conflicting data about the role of MEPE in XLH stem from studies in deletion of MEPE in *hyp* mice (mouse model of XLH) do not supprot rescue of the *hyp* phenotype (Liu *et al.*, 2005). These observations suggest that MEPE is not directly responsible for renal phosphate wasting or aberrant vitamin D metabolism in XLH.

Secreted frizzled related protein 4 (sFRP4) is another highly expressed protein in TIO. sFRP4 is a secreted inhibitor of Wnt signaling. Wnt signal is important in human bone formation and bone mass accrual, suggesting that modulation of Wnt signaling may be important in regulating determinants of bone mass including some aspects of mineral ion homeostasis. Several lines of evidence suggest that sFRP4 has phosphaturic properties. sFRP4 inhibits phosphate transport in cultured renal epithelial cells (Berndt *et al.*, 2003), it reduces fractional excretion of phosphorus when infused into mice and rats, and with longer-term exposure, sFRP4 produces hypophosphatemia with blunting of the compensatory increase in 25-hydroxyvitamin D-1α-hydroxylase expression induced by hypophosphatemia (Berndt *et al.*, 2003).

Similarly, FGF7 is differentially overexpressed in TIO and conditioned media from culture tumor cells inhibited phosphate reabsorption in a renal cell line (Carpenter *et al.*, 2005). Furthermore, this activity was neutralized by an FGF7 antibody. However, the effect on vitamin D metabolism in these experiments was equivocal, leaving unresolved whether FGF7 alone could mediate all aspects of TIO.

Patients with XLH exhibit the same renal phosphate wasting and abnormal vitamin D metabolism as those with TIO. Yet, the genetic defect is not in FGF23 but is caused by loss-of-function mutations in a metalloprotease PHEX (Francis *et al.*, 1997; HYP Consortium, 1995; Sabbagh *et al.*, 2003). Although PHEX does not appear to cleave FGF23 directly (Benet-Pages *et al.*, 2004; Guo *et al.*, 2001; Liu *et al.*, 2003), serum FGF23 levels are elevated in many XLH patients (Jonsson *et al.*, 2003; Weber *et al.*, 2003; Yamazaki *et al.*, 2002) and FGF23 expression is increased in the bones of *hyp* mice (Liu *et al.*, 2003). These observations suggest that PHEX is involved in downregulation and control of FGF23; however, the precise interplay between FGF23 and PHEX is not currently understood.

The genetic defect in the third form of hypophosphatemic rickets, autosomal recessive hypophosphatemic rickets (ARHR), was identified as loss-of-function mutations in Dentin Matrix Protein 1 (DMP-1), a matrix protein related to MEPE and a member of the SIBLING (small integrin-binding ligand N-linked glycoprotein) family (Feng *et al.*, 2006). Interestingly, this protein appears to have two functions, it translocates into the nucleus to regulate gene transcription early in osteocyte proliferation and then, likely in response to calcium fluxes, becomes phosphorylated and is exported to the extracellular matrix to facilitate mineralization by hydroxyapetite (Narayanan *et al.*, 2003) in a process that requires appropriate cleavage of the full-length protein (Tartaix *et al.*, 2004). Loss of DMP-1 function in ARHR leads to modestly and variably increased serum FGF23, dramatically increased expression of FGF23 in bone, defects in osteocyte maturation, and impaired skeletal mineralization (Feng *et al.*, 2003, 2006). It appears that the immature osteocytes overproduce FGF23, which then circulates and acts on the kidney to produce phosphaturia and aberrant vitamin D synthesis.

TREATMENT

TIO is only definitively treated by identification and resection of the causative tumor. Tumor removal results in rapid reversal of the defects in phosphate homeostasis with eventual remineralization of bones. However, in many cases, either because of the difficulty in localizing the tumor or incomplete tumor resection, medical management is indicated.

Medical therapy is directed at increasing serum phosphorus levels with both direct supplementation and the increase of intestinal absorption and renal reabsorption with calcitriol ($1,25(OH)_2D_3$). Calcitriol and phosphorus may be needed in high doses to maintain fasting phosphorus in the low-normal range. Typically 2 g/day of phosphorus in divided doses with 1–3 μg of calcitriol is used, although in some patients calcitriol alone may be sufficient. Therapeutic efficacy can be monitored through resolution of symptoms, normalized serum phosphorus and alkaline phosphatase, and resolution of secondary hyperparathyroidism. Treatment can improve pain and muscle weakness and lead to healing of osteomalacia (Drezner and Feinglos, 1977).

Urine and serum calcium must be monitored to prevent the hypercalcemia, hypercalciuria, nephrolithiasis, and nephrocalcinosis that can result with calcitriol therapy (Lyles *et al.*, 1985). Autonomous parathyroid function with long-term medical therapy is common (Firth *et al.*, 1985; Olefsky *et al.*, 1972) and thus PTH and renal function should be monitored every three months.

The use of octreotide therapy in refractory cases has been reported with variable success. Because some mesenchymal tumors express somatostatin receptors, therapeutic trials with octreotide have been attempted. Of three patients treated with octreotide, one (Seufert *et al.*, 2001) showed improvement whereas two others did not after 8 weeks of therapy (Jan de Beur and Levine, 2002; Kumar, 2000).

Recently, the calcium-sensing receptor agonist, cinacalcet, has been shown to be an effective adjuvant in the treatment of TIO (Geller *et al.*, 2007). Cinacalcet acts on the calcium-sensing receptor to decrease PTH synthesis and secretion. In the absence of PTH, FGF23 is less phosphaturic. Thus, in a small series, treatment with cinacalcet resulted in increased renal phosphate reabsorption, increased serum phosphorus, and reduced oral phosphate supplementation.

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Chapter 72

Osteopetrosis

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INTRODUCTION

Osteopetrosis results from a reduction in bone resorption relative to bone formation, leading to an accumulation of excessive amounts of bone. The relative decrease in resorption is a consequence of inadequate osteoclastic bone resorption. Karsdal *et al.* (2007) have demonstrated that osteoclasts, although unable to resorb bone owing to an inability to acidify the ruffled border, are still capable of stimulating osteoblastic bone formation. del Fattore *et al.* (2006) examined 49 patients with osteopetrosis. Bone formation was increased beyond a normal level. This suggested that a coupling factor was being produced, increasing osteoblastic bone formation. Although a specific coupling factor(s) has not been defined, Karsdal *et al.* proposed that IGF1, TGF-β, BMP-2, IL-7, and IL-6 could be factors that result in the excessive stimulation. These imbalances lead to a thickening of the cortical region and a decrease in the size of the medullary space in the long bones, with sclerosis of the base of the skull (Elster *et al.*, 1992a,b) and vertebral bodies. There are a number of serious consequences resulting from the excessive accumulation of bone. A reduced marrow space results in decrease in hematopoiesis, even to the point of complete bone marrow failure. Extramedullary hematopoiesis occurs but is unable to compensate for the reduction in medullary blood cell production. A decrease in the caliber of the cranial nerve and vascular canals leads to nerve compression and vascular compromise. Dense, poorly vascularized bones are subject to fracture and, being vascularized, are predisposed to necrosis and infection. An increased risk of morbidity and mortality during anesthesia is present in children with osteopetrosis. The increase risk in children with osteopetrosis is primarily related to airway and respiratory factors; however, anemia, hypocalcemia, and airway reactivity have all been noted during anesthesia for surgical procedures. Anesthesia management needs to consider the high rate of adverse events and to prepare a plan for each patient's needs (Burt *et al.*, 1999).

An understanding of osteopetrosis in humans has been intertwined with the description and creation of a variety of animal mutations in osteoclastic function (Seifert *et al.*, 1993). Although the precise genetic defect in most patients remains to be established (one exception being the deficiency in carbonic anhydrase type II Fathallah *et al.*, 1994; Whyte, 1993a), the animal models have contributed substantially to the understanding of osteoclastic function and dysfunction. The osteoclast biology learned from these mutants has led to a variety of treatment strategies that have been used in patients with osteopetrosis. An understanding of the genetic basis of the animal mutations has generated a list of candidate genes that may prove to be the basis for discovering the genetic defects in humans.

CLINICAL DESCRIPTION

Classically, osteopetrosis has been divided into a fatal infantile malignant form, Albers–Schönberg disease, and a milder adult form of osteopetrosis with long-term survival (Grodum *et al.*, 1995; Key, 1987; Shapiro, 1993; Whyte, 1993b). Recently, a variety of intermediate forms have been described. Without knowledge of the genetic defect explaining the osteoclastic dysfunction, it has been difficult to delineate the mechanisms of these different forms (Table I).

Severe

A variety of presentations of osteopetrosis have been seen in infancy. In general, patients have had sporadic forms that appear to have an autosomal recessive inheritance. Although the severe, malignant form predominates, milder forms and autosomal dominant inheritance patterns have also been observed in individuals diagnosed in the neonatal period (Manusov *et al.*, 1993; Whyte, 1993b). In the absence of a known genetic defect in osteoclastic function, histomorphometric and clinical parameters have been established to describe the degree of severity.

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TABLE I Genetic Defects Identified in Human and Animal Osteopetrotic Syndromes

		Genetic defects	Animal correlates	Human disease	Mechanism	Function	References
Severe	TCIRG1	T-cell immunoregulatory 1	osteosclerosis mouse	severe infantile malignant	loss of function	encodes a3 subunit of the vacuolar ATPase, proton pump	Kornak *et al.* (2001)
	ClCN-7	chloride channel 7	ClCN-7$^{-/-}$ mouse (*mi/mi* mouse) Mitf	infantile milder bone disease, but neurodegeneration	loss of function	encodes the chloride channel (ClC-7)	Li *et al.* (1999)
	OSTM1	osteopetrosis-associated transmembrane protein 1	gray lethal mouse	severe infantile malignant	loss of function	possible protein degradation decreasing protein transport	Ramirez *et al.* (2004)
Intermediate	ClCN-7	chloride channel 7		(ADO)II	loss of function	encodes the chloride channel ClC	Waguespack *et al.* (2003)
	LRP5	low-density lipoprotein receptor-related protein 5		(ADO)I	gain of function	putatively upregulates an osteoblastic gene function	Van Wesenbeeck *et al.* (2003)
	PLEKHM1	plekstrin homology domain-containing family M (with RUN domain) member 1	incisor absent rat	intermediate osteopetrosis	loss of function	trafficking acidic vesicles in osteoclasts	Van Wesenbeeck *et al.* (2007)
Mild	CA II	carbonic anyhydrase II		mild osteopetrosis with cerebral calcification and RTA	loss of function	proton production	Fathallah *et al.* (1994)

Patients with osteopetrosis presenting at birth or in early infancy are usually referred to as having the severe, malignant form. The implication is that these patients will have severe sequelae and will die during the first decade of life. This has been the justification for using treatment modalities, such as bone marrow transplantation, that carry a high risk of mortality and morbidity (Gerritsen *et al.*, 1994b; Key and Ries 1993; Schroeder *et al.*, 1992). Patients with this form are characterized by a diffusely sclerotic skeleton with little or no bone marrow space evident, even at birth (Fig. 1) (Gerritsen *et al.*, 1994a). In addition, there is evidence of a severe defect in bone resorption, leading to the presence of the "bone-in-bone" appearance on radiographs (Fig. 2) and in cartilaginous islands within mineralized bone on histology (Fig. 3).

However, some patients with this phenotype will not have a fatal outcome. Indeed, up to 30% of patients diagnosed with severe, malignant osteopetrosis are still alive at age 6 years, with rare patients surviving into the second or third decade (Gerritsen *et al.*, 1994a). Although the quality of life is reported to be poor, up to half of the surviving patients, despite a variety of skeletal and neurological impairments, have normal intelligence and are capable of attending school (Charles and Key, 1998).

A subgroup of patients has an extremely malignant form of the disease. In a group of 33 patients reported by Gerritsen *et al.*, eight patients had hematological and visual impairment before 3 months of age (Gerritsen *et al.*, 1994a). All eight of these patients died before the age of 12 months. Hematological impairment before 6 months of life is prognostic of a markedly reduced survival rate; however, visual impairment alone does not correlate with an early fatal outcome.

Cytological evidence of large osteoclasts with increased numbers of nuclei and a markedly increased amount of ruffled border membrane correlates with a poor prognosis for cure with bone marrow transplantation (Schroeder *et al.*, 1992; Shapiro *et al.*, 1988). Although there is no exact explanation for the reduced success in cure by transplantation, the defect in bone resorption in these patients could be explained if the osteoclast itself were normal but some other aspect of the bone rendered it less resorbable. Thus, histological and electron microscopic analysis of osteoclasts is recommended before a transplantation is undertaken.

Another form of osteopetrosis that has not responded to any therapeutic modality is associated with a neurodegenerative disease (Whyte, 1993b). In some patients, a

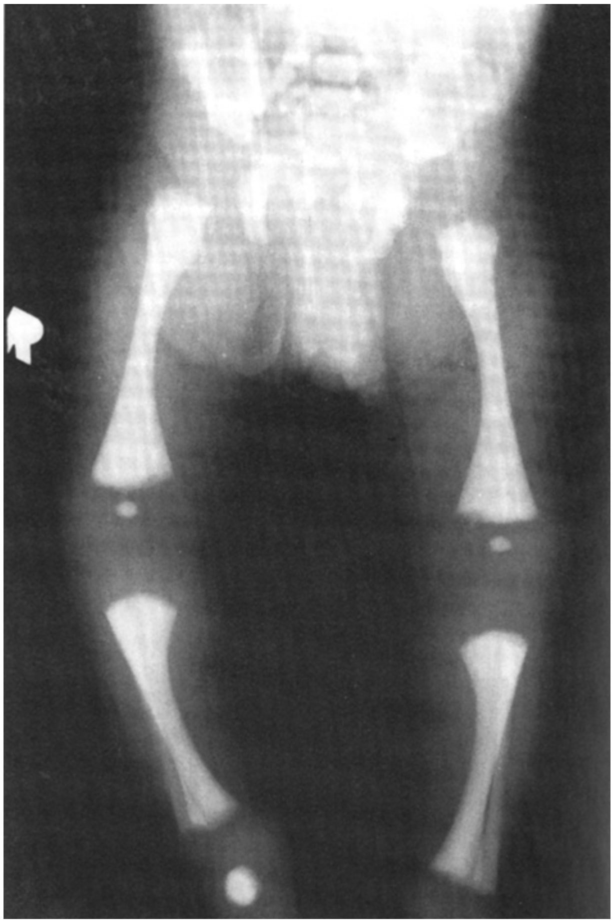

FIGURE 1 The skeleton, both the long bones and the pelvis, is shown to be sclerotic in this radiograph of a 5-day-old infant with anemia and optic nerve compression. Patients with this severe presentation have a 100% chance of death before age 1 year, if untreated. Note that no intramedullary space is seen in the long bones or the pelvis.

neuronal storage disease has been suggested by cytoplasmic inclusions. In most patients, seizures, poor neurological development, abnormalities in the cerebral cortex seen on magnetic resonance imaging, and early development of central apnea characterize this specific presentation. No therapy that has been tried in these patients (bone marrow transplantation, calcitriol, or interferon-γ) has resulted in any significant improvement of the disease. It is likely that even when the bony disease can be reversed (as has occurred by using each of the modalities), the underlying neurological disorder remains unaffected. Death usually occurs before 2 years of age.

Intermediate

The intermediate form of osteopetrosis is frequently "silent" at birth with few or no obvious clinical abnormalities (Key, 1987; Whyte, 1993b). Some cases are diagnosed in infancy, when suspected, suggesting that the defect is present at a subclinical level even from birth. Of interest, the radiographs frequently demonstrate severe manifestation of diffuse sclerotic bone in this form of the disorder, quite similar to those seen in the malignant form (Fig. 4). These patients tend to have fractures toward the end of the first decade and frequently have repetitive fractures with very minor trauma. Infections of the bone can be difficult to eradicate, especially if the mandible is involved. Most patients survive into adulthood. Although anemia and hepatosplenomegaly are rare, at least one patient developed anemia and thrombocytopenia so severe that he was transfusion dependent. His anemia and thrombocytopenia were eliminated by splenectomy.

A subgroup of patients with the intermediate form of osteopetrosis has a carbonic anhydrase II deficiency (Fathal-lah *et al.*, 1994; Whyte, 1993a). These patients have a hyperchloremic metabolic acidosis. Several different mutations have been described in the 50 cases diagnosed. Patients tend to have delayed development with a reduction in intelligence as adults, short stature, fractures, cranial nerve compression, dental malocclusion, and cerebral calcifications. Patients usually have no defects in hematological function and no increased risk of infection.

Transient

Some patients with severe radiographic abnormalities and with anemia have had severely sclerotic bone early, which resolves without specific therapy (Monaghan *et al.*, 1991; Whyte, 1993b; L. L. Key, personal observation). Although long-term follow-up is not available in these patients, no known sequelae resulted from the condition. The patients had no visual impairment, but did have anemia and thrombocytopenia. In one patient (personal experience), there was a history of acetazolamide administration. Whereas osteopetrosis has not been widely associated with acetazolamide administration, the existence of osteopetrosis with naturally occurring mutations in carbonic anhydrase suggests that the therapy may be related to the osteoclastic dysfunction. In both cases, the resolution in the bone disease was apparent early (within 1 month). In addition, patients with other milder sclerosing bony dysplasias are frequently considered to have osteopetrosis in infancy. A classic case is seen in the natural history of severe craniometaphyseal dysplasia where early radiographs are nearly indistinguishable from osteopetrosis (Fig. 5); however, there is no involvement of the vertebral bodies.

ADULT FORMS

In general, patients with the adult forms of the disease have a family history suggesting an autosomal dominant inheritance pattern (Whyte, 1993b). Anemia is not a common manifestation; however, fractures and cranial nerve dysfunction are frequently observed (Bollerslev and Mosekilde, 1993; Bollerslev *et al.*, 1994). When defects have been

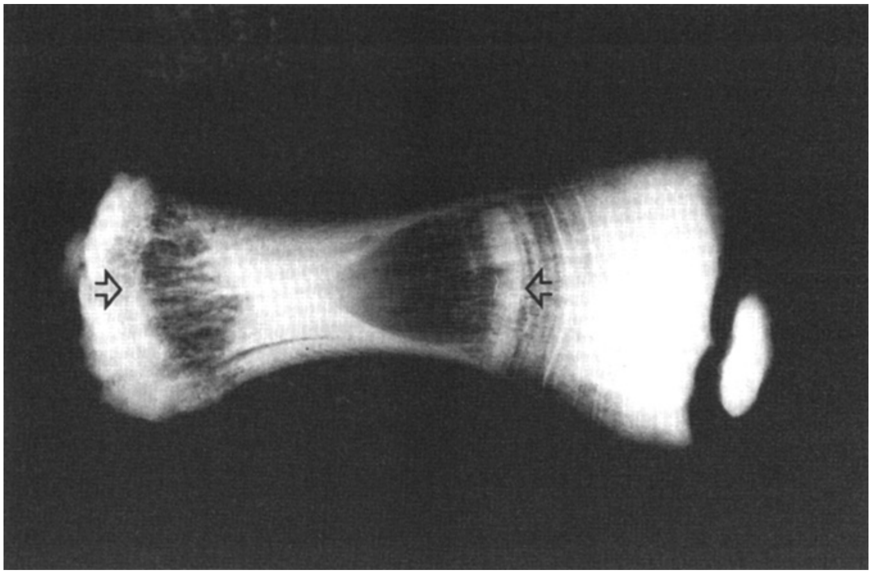

FIGURE 2 This radiograph shows a phalangeal bone, obtained at autopsy from a 7-year-old with malignant osteopetrosis treated with calcitriol. Note the presence of the original bony template (endobone, between the arrows) which was present at birth and never remodeled. The radiograph shows the presence of mineralized bone that has been laid down outside of this original bone/cartilage template without the underlying template having been resorbed.

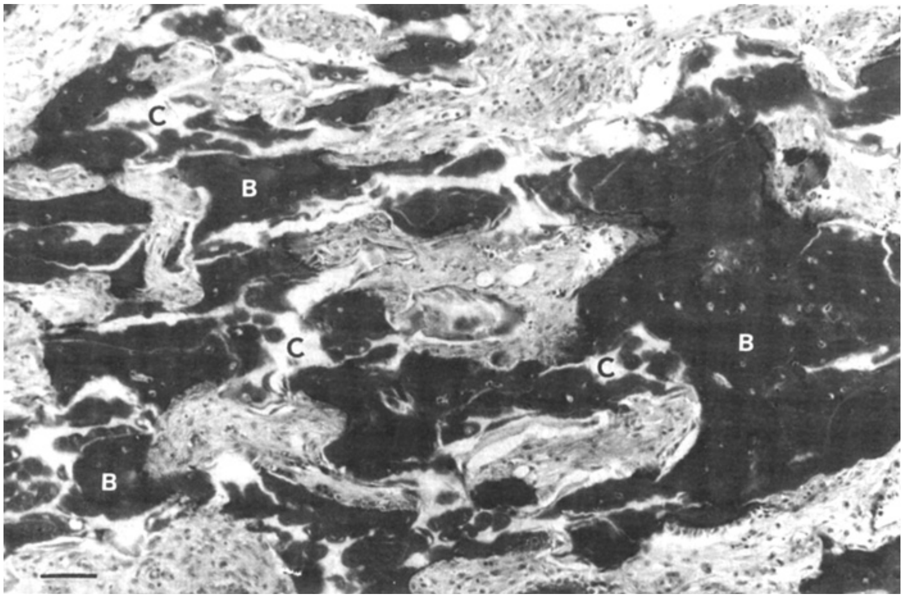

FIGURE 3 The pathognomonic feature of osteopetrosis at the histological level is the presence of unresorbed cartilage (denoted at "C"). Around the cartilage, a highly cellular, embryonic bone (designated as "B") has been laid down. This combination of nonresorbed cartilage and poorly formed bone is the microscopic feature that results in the macroscopic appearance seen in the "bone-in-bone" appearance on radiographs (see Fig. 2). The bar denotes 80.

sought for in infancy, radiographic abnormalities have been found that define the presence of the disease and portend the onset of symptoms in later life.

Severe

We have observed two patients who presented with severe neonatal disease that had a family history suggestive of the presence of an autosomal dominant disorder. In each of these cases, the children were diagnosed with the malignant form of the disease owing to anemia and cranial nerve dysfunction presenting in infancy. In one patient, there was a history of severe infections and failure to thrive. Family members in each case had survived into the fifth and sixth decade (Fig. 6). In each case, the parent transmitting the disease was unaffected.

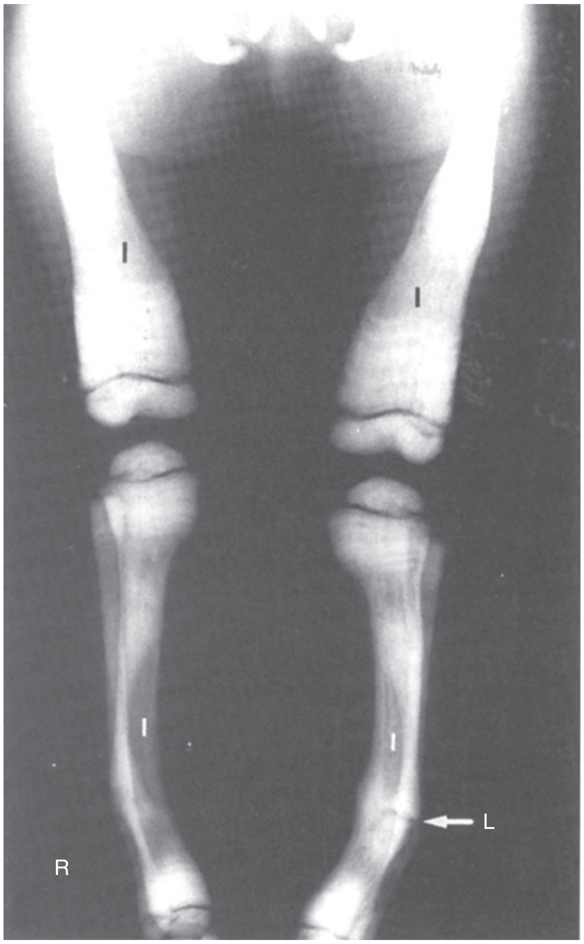

FIGURE 4 A radiograph of the long bones of a 6-year-old with an intermediate form of osteopetrosis shows the presence of some intramedullary space (denoted as "I"). The deformities of modeling result in bowing and thickening of the shaft and metaphyseal regions of the bone. Note a fracture, which was clinically not apparent, is present in the left tibia (arrow).

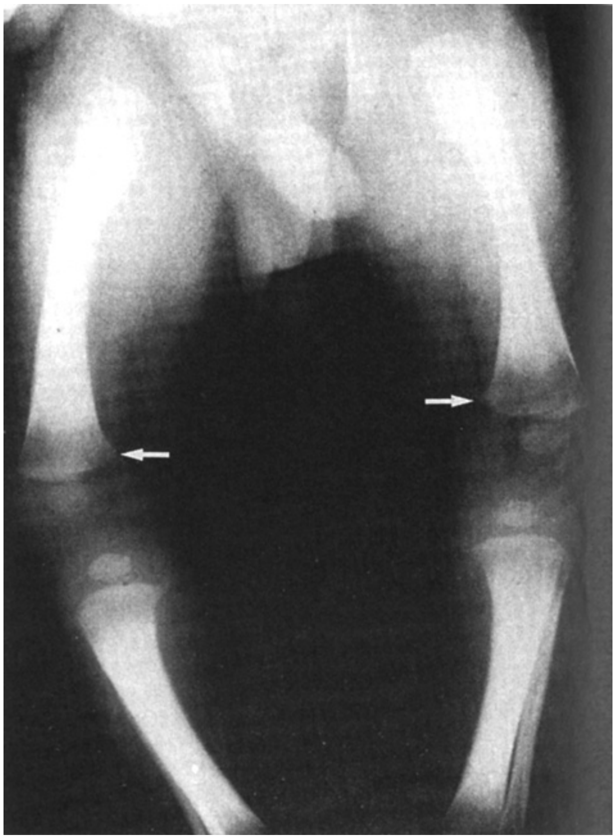

FIGURE 5 This radiograph of a 1 month old with craniometaphyseal dysplasia demonstrates sclerotic long bones with little marrow space seen in the diaphyses. It should be noted that there is already some clearing of the bone in the distal metaphyseal region of both femurs (arrows, which had been sclerotic at birth).

Mild

The "benign" adult form of osteopetrosis is frequently "silent" until later in life (Bollerslev and Mosekidle, 1993; Whyte, 1993b). Two distinct subtypes have been described based on radiographic appearance, symptoms, and biochemical characteristics. Each of these subtypes has been inherited in a variety of kindreds. Each subtype has a distinct natural history, resulting in differing symptomatic presentations. Both types have universally sclerotic bones, primarily involving the axial skeleton. Little or no modeling defects are seen in the long bones. Both types have been observed in some children in the identified families. Approximately 40% of patients with the adult form of osteopetrosis are symptom-free regardless of type. Anemia is rare in either type. Bone pain is common to both types.

Type I is characterized radiographically, by massive sclerosis of the skull with increased thickness of the cranial vault. There is diffusely sclerotic bone in the spine and pelvis. Cranial nerve compression is common in type I. There are few abnormalities seen in the remodeling of bony trabeculae. Indeed, the strength of bone in type I is increased compared with normal. Thus, pathological fractures are rarely observed.

Radiographs from patients with type II disease demonstrate massive sclerosis of the base of the skull, hypersclerotic endplates of the vertebrae resulting in the "rugger jersey" spine, and subcristal sclerotic bands diagnostic of "endobones." Bone turnover is decreased, leading to a reduction in bone tensile strength and resulting in frequent fractures. In type II, creatine phosphokinase, especially the BB isoenzyme, is increased. Histomorphometric analysis shows a defect in trabecular remodeling, resulting in bone that had not been replaced, yielding weakness.

In summary, the adult forms of osteopetrosis are usually diagnosed symptomatically in the second decade. These disorders rarely significantly alter hematopoiesis. Defects in type I disease result in cranial nerve compression, but fractures are rare. In type II disease, fractures are common, but nerve compression is rare.

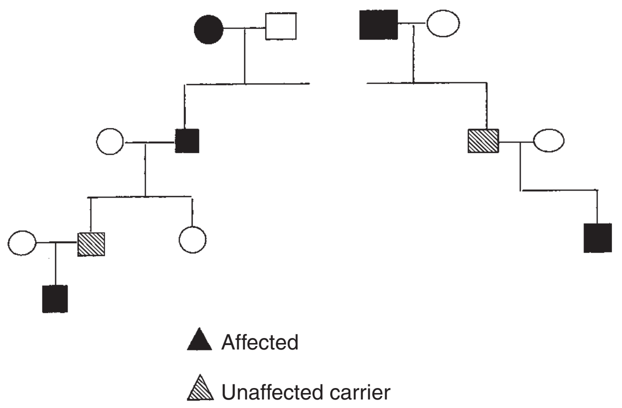

FIGURE 6 This is the family tree of two patients with adult forms of osteopetrosis. The presentation of these patients was severe enough (sclerosis of the long bones in one, anemia, sclerosis of the long bones, and blindness in the second) to result in an initial diagnosis of the severe malignant form.

PATHOPHYSIOLOGY

Osteoclast Dysfunction

Although animal models and a few patients have been found with a profoundly reduced number of osteoclasts, defective function rather than a reduction of formation appears to be the primary pathogenesis (Key and Ries, 1993). This holds true for malignant, intermediate, and benign forms. In the malignant form, there is the possibility of replacing the defective cell and curing the disease with bone marrow transplantation. However, defects in the osteoclasts' environment have also been suspected and may be the explanation for the greater-than-predicted rate of failure in bone marrow transplantation therapy in this disorder (Gerritsen *et al.*, 1994a; Key, 1987). However, to date, no patient has been definitively diagnosed with the one clear-cut stromal defect, a defect in producing macrophage colony-stimulating factor (M-CSF) seen in the *op/op* (osteopetrotic) mouse model (Key *et al.*, 1995a). One patient was reported with a decreased level of M-CSF after a failed transplantation; however, no data were available before the immunomodulation necessary for the transplantation.

A defect in white blood cell and osteoclastic superoxide production has been documented in the majority of patients with osteopetrosis (Key and Ries, 1993). Although the defect in superoxide generation in osteoclasts may represent a more generalized decrease in the ability of the osteoclast to resorb bone, therapy with interferon-γ-1b, designed to increase superoxide production, increases white blood cell superoxide production, thereby reducing infections, and increases bone resorption, enhancing hematopoiesis and enlarging cranial nerve foramina. The result is a reduction in the need for intravenous antibiotics and transfusions and in a deceleration of cranial nerve damage. The net result is an improved survival with reduced morbidity.

Genetic Defects

The search for genetic abnormalities that result in osteopetrosis has recently identified two genetic defects that are related to the osteopetrotic condition. In the first report of a gene localization based on linkage analysis, van Hul *et al.* reported an 8.5-cM region on chromosome 1*p*21 that is associated with the autosomal dominant adult form, type II (van Hul *et al.*, 1997). Although this region was chosen for study because of the presence of the *CSF-1 gene*, a precise defect has not been identified. Indeed, a survey of 20 patients with osteopetrosis found no evidence of defects in the c-*src*, c-*fos*, c-*cbl*, and MITF (human equivalent of the *mi*/*mi*) genes (Yang *et al.*, 1998).

Two studies have identified precise mutations in a subunit of the V-type H^{+}-ATPase protein, OC116 (Kornak *et al.*, 2000; Frattini *et al.*, 2000). Numerous genetic defects have been reported (see Table I). All of these patients appear to have a classic severe osteopetrosis with severe osteosclerosis, hematological failure secondary to reduced bone marrow space, and visual impairment in all but one patient. A history of consanguinity was a common theme for four patients.

Functionally, the defect causes the absence of the *a*3-subunit, OC116, of the V-type proton pump that is present along the ruffled border and responsible for acidification next to the bone surface. In the absence of this acidification, calcium cannot be removed from the surface of the bone. Of interest, a knockout animal has been described (Li *et al.*, 1999), providing the first example of an animal model predicting a human osteopetrotic genetic defect (the details of animal model are presented later). This makes the existing animal models even more exciting as a repository of defects that may potentially explain human disease.

ANIMAL MODELS

Classic

A variety of animal models for osteopetrosis have been explored. The M-CSF deficiency in the *op/op* mouse seems to be the most clearly related defect (Begg *et al.*, 1993; Lowe *et al.*, 1993; Marks *et al.*, 1992; Nilsson and Bertoncello, 1994; Philippart *et al.*, 1993; Wiktor-Jedrezejczak *et al.*, 1994); however, replacing the M-CSF with exogenous cytokine does not result in a complete remission (Sundquist *et al.*, 1995). Several possible explanations have been suggested. The timing of the administration may not be the most advantageous (Hofstetter *et al.*, 1995; Lee *et al.*, 1994; Sundquist *et al.*, 1995). Alternatively, there may be other related factors that must interact. One of the most plausible ideas is that, in addition to circulating levels of M-CSF, there is also the need for membrane-bound M-CSF (Stanley *et al.*, 1994) on the osteoblast or embedded in the bone surface (Ohtsuki *et al.*, 1995) to be presented to the osteoclast or its precursors. A less clear-cut defect, but a role demonstrated for M-CSF therapy in improving the phenotypic abnormalities in the *tl/tl* (toothless) rat (Aharinejad *et al.*, 1995; Marks *et al.*, 1993) has suggested some involvement with the M-CSF production or the M-CSF receptor in the genesis of this mutant phenotype as well. To date the precise defect has not been reported. Thus, animals or humans with few macrophages and osteoclasts may be found to have a defect in M-CSF production or response.

In the *mi/mi* mouse (microphthalmic), there is a defect in the production of a transcription factor (Steingrímsson *et al.*, 1994). This has led to the possibility that a variety of defects, all related to the presence of a defective transcription factor, could exist in the osteoclast and/or in other cells as well. One suggested defect is a decrease in the c-*kit* receptor production, which is necessary for stimulation of tyrosine kinase activity with the stem-cell factor (Ebi *et al.*, 1992). Stem-cell factor and M-CSF activate similar receptor populations, c-*kit* and c-*fims*, which are quite similar in their binding regions. There are also data suggesting that possibly there is a defect in the ability of *mi/mi* stromal cells, failing to support osteoclastic function (Key, 1987) or possibly even inhibiting osteoclastic function *in vitro.*

Both M-CSF and/or interferon-γ have been shown to improve the defect in the *mi/mi* mouse (Key *et al.*, 1995a). Because these cytokines are not deficient in these animals, the studies suggest that these therapies circumvent the specific defects rather than reversing the defects directly. Similar effects of these cytokines have been observed in patients with osteopetrosis where defects are unknown and not directly related to deficiencies in cytokine production or response elements.

Knockout

Perhaps the greatest interest has been in the analysis of man-made knockout mutations that have been found to yield an osteopetrotic phenotype. The effects of a targeted disruption of *Atp6i*, a gene encoding a subunit of the vacuolar pump in the C57BL/6J inbred mouse strain, have been described (Li *et al.*, 1999). The mutant animals resulting from this mutation have extremely dense bones with cartilaginous islands, reduced marrow space (decreased by 80% in the long bones), absent tooth eruption, decreased growth, and severely deformed bones. The condition was lethal in all animals by the fifth week. These animals appear to be quite similar to the patients described with defects in the *TCIRG1* gene of the human vacuolar proton pump described by Frattini *et al.* (2000) and Kornak *et al.* (2000). The results of this animal mutation prove that a defect in the proton pump can cause osteopetrosis, confirming the speculation that the genetic mutations in the vacuolar pump documented in humans is responsible for the associated osteopetrotic phenotype.

The most well studied of these mutants is the c-*src* knockout mutation (Boyce *et al.*, 1993; Lowe *et al.*, 1993). In this mutation, the c-*src* tyrosine kinase is defective and yields a mutant with osteoclasts lacking ruffled borders. This appears to result from the lack of production of phosphorylated proteins necessary to allow fusion of the membrane of endosomes with the cellular membrane within the sealed attachment of the osteoclast to bone. The result is an inactive cell with none of the machinery present at the osteoclast-bone interface necessary to resorb bone. The precise mechanism is not well understood and seems to be more complex than originally thought.

In the c-*fos* knockout mutation (Jacento, 1995; Grigoriadis *et al.*, 1994; Johnson *et al.*, 1992; Wang *et al.*, 1992), there are few mature osteoclasts formed and there is a reduction in the number of osteoclasts and an increase in the number of macrophages. Thus, it appears that c-*fos* is related to the osteoclast lineage's "switch point," which determines whether the progenitor moves in the direction of the osteoclast or the macrophage (Jacento, 1995).

The knockout of PU.1 (Tondravi *et al.*, 1997), an ETS-domain transcription factor essential for the development of myeloid and B-lymphoid cells, is thought to regulate the c-*fms* receptor (receptor for CSF-1). A mutation in this gene results in a nearly complete absence of osteoclasts, resulting in severe osteopetrosis. Bone marrow transplantation with PU.1 competent stem cells rescued the animals, completely reversing the defects. No human correlate has been found. Knocking out the gene for cathepsin K generated an additional osteopetrotic mouse model (Saftig *et al.*, 1998; Gowen *et al.*, 1999). This mutant was found to have dense bones with a reduction in bone marrow space, and extramedullary hematopoiesis. Although these animals shared features of osteopetrosis, it was noted that the defective bone resorption varied from bone to bone. Recently, a similar mutation was identified in humans with pycnodysostosis, a disorder resembling osteopetrosis, but having osteoacrolysis, poor fusion of the sagittal suture, micrognathia, and a predisposition for fracture (Ho *et al.*, 1999). The osteoclastic defect appears to be an inability of the osteoclast to degrade matrix, although removal of calcium appears to be normal. Thus, it is likely that some of the knockout mutations may be genes responsible for other sclerosing bony dysplasias. It appears that mutations resulting in osteopetrosis are those that completely inactivate the osteoclastic bone resorption by either leading to a failure to generate the osteoclast itself or the failure to form the ruffled border. This suggests that there is some redundancy of function in the osteoclast's bone-resorbing process such that knocking out a single enzyme system does not completely inactivate bone resorption.

At the present time, knockout mutations have been much more important in understanding osteoclastic function than in explaining the human osteopetrotic condition. However, these studies do provide a variety of candidate genes and are beginning to suggest genetic defects that will allow us to diagnose certain types of osteopetrosis.

Avian Osteopetrosis (Increased Osteoblastic Function)

Osteopetrosis has been described in the chicken and appears to result from infection with the avian leukosis virus. This sclerosing condition is characterized by an increase in bone formation by infected osteoblasts (Smith and Ivanyi, 1980) with normal osteoclastic function. Avian osteopetrosis, although similar to the sclerosis seen in a variety of human diseases, has not been shown to occur in humans with one possible exception. In "transient osteopetrosis," Ozsoylu and Besim have suggested that the etiology may be bony sclerosis secondary to excessive osteoblastic function (Ozsoylu and Besim, 1992).

THERAPY OF HUMAN DISEASE

Even with the marked advances in understanding the osteoclastic defects that could result in osteopetrosis, we are still left today with the need to treat somewhat blindly in trying to combat this disorder. Transplantation has been the mainstay of treatment; however, somewhat disappointing results and, at present, the lack of available donors have made it difficult to rely on transplantation as the sole therapy for this disease. Thus, a variety of other therapies have been attempted with some success. Because the mortality associated with failed transplantations is high, other treatment alternatives and/or stopgap measures remain important despite the inability of these therapies to completely cure the condition in most patients. These therapies may be the sole therapy of the patient or may result in a milder course of the disease while awaiting a definitive cure.

Transplantation

Despite the fact that, at present, transplantation is available in only $\sim$50% of children with osteopetrosis and is successful in $\sim$45% of patients who receive grafts; the allure of this therapy is that a successful transplantation yields a nearly complete cure (Gerritsen *et al.*, 1994b). If this cure can be obtained before there are too many physical disabilities, children with osteopetrosis tend to grow and develop normally. In transplantations where the donor graft is harvested from young siblings who have a favorable HLA match, there appears to be 70%, or slightly greater, survival. Despite the fact that not all engraftments are maintained, even the retention of a small number of donor cells seems to correct the defect significantly and stave off recurrence of symptomatic disease. However, beyond this group, the success rate is less favorable. In transplantations where the graft is obtained from HLA haploidentical (mismatched) relatives, the engraftment and survival rates for transplants are only $\sim$15%. Given the natural history of a survival of almost 30% for the malignant form of osteopetrosis as a whole, this is considered by most transplantation experts to be an unacceptable rate of failure. However, it should be noted that in the 15% of patients where transplantations have been successful, cures have been achieved. The great hope for the future is transplants with matched unrelated donors and possibly, cord blood stem cells. (Information on cord blood transplantation in children was communicated personally by M. Klemperer, St. Petersberg, FL.) With the advent of DNA typing to ensure the adequacy of the matches, success rates of = 80% are being achieved in most diseases. However, success rates of only $\sim$40% have been found in osteopetrosis. The exact reason for this discrepancy is not clear at this time. The possibility that cord blood or placental transplants may improve chances for engraftments is just beginning to be explored. Nonetheless, at present, in the absence of a matched sibling donor, survival can be expected to be well below 50%.

Other Therapies

Many families have found that transplantation carries an unacceptable risk, and thus, there has been a search for alternative therapies. The use of high-dose calcitriol has been tried in a large number of children with some period of stabilization, and in some instances, a cure (Key and Ries, 1993; van Lie Peters *et al.*, 1993). Nonetheless, long-term cures have been achieved in 25% of patients treated.

Interferon-γ has been used as a treatment of osteopetrosis (Key *et al.*, 1995b; Kubo *et al.*, 1993). The intent of the therapy was to enhance the production of oxygen radicals by white blood cell phagocytes as a means of fighting off life-threatening infections. In addition, therapy with interferon-γ-1b along with calcitriol increases both bone remodeling and the amount of bone marrow space in patients (Fig. 7 and Fig. 8). To determine whether interferon-γ-1b (1.5 μg/kg/dose subcutaneously, three times per week, Actimmune, Intermune Pharmaceuticals, Inc., Palo Alto, CA) plus calcitriol (1 μg/kg/day orally) or calcitriol alone is effective in delaying the time to treatment failure, 16 patients with congenital osteopetrosis were recruited from all over the world to participate in a randomized, controlled trial. Patients were randomized in a 2:1 ratio onto interferon-γ-1b plus calcitriol or calcitriol alone, respectively. The drug was administered subcutaneously to patients three times a week. The patients receiving interferon-γ-1b plus calcitriol had their first evidence of failure at a mean of 452 days compared with those on calcitriol alone, who failed at 130 days ($P = 0.016$). Only 10% of the patients treated with interferon-γ-1b experienced a serious infection compared with 67% of the patients on

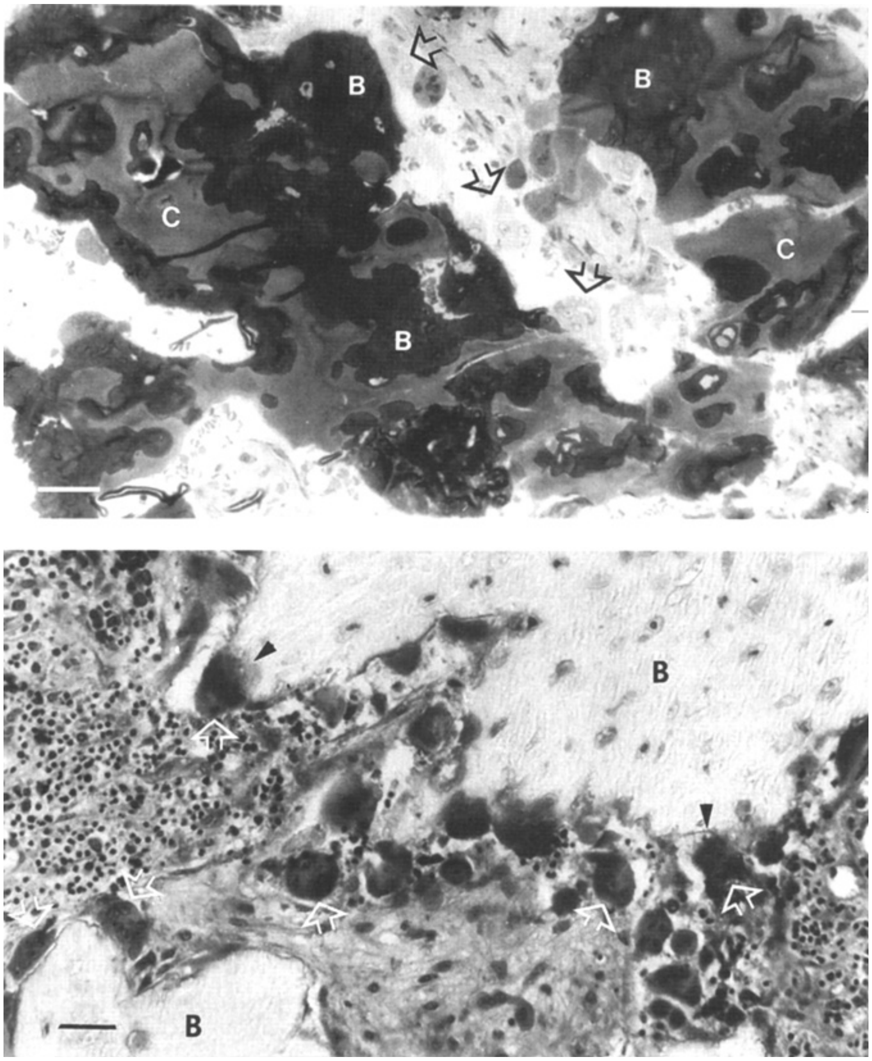

FIGURE 7 Bone biopsies from age 11 and 29 months in a patient with severe infantile osteopetrosis. (*Top*) Contains a 1-μm Epon-embedded section obtained from the patient at 11 months of age, prior to therapy with interferon-γ-1b and calcitriol. Coarse trabecular bone (denoted as "B") with cartilaginous streaks (denoted as "C") is evident. Lacunae are absent even though osteoclasts (arrows) adjoin the bone surface. Bone marrow cells are absent from this section. (*Bottom*) A 5-μm-thick methyl-methacrylate-embedded section obtained by biopsy of the same patient after 18 months of therapy. A combination of mineralized coarse trabecular bone and porous cortical bone (designated by "B") lined with numerous osteoclasts (open arrows) with brush borders and adjoining lacunae (arrowheads) is visible. Islands and streaks of cartilaginous tissue are absent. Bar = 40μm.

calcitriol alone ($P < 0.0001$). In limited data, biopsies obtained from patients treated with interferon-γ-1b had a 50% reduction in bone mass; while no reduction was seen in one patient treated with calcitriol alone. The size of both the optic nerve foramina and auditory canals increased in subjects treated with interferon-γ-1b, but not in those treated with calcitriol only.

SUMMARY

In summary, significant progress has been made in classifying osteopetrosis. There are at least three major categories (malignant, intermediate, and mild) of the disease with a variety of subtypes. This classification allows prognostic information to be provided to the parent or patient, improving decision making concerning the appropriate therapy. The genetics of osteopetrosis has become clearer with the discovery of the etiology of the autosomal recessive disorders (severe) is related to defects in acid production and possibly, an increased bone formation. A defect in the *osteopetrosis associated transmembrane protein 1*, previously described in the grey lethal mouse (Ramirez *et al.*, 2004) and in human patients with severe infantile osteopetrosis. Additional knowledge has been gained in understanding the milder forms. ADO II, the predominant type of the "Adult" or mild osteopetrosis, appears to be related primarily to chloride channel defects (Waguespack *et al.*, 2003). Cases of intermediate severity

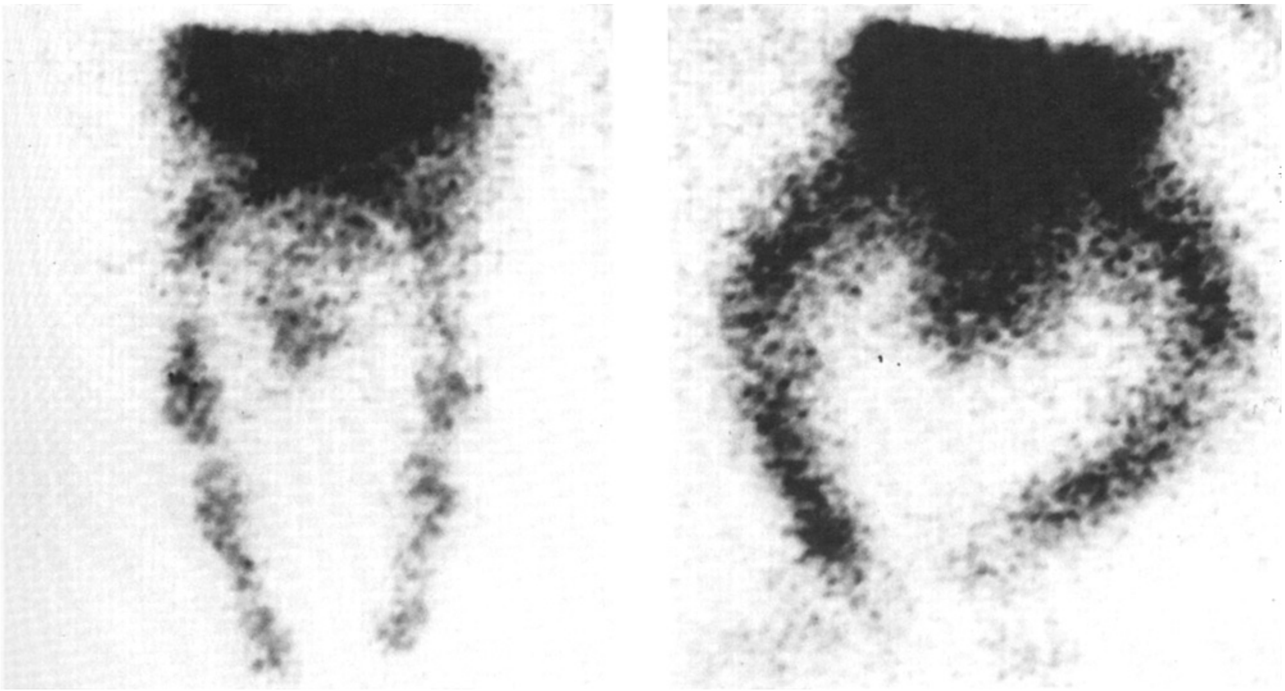

FIGURE 8 f0080^{999}Tc-labeled sulfur colloid bone marrow scans are shown from the same patient whose biopsies are shown in Fig. 7. The initial scan (*left*) shows abnormal uptake of label in the long bones of the lower extremities. Areas with little or no radionuclide are seen in the diaphyses with the uptake of the radionuclide being primarily concentrated to the subepiphyseal regions. (*Right*) After interferon γ-1b therapy, there is an increase in the amount of radionuclide labeling and there are no longer any discreet areas with reduced uptake.

also result from mutations in low-density lipoprotein receptor-related protein 5 (van Wesenbeeck *et al.*, 2003) and Plekstrin Homology Domain-containing Family M 1 (PLEKHM 1) (van Wesenbeeck *et al.*, 2007). Finally, treatment is largely still directed toward enhancing osteoclastic function in general rather than correcting specific defects. Based on the work of Karsdal *et al.* (2007) and del Fattore *et al.* (2006), we speculate that strategies for decreasing bone formation may also improve bone density and severity. Bone marrow transplantation remains the main hope for complete cure. Until now we have focused solely on osteoclastic precursors. Attention should be directed to stromal elements of marrow cells and to osteoblasts to examine the role that osteoblastic bone formation contributes to osteopetrosis. Through this route of investigation, the contribution of bone resorption in creating excessive bone formation can be explored and exploited for a more complete cure. The introduction of calcitriol, M-CSF, and interferon-γ-1b**,** along with inhibitors of bone formation may provide promise for the future survival and hopefully, the cure of osteopetrosis. In an article by Iacobini *et al.* (2001) a patient was treated with prednisone at 2mg/kg/day with apparent resolution of osteopetrosis. This may be similar to the "transient" form of osteopetrosis, but it also could be related to the inhibition of cartilage and bone formation by high-dose steroids (van der Eerden *et al.*, 2007). Thus, treatment with osteoclastic stimulators, such as high-dose calcitriol (1–1.5 μg/kg/day) and prednisone (1–2 mg/kg/day) could provide an excellent regimen. As in the past, this very interesting family of disorders continues to lead the way in developing our understanding of osteoclastic function., and now appears to be important in our understanding of formation–resorption coupling.

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Chapter 73

Hypophosphatasia

Nature's Window on Alkaline Phosphatase Function in Humans

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INTRODUCTION

Alkaline phosphatase (ALP) was discovered by Robert Robison in 1923 (Robison, 1923). From that time, he advanced the hypothesis that this enzyme functions critically in skeletal mineralization, emphasizing the liberation of inorganic phosphate (Pi) for hydroxyapatite crystal formation (Robison, 1932).

Beginning in the 1930s, physicians appreciated that significant clinical insight can come from measuring ALP activity in serum, because elevated levels (hyperphosphatasemia) usually indicate skeletal or hepatobiliary disease (Wolf, 1978). Since then, quantitation of serum ALP activity has been routine in clinical laboratories, and perhaps it is still (McComb *et al.*, 1979) the most frequently performed enzyme assay.

Although we now understand that ALP is ubiquitous throughout Nature (McComb *et al.*, 1979; Harris, 1990; Moss, 1992; Whyte, 1994), there is considerable uncertainty about the physiological function(s) of the four ALP isoenzymes in humans (Millán, 2006). Nevertheless, we have proof of Robison's hypothesis, showing that the "tissue nonspecific" isoenzyme of ALP (TNSALP) is essential for skeletal mineralization. Confirmation came from the discovery (Rathbun, 1948) and subsequent investigation of people with the inborn error of metabolism, "hypophosphatasia" (HPP) (Whyte, 1994, 2001).

As reviewed in this chapter, documentation beginning in 1988 that HPP is caused by loss-of-function mutations in *TNSALP,* the gene that encodes the TNSALP isoenzyme, confirmed Robison's hypothesis. However, the pathogenesis of the defective skeletal mineralization in HPP seems primarily to result from deficient TNSALP hydrolysis of inorganic pyrophosphate (PP_i), an inhibitor of mineralization (Weiss *et al.* 1988a; Whyte, 1994). Following a brief history of the proposed function of ALP in humans, and review of the molecular and biological chemistry of human ALPs, HPP is described in some detail in this chapter, and the insights gained from this "experiment-of-nature" are discussed. Then, refinements concerning the role of TNSALP obtained from knockout mouse studies are summarized (Millán, 2006).

HISTORY AND PROPOSED PHYSIOLOGICAL ROLE OF ALKALINE PHOSPHATASE

In 1923, Robert Robison (Fig. 1) discovered that there was considerable phosphatase activity in the bone and cartilage of growing rats and rabbits (especially those with rickets) and suggested that this new enzyme might act in skeletal mineralization by hydrolyzing hexosephosphoric esters to increase local concentrations of P_i (Robison, 1923). In 1924, with K. Soames, Robison reported that this phosphatase precipitated mineral into rachitic rat bone when monophosphate esters were the only source of P_i, and demonstrated that the enzyme had a distinctly alkaline pH optimum *in vitro* (Robison and Soames, 1924). However, Robison knew the apparent pH optimum for ALP in his laboratory was not physiological, and never referred to his discovery as "alkaline phosphatase" (Robison, 1932); the term was introduced by others (McComb *et al.*, 1979). Robison called the enzyme "bone phosphatase" (Robison, 1932). Soon after the identification and early characterization of ALP, evidence emerged that questioned Robison's hypothesis (McComb *et al.*, 1979). ALP was found to be abundant in tissues that normally do not calcify (e.g., liver, intestine, and placenta). Furthermore, extracellular fluids were supersaturated with calcium and P_i, and Robison had not isolated the enzyme's physiological substrate(s) (Neuman and Neuman, 1957). Instead, other biological purposes for ALP were proposed (see later).

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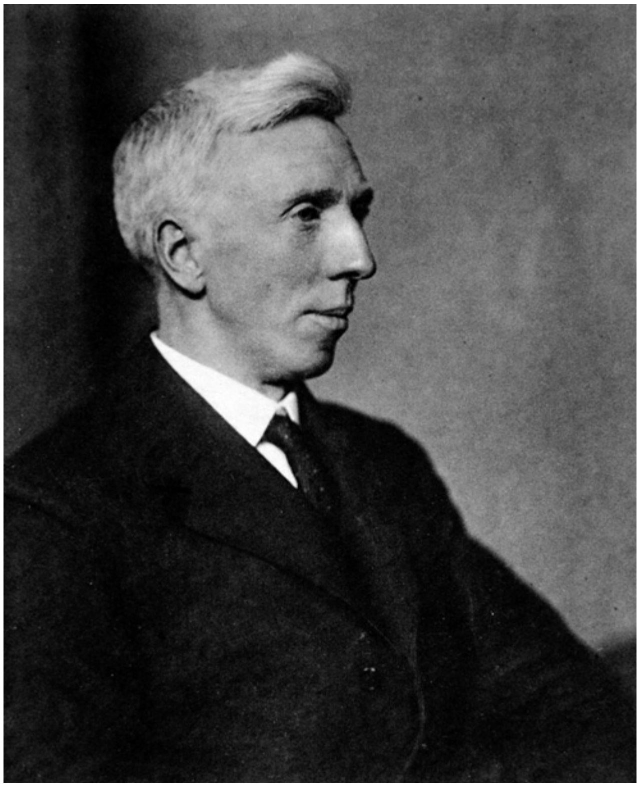

FIGURE 1 Robert Robison, Ph.D., D.Sc., F.R.S. (1883–1941), who discovered alkaline phosphatase in 1923 (reproduced with permission from the Godfrey Argent Studio, as published in Obituary Notices of Fellows of The Royal Society, Vol. 3, 1941, p. 929).

By the end of the 1960s, however, electron microscopy helped rejuvenate Robison's hypothesis when the earliest site of hydroxyapatite crystal deposition in the skeleton was discovered by H. C. Anderson to be unique, extracellular structures he called "matrix vesicles" (MVs) (Anderson, 1969). MVs seem to be buds of the plasma membrane of chondrocytes and osteoblasts, and are especially rich in ALP (Ali, 1986). They possess many enzyme activities [including inorganic pyrophosphatase (PP_i-ase) and ATPase], as well as phospholipids, polysaccharides, and glycolipids (Anderson, 1992). During the earliest ("primary") phase of skeletal mineralization, hydroxyapatite crystals appear and then grow within MVs. When the MV ruptures, extravesicular ("secondary") mineralization proceeds as hydroxyapatite crystal propagation continues with their eventual deposition into skeletal matrix (Ornoy *et al.*, 1985). In 1975, nucleoside phosphate (released by dying cells) was proposed as the substrate source for P_i necessary to fulfill Robison's hypothesis (Majeska and Wuthier, 1975).

Further evidence that ALP functions in skeletal mineralization emerged from studies using stereospecific inhibitors of ALP activity, such as L-tetramisole, which block calcification i*n vitro* (Fallon *et al.*, 1980). However, the noninhibitory stereoisomers were later shown to also impair mineralization (Whyte, 1994).

TABLE I Suggested Roles for ALP in Skeletal Mineralization

Locally increase P_i levels
Destruction of inhibitors of hydroxyapatite crystal growth
Transport of P_i
Ca^{2+}-binding protein (Ca^{2+} uptake by cells)
Ca^{2+}/Mg^{2+}-ATPase
Tyrosine-specific phosphoprotein phosphatase

Reproduced with permission from M. P. Whyte (1989). In "*Bone and Mineral Research*" (W. A. Peck, *ed.*), *Vol.* 6, *pp.* 175–218. *Elsevier Science Publishers* BV (*Biomedical Division*), *Amsterdam*.

By the 1990s, the proposed biological roles for ALP were many (McComb *et al.*, 1979; Harris, 1990; Moss, 1992; Whyte, 1989, 1994) and included hydrolysis of P_i esters to provide the non-P_i moiety, transferase action for the synthesis of P_i esters, regulation of P_i metabolism, maintenance of steady-state levels of phosphoryl metabolites, and action as a phosphoprotein phosphatase (McComb *et al.*, 1979; Alpers *et al.*, 1990; Harris, 1990; Muller *et al.*, 1991; Simko, 1991; Moss, 1992; Whyte, 1994). At cell membranes, ALP perhaps conditioned the active transport of P_i, but also calcium, fat, protein, carbohydrate, and Na^+/K^+(Muller *et al.*, 1991; Simko, 1991). Sequence analyses of ALPs suggested they coupled to other proteins, including collagen (Wu *et al.*, 1992) (see later). In the placenta, ALP was found to bind the Fc receptor of IgG where perhaps it transcytosed this immunoglobulin (Makiya *et al.*, 1992). During embryogenesis, ALP seemed to act intracellularly (Narisawa *et al.*, 1992), although we now know that ALP functions primarily on cell surfaces (see below).

Additional proposals had emerged for an ALP role in skeletal mineralization (Table I) (Whyte, 1989, 1994). Perhaps ALP was a plasma membrane transporter for P_i (Wuthier and Register, 1985), an extracellular Ca^{2+}-binding protein that stimulates calcium-P_i precipitation and orients mineral deposition into osteoid (DeBarnard *et al.*, 1986), a Ca^{2+}/Mg^{2+}-ATPase (Birge and Gilbert, 1974), or a phosphoprotein phosphatase that conditions skeletal matrix for ossification (Lau *et al.*, 1985; Tsonis *et al.*, 1988). Furthermore, it was possible that certain structural domains enabled ALP to bind to types I, II, and X collagen in cartilage and bone (Tsonis *et al.*, 1988; Wu *et al.*, 1992). However, an early theory gained preeminence; i.e., ALP hydrolyzes an inhibitor of calcification (Neuman and Neuman, 1957; Caswell *et al.*, 1991; Moss, 1992; Whyte, 1994; Millán, 2006). The principal candidate for such inhibition was inorganic pyrophosphate (PP_i). High concentrations of PP_i impair hydroxyapatite crystal growth, and ALP can hydrolyze PP_i (Moss *et al.*, 1967). In fact (see later), plasma and urine levels of PP_i are increased in HPP (Russell, 1965; Russell *et al.*, 1971).

Ironically, approaching almost a century after its discovery, current methods used to assay ALP activity do not capture Robison's understanding of the physiological role of this enzyme (McComb *et al.*, 1979; Harris, 1990; Moss, 1992; Whyte, 1994; Coburn *et al.*, 1998). In both clinical and research laboratories, to maximize catalytic activity, ALP continues to be measured using distinctly nonphysiological alkalinity (e.g., pH 9.2 to 10.5) and high concentrations (millimolar) of artificial substrates whose products can be detected colormetrically (e.g., *p*-nitrophenylphosphate) (McComb *et al.*, 1979). Furthermore, biological specimens for analysis are diluted into buffers without P_i, although P_i competitively inhibits ALP (Coburn *et al.*, 1998). These assays were devised especially for their utility in clinical medicine (Wolf, 1978), yet it has been known for decades that the pH optimum for ALP is considerably less alkaline for lower concentrations of potential physiological substrates, although hydrolytic rates are reduced (McComb *et al.*, 1979; Moss, 1992). Until the discovery of several natural substrates for TNSALP in studies of HPP, the significance of this observation was unknown (McComb *et al.*, 1979; Harris, 1990; Moss, 1992; Whyte, 1994).

To understand HPP and to appreciate how this disorder teaches us about ALP, it is first necessary to briefly review ALP genomic structure and protein chemistry.

GENOMIC STRUCTURE AND PROTEIN CHEMISTRY OF ALKALINE PHOSPHATASE

ALP (orthophosphoric-monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1) is found throughout Nature in plants and animals (McComb *et al.*, 1979). In humans, four ALP isoenzymes are encoded by four separate genes (Millán, 1988, 2006; Harris, 1990; Moss, 1992). Three of the isoenzymes are expressed in essentially a tissue-specific distribution and are called intestinal, placental, and germ-cell (placental-like) ALP. The fourth ALP isoenzyme is ubiquitous in the body, and therefore designated tissue-nonspecific ALP (TNSALP) (Stigbrand and Fishman, 1984; Harris, 1990; Moss, 1992). Hepatic, skeletal, and renal tissue is especially rich in TNSALP. The gene mapping symbol for TNSALP is *ALPL* ("ALP-liver"), although the function of the bone, but not liver, isoform is known (see later). The distinctive physicochemical properties (e.g., heat stability, electrophoretic mobility, etc.) among the ALPs purified from human liver, bone, and kidney are lost upon exposure to glycosidases (Moss and Whitaker, 1985). In fact, the TNSALPs are a family of "secondary" isoenzymes (isoforms) with the identical polypeptide sequence encoded by the same gene, and differ only by post-translational modifications involving carbohydrates (Harris, 1980).

The TNSALP gene is located near the tip of the short arm of chromosome 1 (1*p*36.1–*p*34), whereas the genes for intestinal, placental, and germ-cell ALP are at the tip of the long arm of chromosome 2 (2*q*34–*q*37) (Harris, 1990; Millán, 2006). *TNSALP* seems to represent the ancestral gene, whereas the tissue-specific ALPs were likely formed by gene duplication (Harris, 1990).

TNSALP is somewhat larger than 50 kb and contains 12 exons, 11 of which are translated to form the mature enzyme consisting of 507 amino acid residues (Weiss *et al.*, 1988b). TATA and Sp1 sequences may be regulatory elements, but basal expression seems to reflect "housekeeping" promoter effects, whereas differential expression in various tissues may be mediated by a post-transcriptional mechanism (Kiledjian and Kadesch, 1990); the 5′-untranslated region differs between bone and liver TNSALP (Nosjean *et al.*, 1997). *TNSALP* has two promoters and two corresponding 5′ noncoding exons, 1a and 1b. Expression from each promoter results in the use of either exon 1a or 1b, resulting in two different mRNAs with differing 5′-untranslated regions. Transcription from the upstream promoter (1a) is preferentially used in osteoblasts, whereas the downstream promoter (1b) is preferentially used in liver and kidney (Millán, 2006).

The tissue-specific ALP genes are smaller than *TNSALP*, primarily owing to shorter introns. Amino acid sequences deduced from their cDNAs suggest 87% positional identity between placental and intestinal ALP, but only 50% to 60% identity between the tissue-specific ALPs and TNSALP (Harris, 1990).

The cDNA sequence of TNSALP reveals five potential N-linked glycosylation sites (Weiss *et al.*, 1988). *N*-Glycosylation is necessary for catalytic activity. *O*-Glycosylation characterizes the bone, but not the liver, isoform (Nosjean *et al.*, 1997).

In 2000, the crystal structure for human placental ALP was detailed at 1.8-Å resolution (Le Due *et al.*, 2000). The active site of TNSALP would be encoded by six exons, comprising 15 amino acid residues (Zurutuza *et al.*, 1999), and feature a nucleotide sequence conserved in ALPs throughout nature (Henthorn and Whyte, 1992).

The ALPs are Zn^{2+}-metalloenzymes (McComb *et al.*, 1979). Catalytic activity requires a multimeric configuration of identical subunits with each monomer having one active site and two Zn^{2+} atoms that stabilize the tertiary structure (Kim and Wycoff, 1991).

The ALPs are generally considered homodimeric in the circulation (McComb *et al.*, 1979). TNSALP, in symmetrical dimeric form, has $\alpha\beta$ topology for each subunit with a ten-stranded β-sheet at its center (Hoylaerts and Millán, 1991). However, in tissues, ALPs are tethered (see later) to cell surfaces, probably as homotetramers (Fedde *et al.*, 1988).

ALPs exhibit broad substrate specificities and pH optima *in vitro* depending on the type and concentration of phosphocompound undergoing catalysis (McComb *et al.*, 1979). Catalytic activity requires Mg^{2+} as a cofactor (McComb *et al.*, 1979). PP_i as well as phosphoesters can be hydrolyzed (Xu *et al.*, 1991). The reaction involves phosphorylation-dephosphorylation of a serine residue. Dissociation of the covalently linked P_i seems to be the rate-limiting step. In fact, P_i is a potent competitive inhibitor of ALP (McComb *et al.*, 1979; Kim and Wyckoff, 1991; Coburn *et al.*, 1998). However, P_i may stabilize the enzyme (Farley, 1991).

Uncertainties remain about the biosynthesis of ALP in higher organisms. Analysis of the gene sequences of the human ALP isoenzymes indicates that the nascent polypeptides have a short signal sequence of 17 to 21 amino acid residues (Harris, 1990), and a hydrophobic domain at their carboxyl terminus (Weiss *et al.*, 1988b). ALPs link to the plasma membrane surface, tethered to the polar head group of a phosphatidylinositol-glycan moiety (Whyte *et al.*, 1988; Whyte, 1994). They can be released by phosphatidylinositol-specific phospholipase (Fedde *et al.*, 1988). However, the precise interaction with phosphatidylinositol may differ among the ALP isoenzymes (Seetharam *et al.*, 1987). Intracellular degradation of ALPs can involve proteosomes (Cai *et al.*, 1998).

Lipid-free ALP is the moiety normally found in the circulation. Yet, the mechanism(s) for ALP release from cell surfaces is not known. The process could involve a phosphatidase of the C or D type, detergent action, proteolysis, membrane fractionation, or lipolysis (Alpers *et al.*, 1990).

In healthy men and women, nearly all ALP in serum or plasma consists of approximately equal amounts of the bone and liver isoforms of TNSALP (Millán *et al.*, 1980). Infants and children, and particularly newborns and adolescents, have higher circulating levels of the bone isoform (McComb *et al.*, 1979). Some individuals (with B and O blood types who are "secretors") increase intestinal ALP in the circulation after ingesting a fatty meal (Langman *et al.*, 1966; McComb *et al.*, 1979). Intestinal ALP usually contributes just a few percent to serum total ALP levels (maximum 20%) (McComb *et al.*, 1979; Mulivor *et al.*, 1985). Placental ALP is typically expressed only during the last trimester of pregnancy (Birkett *et al.*, 1966). Various cancers, however, release placental or germ-cell ("placental-like") ALP (Millán, 1988) into the blood. Clearance of circulating ALP, as for many other glycoproteins, is probably caused by uptake and degradation by the liver (Young *et al.*, 1984).

HYPOPHOSPHATASIA

Subnormal extracellular levels of calcium and P_i, or P_i alone, cause nearly all forms of rickets or osteomalacia (Whyte, 2002). HPP is a remarkably instructive exception. In HPP, circulating levels of calcium and P_i are usually normal or elevated, yet the skeleton does not mineralize properly (Whyte, 1994, 2001). HPP has been "Nature's window" for understanding the physiological role of TNSALP in humans. With the discovery, beginning in 1988 (Weiss *et al.*, 1988a), of loss-of-function mutations in TNSALP in HPP, Robison's hypothesis that ALP acts in biomineralization was confirmed. Additionally, this observation revealed that TNSALP was necessary for the development of primary teeth (Whyte, 1994). However, undisturbed function in HPP of other organs/tissues, especially the liver, questioned the biological significance for TNSALP elsewhere (Whyte, 1994, 2001).

History

John C. Rathbun (Fig. 2), a Canadian pediatrician, coined the term "hypophosphatasia" in 1948 when he reported an infant boy who died of seemingly acquired rickets complicated by epilepsy whose ALP activity in serum, bone, and other tissues was paradoxically subnormal (Rathbun, 1948). Approximately 300 cases of HPP have now been described in the medical literature (Whyte, 1994, 2001). A PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez/) search from 1955 onward shows 417 articles where

FIGURE 2 John C. Rathbun, M.D. (1915–1972) who characterized hypophosphatasia in 1948.

"hypophosphatasia" is in the title, and 648 where it appears in the text.

Discussed in detail subsequently, our understanding of both the metabolic basis for HPP and the physiological function of TNSALP were advanced by the discoveries of elevated levels of three phosphocompounds in affected individuals. In 1955, in the era of paper chromatography to diagnose inborn errors of metabolism, identification of increased amounts of phosphoethanolamine (PEA) in urine provided a second biochemical marker for HPP in addition to hypophosphatasemia (Fraser *et al.*, 1955; McCance *et al.*, 1955). In 1965 and 1971, respectively, discovery of elevated levels of PP_i in the urine (Russell, 1965) and plasma (Russell *et al.*, 1971) of HPP patients would reveal the mechanism for the characteristic defective skeletal mineralization, because PP_i was increasingly documented to inhibit this process (Heinonen, 2001). In 1985, identification of high plasma concentrations of pyridoxal 5′-phosphate (PLP) in HPP, together with understanding of vitamin B_6 metabolism, revealed an ectoenzyme function for TNSALP, and explained how these three phosphocompounds could accumulate extracellularly in this "inborn-error-of-metabolism" (Whyte *et al.*, 1985).

Clinical Features

Hypophosphatasia (OMIM numbers 146300, 171760, 241500, and 241510) has been reported throughout the world and seems to affect all races (Whyte *et al.*, 2006). However, it is especially prevalent in Mennonites in Manitoba, Canada, where about 1 in 25 individuals is a carrier, and 1:2500 newborns manifests severe disease (Greenberg *et al.*, 1993). Canadian Hutterites also manifest a relatively high prevalence of HPP. In Toronto, Canada, the incidence for severe forms of HPP was estimated in 1957 to be 1 per 100,000 live births (Fraser, 1957). Conversely, HPP seems to be particularly rare in people of black ancestry in the United States (Whyte *et al.*, 2006).

Despite relatively high levels of TNSALP in bone, cartilage, liver, kidney, and adrenal tissue (and at least some TNSALP ubiquitously) in healthy individuals (McComb *et al.*, 1979), HPP seems to directly disrupt only the formation of the skeleton and dentition (Whyte, 2001). Discussed later, vitamin B_6-dependent seizures are a further metabolic consequence of severe HPP (Baumgartner-Sigl *et al.*, 2007).

One of the most remarkable features of HPP is the extraordinarily wide-ranging expressivity of the disease, spanning from death *in utero* with nearly an unmineralized skeleton to early shedding of "baby teeth" without skeletal disturbances (Fraser, 1957; Whyte, 2001). In fact, some individuals with the biochemical characteristics of HPP and one defective *TNSALP* allele, seem to escape symptoms or complications and can be considered "carriers" (Whyte *et al.*, 1982a).

Many (≈200) different deactivating mutations in *TNSALP*, usually transmitted in various combinations to compound heterozygotes by autosomal recessive inheritance (see later), have been documented in HPP (Weiss *et al.*, 1988; Henthorn *et al.*, 1992; Mornet *et al.*, 1998; Whyte, 2000; Mumm *et al.*, 2002). Understandably, therefore, a TNSALP mutation-based nosology for clinical characterization and prognostication would be extremely challenging, especially for severe instances of this rare disorder (Whyte, 2001). Furthermore, it is increasingly apparent that other genetic and nongenetic factors can significantly condition HPP expressivity. This fact is illustrated by the variable HPP severity and complications noted among siblings sharing identical TNSALP defects (Henthorn *et al.*, 1992; Whyte *et al.*, 2006; Mumm *et al.*, 2006) (Fig. 3). Accordingly, the prevailing classification scheme for patients (Whyte, 2001) remains a clinical one based on the proposals of Fraser in 1957 (Fraser, 1957).

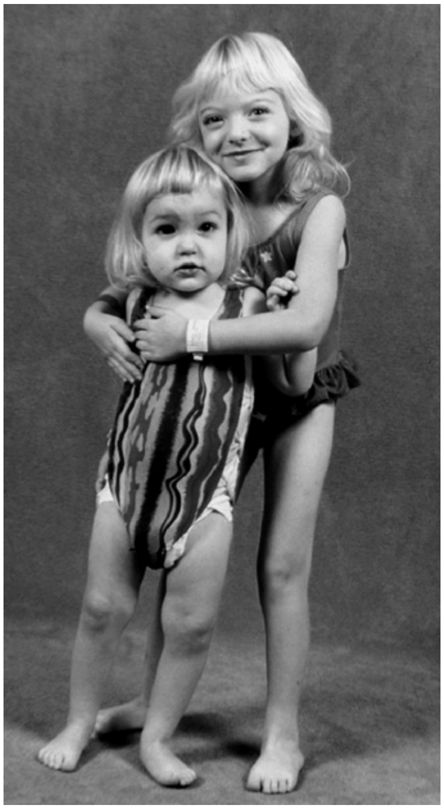

FIGURE 3 Variable clinical expressivity of hypophosphatasia is exemplified by these sisters who are compound heterozygotes sharing the same two *TNSALP* missense mutations. The proposita (*Right*) at age 5–3/12 years survived infantile HPP featuring poor weight gain, hypercalcemia with nephrocalcinosis, and severe rickets (Barcia *et al.*, 1997) that was followed by short stature, premature loss of teeth, and craniosynostosis. Her younger sister (*Left*) at age 2–2/12 years appears well and has mild rickets despite very similar hypophosphatasemia and endogenous elevations of the TNSALP substrates.

Five principal forms of HPP are usually discussed. The age at which HPP is diagnosed because of skeletal disease distinguishes the perinatal, infantile, childhood, and adult types (Fraser, 1957; Whyte, 2001). Individuals who do not have skeletal disease, but instead manifest dental features only, are said to have "odontohypophosphatasia" (Whyte, 2001). However, this nosology does not unambiguously classify all patients, and it is better to appreciate that HPP manifests a continuum of expressivity. An extremely rare form of HPP called "pseudohypophosphatasia" resembles infantile HPP except that serum ALP activity is not subnormal, but instead normal or increased, in the clinical laboratory (see later) (Scriver and Cameron, 1969; Whyte, 2001). A "benign *in utero*" form of HPP, characterized in 1999 from fetal sonography, demonstrates that skeletal deformity *in utero* owing to HPP may not always predict a lethal outcome (Moore *et al.*, 1999; Pauli *et al.*, 1999).

The prognoses for the five major forms of HPP are determined by the severity of the skeletal disease. Typically, the earlier the skeletal signs and symptoms, the worse the outcome (Fraser, 1957; Whyte, 2001).

Perinatal Hypophosphatasia

This most severe form of HPP, defined by generalized skeletal disease obvious in a neonate, manifests *in utero* with profound hypomineralization of cartilage and bone and typically causes death at, or soon after, birth. Remarkable skeletal undermineralization results in caput membraneceum and limbs that are shortened and deformed. Some affected newborns survive a few days or weeks, but then suffer increasing respiratory compromise owing to rachitic disease of the chest and possibly lungs that are hypoplastic (Silver *et al.*, 1988). There may be vitamin B_6-dependent seizures (Baumgartner-Sigl *et al.*, 2007). Myelophthisic anemia perhaps follows encroachment on the marrow space by excessive osteoid (Terheggen and Wischermann, 1984). Extended survival is very rare.

Skeletal radiographs (Fig. 4) readily distinguish perinatal HPP from even the most severe forms of osteogenesis imperfecta or congenital dwarfism, and the findings may be considered diagnostic. Nevertheless, there is considerable patient-to-patient variability (Shohat *et al.*, 1991). In some stillborns, the bones are nearly devoid of mineral (see Fig. 4A). In others, there are severe rachitic changes (see Fig. 4B). Occasionally, individual vertebrae appear to be completely or partially missing (Shohat *et al.*, 1991). In the skull, these membranous bones may calcify only at their centers, giving the illusion that the cranial sutures are widely separated ("open"), although they may be functionally closed (see Fig. 4C). Other unusual radiographic features (Whyte, 1988) include bony protrusions (Bowdler spurs) extending from the midshafts of the ulnas and fibulas (see Fig. 4D).

Infantile Hypophosphatasia

This form of HPP was described by Rathbun (Rathbun, 1948). Skeletal disease manifests after birth, but before 6 months of age (Fraser, 1957; Whyte, 2001). Development may seem normal until there is poor feeding, hypotonia, and inadequate weight gain. At this time, the clinical manifestations of rickets appear, leading to the diagnosis. Vitamin B_6-dependent epilepsy can be the first manifestation of infantile HPP and predicts a lethal outcome (Baumgartner-Sigl *et al.*, 2007). A flail chest from rib fractures, rachitic deformity, etc., often leads to pneumonia. Hypercalcemia and hypercalciuria are common, and may explain episodes of recurrent vomiting, as well as nephrocalcinosis and renal compromise (Fraser, 1957; Whyte *et al.*, 1982b).

Although the striking radiographic features of the skeletal disease of infantile HPP are diagnostic (Fig. 5A), they are less severe than in perinatal HPP. Radiographs may suggest that cranial sutures are wide open, but this is an illusion from hypomineralization of the skull, and there can instead be "functional" craniosynostosis. Later, true, early sutural fusion can occur if the patient survives infancy, causing symptoms and complications of craniosynostosis (see Fig. 5B). In some babies, abrupt transition from relatively normal-appearing diaphyses to poorly mineralized metaphyses (see Fig. 5C) suggests that metabolic deterioration has occurred suddenly (Fraser, 1957). This observation is supported by the acquired hypercalciuria and hypercalcemia that can develop in this form of HPP. Serial radiographs may reveal not only impaired skeletal mineralization (i.e., rickets), but also gradual, generalized demineralization of osseous tissue and fractures (Whyte *et al.*, 1982b) indicating a lethal outcome (Whyte *et al.*, 2003; Cahill *et al.*, 2007) (see Fig. 5D). Alternatively, there can be spontaneous but unexplained improvement (Ish-Shalom *et al.*, 1986).

Childhood Hypophosphatasia

This form of HPP has especially variable expressivity (Fallon *et al.*, 1984; Whyte, 2001). The diagnosis is made because of bone disease discovered after 6 months of age, but before skeletal maturity. In 1953, premature loss of deciduous teeth was found to be a major clinical feature (Sobel *et al.*, 1953). Early shedding of "baby" teeth (i.e., at less than 5 years of age) results from hypoplasia or aplasia of dental cementum (Van den Bos *et al.*, 2005). Consequently, the roots are not bound sufficiently by the periodontal ligament (Lundgren *et al.*, 1991) and entire teeth come out painlessly without trauma (Fig. 6A). The incisors are typically lost first, and occasionally almost the entire primary dentition is exfoliated prematurely. We have encountered early loss of at least one deciduous tooth in 98% of our pediatric patients with HPP (Whyte *et al.*, in

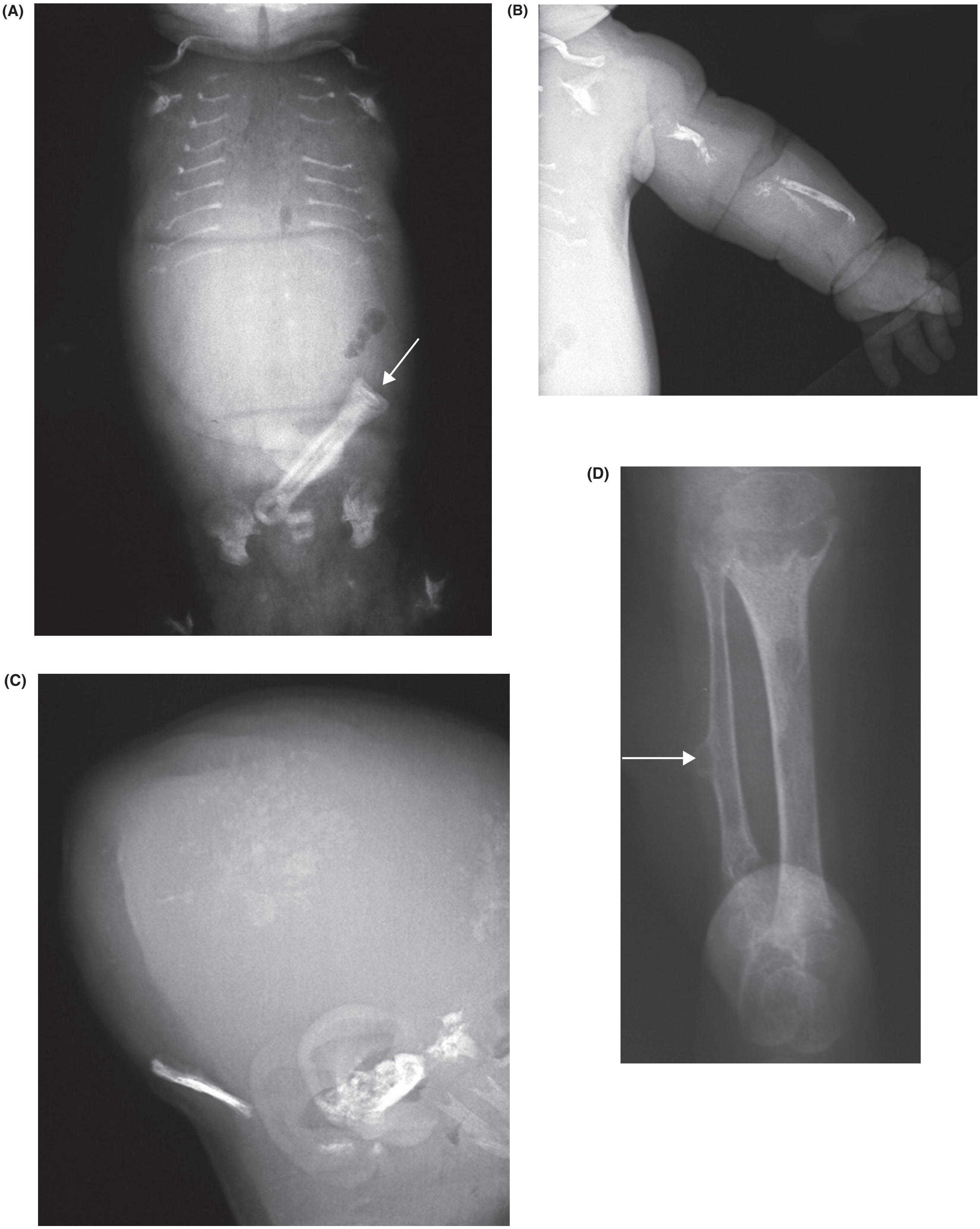

FIGURE 4 Perinatal hypophosphatasia. (**A**) Profound skeletal hypomineralization is obvious at birth (arrow points to umbilical cord clip). (**B**) The ends of the upper extremity long bones at 1 day of age show characteristic, extreme, rachitic changes. (**C**) Severe hypomineralization of the calvarium is present at 1 day of age. (**D**) A Bowdler spur (arrow) is found in some patients.

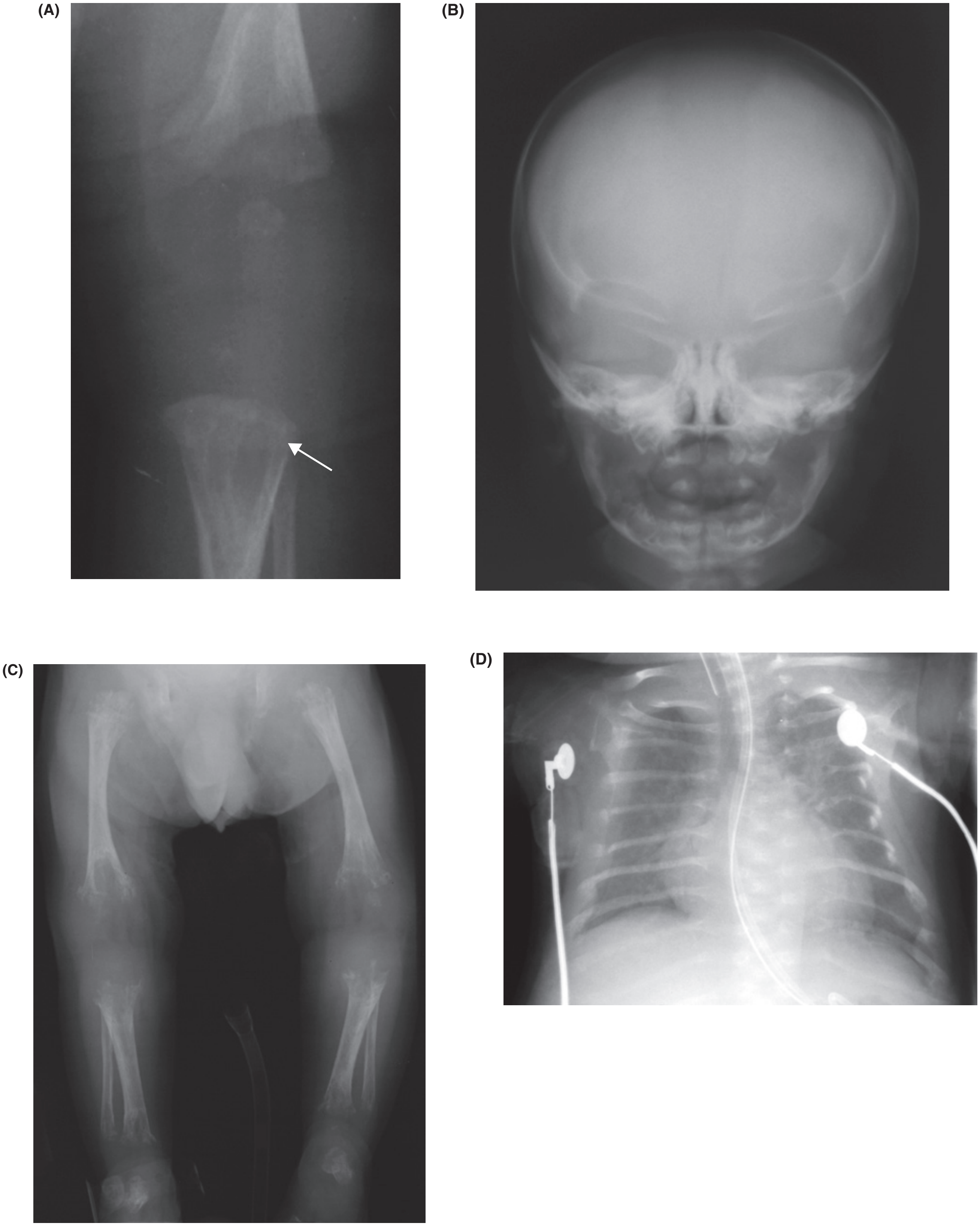

FIGURE 5 Infantile hypophosphatasia. (**A**) Characteristic tongues of radiolucency (arrow) extend from the growth plate into the metaphysis. (**B**) Cranial sutures appear widened in this hypomineralized skull at 1 month of age. (**C**) An abrupt transition seems to have occurred from well mineralized diaphyses to poorly mineralized metaphyses by age 3 months. (**D**) The "bell-shaped" configuration of the chest from a soft thorax together with rib fractures will predispose to respiratory disease at 23 months of age.

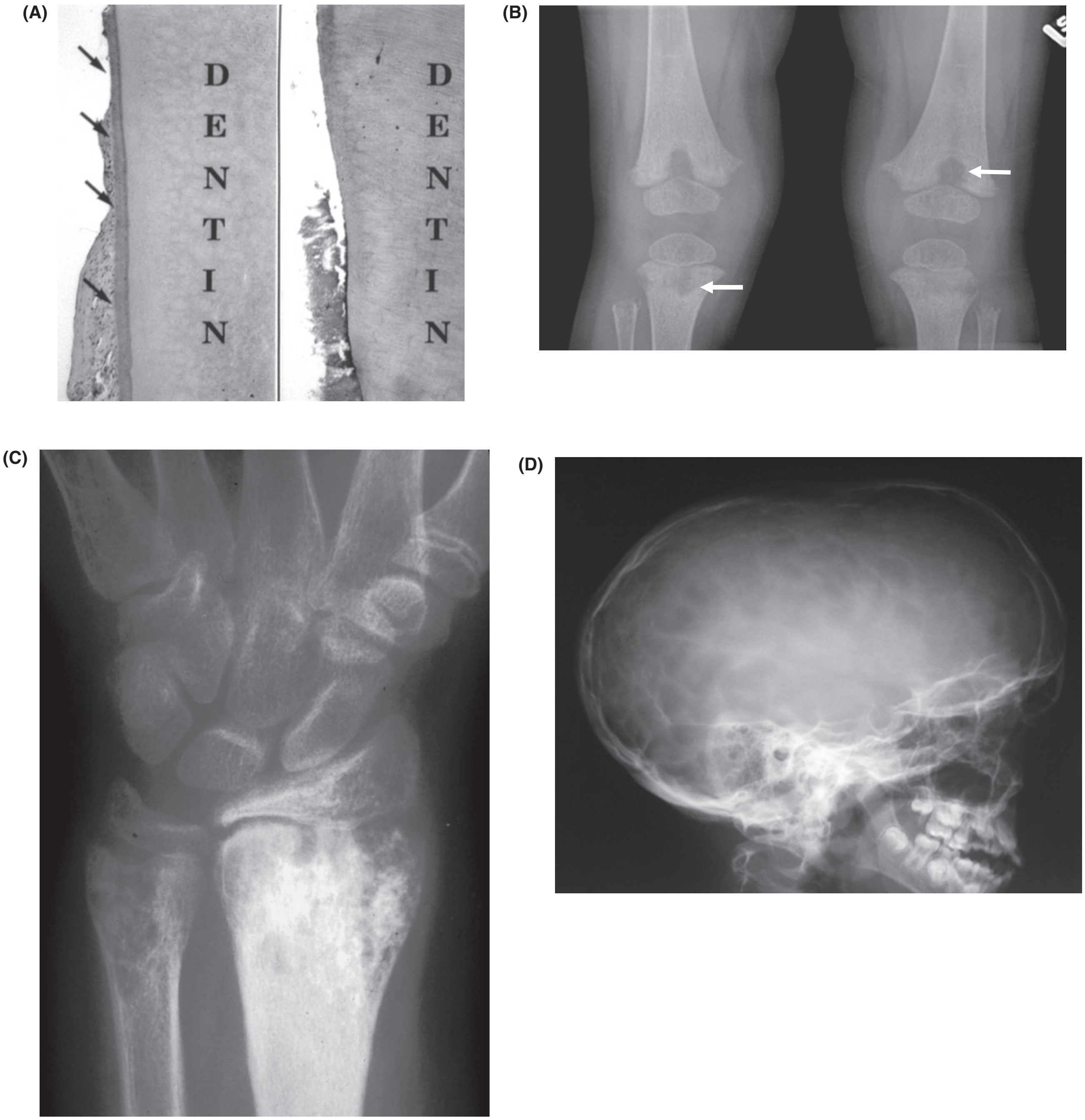

FIGURE 6 Childhood hypophosphatasia. (**A**) Dental findings. (*Left*) Decalcified section of part of the root of a maxillary incisor from a child with X-linked hypophosphatemia is essentially normal and shows primary cementum (delineated by arrows) at its surface (original magnification, ×150). (*Right*) In hypophosphatasia, cementum is absent. Original magnification, × 150. [Reproduced with permission from Whyte (2001).] (**B**) Characteristic tongues of radiolucency (arrows) project from growth plates into metaphyses at 16 months of age. (**C**) Idiopathic, patchy, metaphyseal osteosclerosis is a common finding. (**D**) A "beaten copper" appearance in the calvarium, here at age 11 years, signifies premature closure of cranial sutures (craniosynostosis) that can lead to raised intracranial pressure.

manuscript). Delayed walking and a characteristic waddling gait are also typical of childhood HPP. These problems seem to reflect the degree of skeletal disease. Patients can complain of stiffness and pain and have appendicular muscle weakness (especially in the thighs) consistent with a nonprogressive myopathy (Seshia *et al.*, 1990). There may also be delays in speech and language acquisition (Ruzicka *et al.*, in manuscript).

Radiographs usually show characteristic focal "tongues" of radiolucency that project from rachitic growth plates into the metaphysis (see Fig. 6B). There can be patchy areas of metaphyseal osteosclerosis (see Fig. 6C). True premature bony fusion of cranial sutures may raise intracranial pressure (see Fig. 6D). Dental radiographs sometimes show enlarged pulp chambers and root canals that characterize the "shell teeth" of rickets.

Adult Hypophosphatasia

This form of HPP typically presents after middle age (Whyte, 2001; Whyte *et al.*, 1982a). Not infrequently, however, patients recall being told of rickets and/or premature loss of their deciduous teeth in childhood. Then, there is relatively good health during young adult life (Weinstein and Whyte, 1981). Some of these individuals may have been considered "carriers" of HPP until they manifested the skeletal disease.

In adult HPP, osteomalacia often presents as recurrent, painful, poorly healing, metatarsal stress fractures (Fig. 7A) (Whyte *et al.*, 2007). With more advanced disease, persistent aching or tenderness in the thighs or hips is explained by femoral pseudofractures (see Fig. 7B) that will not heal spontaneously unless they progress to complete fractures (Khandwala *et al.*, 2006). Early loss or extraction of the secondary dentition is not uncommon, although what may be wrong with the adult teeth is not well understood (Whyte *et al.*, 1982a). Calcium pyrophosphate dihydrate (CPPD) crystal deposition, occasionally with overt attacks of arthritis (pseudogout), troubles some patients and stems from increased endogenous levels of PP_i (see later) (O'Duffy, 1970; Whyte *et al.*, 1982a). Some affected individuals suffer degeneration of articular cartilage and "pyrophosphate arthropathy" (Whyte *et al.*, 1982a). Radiographs may reveal osteopenia and chondrocalcinosis (Lassere and Jones, 1990; Whyte *et al.*, 1982a). In certain families with hypophosphatasemia, calcium phosphate deposition manifests as calcific periarthritis with ossification of ligaments (syndesmophytes) resembling spinal hyperostosis (Forestier's disease) (Lassere and Jones, 1990).

Odontohypophosphatasia

This form of HPP is diagnosed when there are dental manifestations, but radiographic or histological studies reveal no evidence of rickets or osteomalacia.

Pseudohypophosphatasia

This especially rare variant of HPP has been documented convincingly in two infants (Scriver and Cameron, 1969;

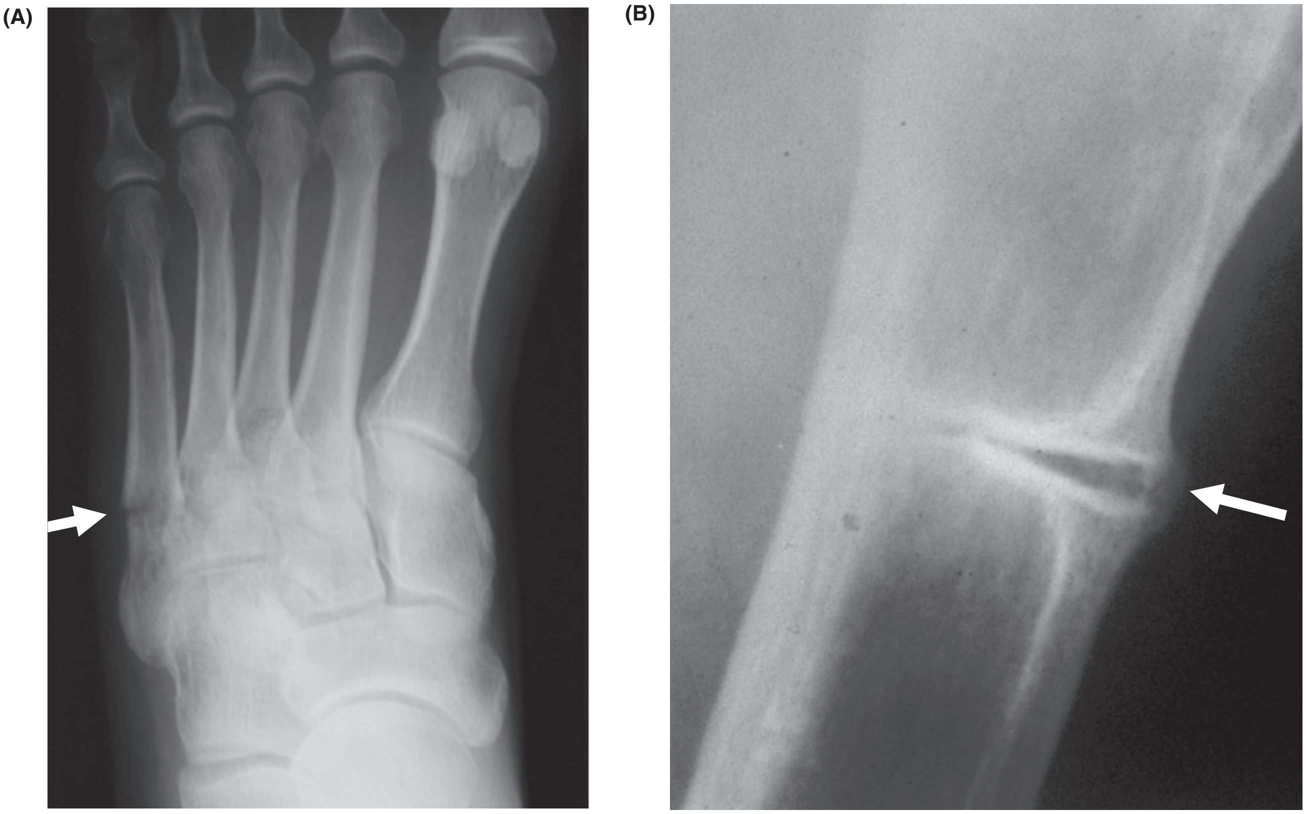

FIGURE 7 Adult hypophosphatasia. (**A**) Recurrent, poorly healing, metatarsal stress fractures (arrow) may be the most common skeletal manifestation of the adult form of hypophosphatasia. (**B**) Femoral pseudofractures (arrow) are often painful and characteristically occur on the lateral aspect proximally in adult hypophosphatasia. They do not heal spontaneously, unless a through-and-through break occurs.

Whyte, 2001). The clinical, radiographic, and biochemical findings are those of infantile HPP with one key exception—serum total ALP activity is not low, but instead consistently normal or increased (Whyte, 1994, 2001). The enzymatic defect causing pseudo-HPP involves a TNSALP with diminished hydrolysis for PEA, PP_i, and PLP endogenously, but normal or even increased catalysis for artificial substrates in the nonphysiological conditions of ALP assays used in clinical laboratories (see later) (Fedde *et al.*, 1990).

Benign *in Utero* Hypophosphatasia

Increasing use of sonography is revealing fetuses with bowing of major long bones that do not have a lethal form of HPP or skeletal dysplasia, but instead show spontaneous improvement in their deformities after birth and prove to have a relatively mild form of HPP (Moore *et al.*, 1999; Pauli *et al.*, 1999; Wenkert *et al.*, 2005)

Laboratory Diagnosis

Biochemical Findings

HPP can be diagnosed with confidence from a consistent medical history and physical findings, typical skeletal radiographic changes, and serum ALP activity that is subnormal for the patient's age (Whyte, 2001). Even individuals with odonto-HPP are distinguishable from healthy subjects by their low serum ALP activity, although values for odonto-HPP can approach the lower end of carefully established reference ranges (Fig. 8). In general, HPP severity correlates inversely with age-appropriate serum ALP activity. In perinatal and infantile HPP, hypophosphatasemia is detectable at birth in umbilical cord blood (Whyte, 2001).

Hypophosphatasemia can occur from other conditions (starvation, hypothyroidism, scurvy, severe anemia, Wilson's disease, celiac disease, multiple myeloma, hypomagnesemia, Zn^{2+} deficiency), certain drugs (glucocorticoids, clofibrate, chemotherapy, vitamin D intoxication, milk-alkali syndrome), and exposure to radioactive heavy metals or massive transfusion of blood or plasma (Macfarlane *et al.*, 1992). These clinical situations should, however, be readily recognized. Especially rare cases of lethal osteogenesis imperfecta (Royce *et al.*, 1988) and some infants with cleidocranial dysplasia (Unger *et al.*, 2002; Wycoff *et al.*, 2005) can also have hypophosphatasemia (apparently from little skeletal mass together with impaired cellular processing of bone ALP, or osteoblast hypofunction, respectively). Severe cleidocranial dysplasia is the most likely disorder to be confused with HPP (Unger *et al.*, 2002; Wycoff *et al.*, 2005).

Transient increments in serum ALP activity (probably the bone isoform of TNSALP) can occur in HPP after orthopedic surgery or significant fracture. In theory, circumstances that increase serum levels of any type of ALP (e.g., pregnancy, liver disease) could obscure the biochemical diagnosis of HPP. Thus, documenting hypophosphatasemia on more than one occasion during clinical stability seems advisable for the exceptional, confusing case. Quantitation in serum of the ALP isoenzymes (Mulivor *et al.*, 1985), isoforms of TNSALP (Whyte *et al.*, 1996), or leukocyte ALP (Iqbal *et al.*, 2000)—all available in clinical laboratories—may also be helpful.

Mineral homeostasis

Neither circulating calcium nor P_i levels are subnormal in HPP. In fact, in infantile HPP there may be hypercalcemia and hyperphosphatemia together with hypercalciuria (Fraser, 1957; Shohat *et al.*, 1991; Whyte *et al.*, 1982b). The disturbed mineral homeostasis is incompletely understood, but seems, in part, to be from impaired calcium and P_i uptake by a poorly growing skeleton, sometimes together with progressive bone demineralization. In childhood HPP, patients often manifest hypercalciuria, but usually without hypercalcemia. Serum levels of 25(OH)D,

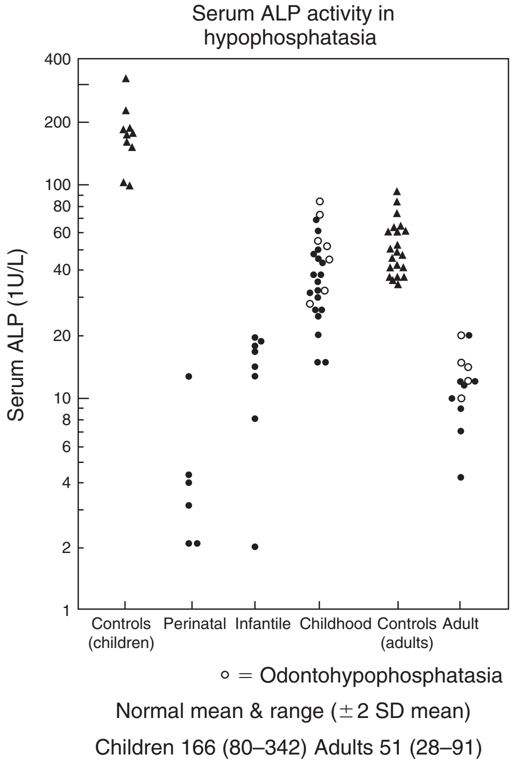

FIGURE 8 Serum ALP activity in hypophosphatasia. Serum total ALP activity in healthy children and adults and in 52 patients from 47 families with the various clinical forms of hypophosphatasia (note the logarithmic scale). All assays were performed at the Center for Metabolic Bone Disease and Molecular Research, Shriners Hospitals for Crippled Children, St. Louis, MO. [Reproduced with permission from Whyte (2001)].

$1,25(OH)_2D$, and parathyroid hormone (PTH) are typically normal (Whyte and Seino, 1982), unless altered physiologically by hypercalcemia or by renal failure (Fallon *et al.*, 1984). Several HPP patients have had elevated serum PTH levels, but impaired renal function (causing retention of immunoreactive PTH fragments) may have been the explanation (Whyte, 1994). Others had primary hyperparathyroidism for which there may be a predisposition in adult HPP (Whyte, 2001). Conversely, a low circulating level of intact PTH (likely from feedback of increased ionized calcium levels on the parathyroid glands) is not uncommon in our pediatric patients (unpublished work).

Individuals with the childhood or adult forms of HPP have serum P_i levels above average values for age-matched controls (Whyte, 2001). Indeed, ≈50% are distinctly hyperphosphatemic. Enhanced renal reclamation of P_i (increased TmP/GFR) underlies this poorly understood finding (Whyte and Rettinger, 1987). In some, but not all, instances, suppressed PTH seems contributory. Rarely, hypophosphatasemia is reported with hypophosphatemia from renal P_i wasting (Juan and Lambert, 1981).

Phosphoethanolamine

Elevated urine levels of PEA support a diagnosis of HPP (Rasmussen, 1968), but are not pathognomonic. Licata *et al.* (1978) demonstrated that phosphoethanolaminuria occurs in other conditions, including several metabolic bone diseases. Reference ranges for urine PEA vary according to age and somewhat by diet, and follow a circadian rhythm. PEA values can be unremarkable in mildly affected HPP patients. Age-adjusted normal ranges (expressed as micromoles of PEA per gram of creatinine) are: for less than 15 years, 83 to 222; for 15 to 30 years, 42 to 146; for 31 to 41 years, 38 to 155; and for more than 45 years, 48 to 93 (Licata *et al.*, 1978).

Pyridoxal 5′-phosphate

An increased plasma concentration of PLP is a sensitive marker for HPP (including pseudo-HPP) (Coburn and Whyte, 1988) (Fig. 9). Testing is available at several commercial laboratories, sometimes designated "vitamin B_6". Even patients with odonto-HPP manifest this biochemical finding (Whyte *et al.*, 1985). Vitamin supplements containing pyridoxine should not be taken, if possible, for one week before blood is obtained in order to avoid false-positive results. HPP disease severity correlates positively with the elevation in the plasma PLP concentration (Whyte, 2001). Nevertheless, values overlap between the different clinical types (see Fig. 9). Quantitation of plasma PLP after challenge with pyridoxine given orally once each day for six days seems to distinguish HPP patients especially well (Whyte, 1994), and identifies Canadian Mennonite carriers of severe HPP (Chodirker *et al.*, 1990).

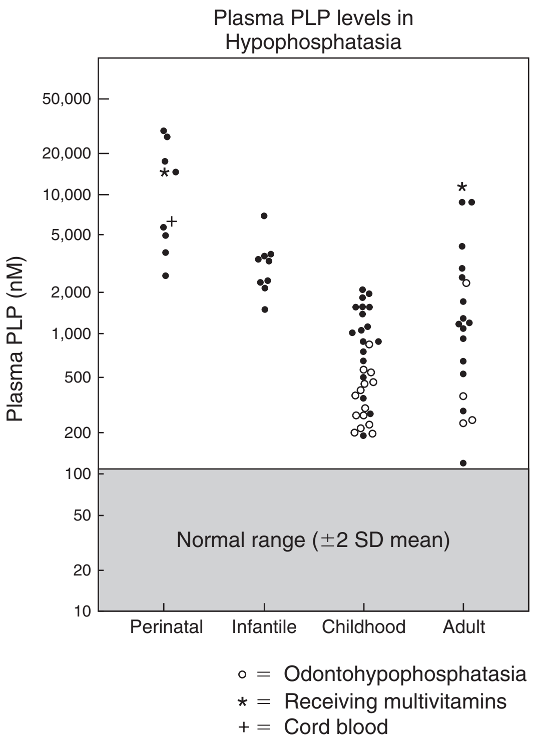

FIGURE 9 Plasma PLP levels in hypophosphatasia. PLP concentrations in plasma in various clinical forms of hypophosphatasia (hatched area is the normal range for children and adults). Note the logarithmetic scale with some overlap between the clinical forms (assays performed courtesy of Dr. Stephen P. Coburn, Fort Wayne State Developmental Center, Fort Wayne, IN). [Reproduced with permission from Whyte (2001)].

Inorganic pyrophosphate

Assay of PP_i in plasma or urine is not commercially available. Urine PP_i levels are increased in most HPP patients, but can be unremarkable in mildly affected individuals (Caswell *et al.*, 1991). Nevertheless, this test may help with carrier detection (Whyte, 2001).

Radiographic Findings

The radiographic features of HPP (see Figs. 4 to 7) have been illustrated for the principal clinical forms (see earlier).

Histopathological Findings

Histopathological disturbances in HPP that are a direct outcome of TNSALP deficiency seem to be limited to hard tissues. In severe cases, extramedullary hematopoiesis (Fallon *et al.*, 1984; Ornoy *et al.*, 1985) may derive from marrow space crowding owing to the osteomalacia. The cause for any pulmonary hypoplasia is not clear (Silver *et al.*, 1988), but perhaps involves a small thorax.

Skeleton

In all but the mildest cases of HPP (i.e., odonto-HPP), nondecalcified sections of the skeleton reveal defective mineralization (Fallon *et al.*, 1984; Ornoy *et al.*, 1985). The degree of rickets or osteomalacia generally reflects the clinical severity (Fallon *et al.*, 1984). Features of secondary hyperparathyroidism (present in rickets or osteomalacia associated with hypocalcemia) are typically absent (Fallon *et al.*, 1984; Anderson *et al.*, 1997).

In growth plates and osseous tissue, the cellular sources of the bone isoform of TNSALP (chondrocytes and osteoblasts) are present (Anderson *et al.*, 1997), but their TNSALP activity is deficient (Fallon *et al.*, 1984; Ornoy *et al.*, 1985). Other forms of rickets or osteomalacia cannot be excluded by their histopathological features unless ALP activity is assessed. In HPP, ALP activity in bone inversely reflects the degree of osteoid accumulation, and therefore the severity of the skeletal disease (Fallon *et al.*, 1984).

Electron microscopy of perinatal HPP has shown normal distribution of MVs, proteoglycan granules, and collagen fibers in the extracellular space of cartilage (Fallon *et al.*, 1984; Ali, 1986), although the MVs lacked ALP activity. Early reports described only isolated or tiny groups of hydroxyapatite crystals (calcospherites) that frequently were not associated with vesicular structures (Ali, 1986; Shohat *et al.*, 1991). Actually, HPP MVs do contain hydroxyapatite, but the crystals fail to enlarge after these structures rupture (Anderson *et al.*, 1997). Secondary, but not primary, mineralization is compromised.

Dentition

Premature exfoliation of primary teeth occurs in a few diseases, such as cyclical neutropenia and Papillon–Lefevre syndrome (Van den Bos *et al.*, 2005). In HPP, a paucity of cementum (despite presence of cells that look like cementoblasts) seems to explain this complication (see Fig. 6A) (el-Labban *et al.*, 1991; Lundgren *et al.*, 1991). Desiccated teeth can still be useful for histopathological examination. What cementum may be present appears afibrillar (el-Labban *et al.*, 1991). The severity of this defect varies from tooth to tooth, but typically reflects the degree of skeletal disease. Incisors are usually most affected, and the first to be shed.

In addition to defects in cementum, dentinogenesis seems to be impaired, as shown by big pulp chambers. Dentin tubules may be enlarged although reduced in number. The excessive width of predentin, increased amounts of interglobular dentin, and impaired calcification of cementum are analogous to the osteoidosis found in bone. The enamel is not impacted directly (Lundgren *et al.*, 1991). The histopathological changes observed in the permanent teeth are similar to those in the deciduous teeth (el-Labban *et al.*, 1991), but their prognosis is better (Lepe *et al.*, 1997).

Biochemical and Genetic Defect

TNSALP Deficiency

Early on, postmortem studies of perinatal and infantile HPP revealed the enzymatic defect defining this inborn error of metabolism, and thereby suggested its genetic basis. Profound deficiency of ALP activity was discovered in liver, bone, and kidney tissue, but not in intestine or placenta (fetal trophoblast) (Vanneuville and Leroy, 1981). This observation matched amino acid sequence analyses of ALP purified from various healthy human tissues (Stigbrand and Fishman, 1984)—arguing for selective deficiency of the enzymatic activities of all of the isoforms comprising the TNSALP family.

Postmortem studies of children or adults with HPP have not been reported, but globally diminished TNSALP activity is suggested by deficiencies in serum, bone, circulating granulocytes (Fallon *et al.*, 1984), and skin fibroblasts (Vanneuville and Leroy, 1981).

Hypophosphatasemia in HPP does not involve accelerated clearance of unaltered TNSALP from the circulation (Gorodischer *et al.*, 1976). Purified placental ALP (Whyte *et al.*, 1992), as well as the bone isoform of TNSALP contained in the plasma of patients with Paget's bone disease (Whyte *et al.*, 1982b), showed normal circulating half-lives of several days when infused intravenously into severely affected infants during attempted ALP enzyme replacement therapy (see later).

Furthermore, coincubation experiments with mixtures of serum, and coculture studies with fibroblasts from severely affected patients, provided evidence against an inhibitor or absence of an activator of TNSALP in HPP (Fraser, 1957; O'Duffy, 1970; Whyte and Vrabel, 1985). In 1985, heterokaryon studies using skin fibroblasts in culture indicated a defect at a single gene locus (Whyte and Vrabel, 1985).

ALP immunoreactivity has been studied in bone, liver, and kidney as well as in skin fibroblasts from patients with severe HPP (Fallon *et al.*, 1989; Fedde *et al.*, 1996). The preliminary report by Fallon and coworkers, using a polyclonal antibody to the liver isoform of TNSALP, described normal amounts of immunoreactive TNSALP in bone, liver, and kidney from five HPP patients (Fallon *et al.*, 1989). Others, using isoelectric focusing and enzyme inhibition studies, suggested that the low ALP activity remaining in one HPP patient was intestinal ALP (Mueller *et al.*, 1983). In fibroblasts from severely affected individuals, ALP had somewhat different physicochemical and immunological properties compared with ALP from healthy controls, but nevertheless seemed to be a form of TNSALP (Fedde *et al.*, 1996). Leukocyte ALP activity can be subnormal in any clinical type of HPP, except perhaps pseudo-HPP, and therefore likely represents an isoform of TNSALP (Fallon *et al.*, 1984). The nature of ALP in the circulation in HPP requires further study. Monoclonal antibody-based immunoradiometric assays (IRMAs) for polymeric TNSALP demonstrated low levels of the bone and liver isoforms of TNSALP in sera from patients with all clinical types of HPP, except pseudo-HPP (Whyte *et al.*, 1996). Upon release from plasma membranes and entry into blood, TNSALP in HPP seemed to be altered in such a way that immunoreactivity was diminished and/or clearance was accelerated (Whyte *et al.*, 1996). Fedde *et al.* (1996) concluded that the precise impact of the underlying *TNSALP* mutation(s) (see later) must be understood to fully appreciate its effect on the enzyme's structure in tissues, and thereby HPP disease expression in patients. These cumulative findings have been clarified by the subsequent identification of ≈200, usually missense, mutations of *TNSALP* in HPP (see later).

Inheritance

Mutation analysis of *TNSALP* coupled with clinical studies of families has established that both autosomal dominant and autosomal recessive patterns of inheritance account for HPP. Although perinatal and infantile HPP represent autosomal recessive disease (Weiss *et al.*, 1988; Henthorn *et al.*, 1992; Mumm *et al.*, 2001; Whyte *et al.*, 2003, 2006), milder forms of HPP can result from either autosomal recessive (Henthorn *et al.*, 1992) or autosomal dominant transmission of *TNSALP* defects (Mumm *et al.*, 2006; Whyte *et al.*, 2007).

Certain *TNSALP* loss-of-function mutations seem to have dominant-negative effects (Henthorn, *et al.*, 1996; Mumm *et al.*, 2006) (see later).

Attempts to identify carriers for HPP by using conventional biochemical testing can have uncertainties. The utility of quantitating several such parameters has been discussed (Sorensen *et al.*, 1978). A comprehensive evaluation of vitamin B_6 loading has not been published.

TNSALP Gene Defects

In 1988, proof that HPP can be caused by deactivating mutation within the candidate (TNSALP) gene came with discovery by Weiss and coworkers of a homozygous *TNSALP* missense defect in a consanguineous boy with perinatal HPP (Weiss *et al.*, 1988). Transfection studies showed that the single-base transition compromised the enzyme's activity, perhaps by impairing metal ligand binding to an important arginine residue at the catalytic pocket (Weiss *et al.*, 1988). In 1992, Henthorn *et al.* reported eight further missense mutations in four additional, unrelated patients with perinatal or infantile HPP (Henthorn *et al.*, 1992). In this study, two siblings with childhood HPP and one unrelated woman with adult HPP were compound heterozygotes for identical *TNSALP* defects.

In 1993, Greenberg and coworkers found that homozygosity for a tenth *TNSALP* missense mutation accounts for the especially high prevalence of HPP in Mennonites in Canada (Greenberg, *et al.*, 1993).

Now, all clinical forms of HPP have been shown to involve loss-of-function mutations in *TNSALP* (Whyte, 2000), and all patients studied to date have carried one or two defective *TNSALP* alleles. A web site organized by E. Mornet summarizes the *TNSALP* mutations identified in HPP patients worldwide (http://www.sesep.uvsq.fr/Database.html). Currently, ≈200 specific *TNSALP* mutations are recorded, of which a considerable majority (≈80 %) are missense. Some have regional or national prevalence (Mumm *et al.*, 2006).

TNSALP Structural Defects

Many of the *TNSALP* mutations causing HPP (Taillander *et al.*, 2001; Mumm *et al.*, 2002) alter an amino acid residue that is conserved in mammalian TNSALPs (Henthorn and Whyte, 1992; Mornet *et al.*, 1998). In fact, several are conserved in bacteria (Henthorn *et al.*, 1992).

Revelation of the three-dimensional structure of *Escherichia coli* ALP (Kim and Wyckoff, 1991) and human placental ALP (Le Due *et al.*, 2000) by x-ray crystallography has greatly advanced our understanding the biochemical basis for HPP (Henthorn *et al.*, 1992; Kim and Wyckoff, 1991; Millán, 2006). Missense mutations in *TNSALP* can be examined for their impact on the catalytically active dimeric TNSALP molecule [Chemscape Chime version 1.02 by MDL Information Systems, Inc. (San Leandro, CA) at http://www.mdli.com and RasWin Molecular Graphics, Windows version 2.6 by Roger Sayle, Glaxo-Wellcome Research and Development (Stevenage, Hertfordshire, UK) at http://www.umass.edu/microbio/rasmol/index.html]. Some *TNSALP* mutations disturb the enzyme's catalytic pocket or structurally important metal ligand-binding sites; others possibly affect dimer formation (Mornet *et al.* 1998; Millán, 2006). Nevertheless, how all such *TNSALP* mutations are deleterious is not completely understood (Whyte, 2000). For example, site-directed mutagenesis with the Ala^{161}-Thr substitution, first discovered to cause HPP (Weiss *et al.*, 1988), did not compromise the catalytic activity of *E. coli* ALP (Chaidaroglou and Kantrowitz, 1993). Some mutations alter intracellular movement of TNSALP (Mornet *et al.*, 1998; Shibata *et al.*, 1998; Fukushi *et al.*, 1998). Transient transfection studies have examined the impact of specific mutations on TNSALP catalytic activity (Weiss *et al.*, 1988; Brunt-Heath, *et al.*, 2005) Clinical, radiological, and biochemical studies of heterozygous carriers of HPP may prove especially helpful for understanding the impact of particular *TNSALP* mutations on HPP disease severity (Whyte, 2000).

Prognosis

Perinatal HPP is almost always fatal. With intensive life support, affected neonates may live for a short time. Rarely, there is long-term survival.

Infantile HPP has a less certain outcome. Often there is clinical and radiographic deterioration with ≈50% of babies dying of pneumonia and respiratory compromise owing to worsening skeletal disease, particularly in the chest (Fraser, 1957; Whyte *et al.*, 2003; Cahill *et al.*, 2007). Sometimes, considerable spontaneous improvement occurs. The prognosis seems to improve if there is survival past infancy. In fact, a preliminary report from Canada (Ish-Shalom *et al.*, 1986) suggests that the stature of adult survivors of infantile HPP may be normal (I am aware of many significant exceptions in the United States) (Whyte, 2001). Childhood HPP may also spontaneously improve, but this seems to usually occur after adolescence when growth plates fuse. Unfortunately, recurrence of skeletal symptoms and complications later in adulthood is likely (Fraser, 1957; Weinstein and Whyte, 1981; Khandwala *et al.*, 2006; Whyte *et al.*, 2007).

Adult HPP causes recurrent and lingering orthopedic difficulties (Whyte *et al.*, 1982a). Worsening osteomalacia, associated with osteopenia and fractures, was not prevented in two affected women by estrogen replacement at menopause (personal observation).

Treatment

Medical

There is no established medical treatment for HPP, although several approaches have been attempted (Fraser *et al.*, 1955) including trials of ALP enzyme replacement (Whyte *et al.*, 1982b, 1984, 1992), marrow cell transplantation (Whyte *et al.*, 2003; Cahill *et al.*, 2007), and teriparatide administration (Whyte *et al.*, 2007). Cortisone given to a few pediatric patients with severe disease reportedly coincided with periods of normalization of serum ALP activity and radiographic improvement (Fraser and Laidlaw, 1956), but this has not been a consistent finding. Brief supplementation with Mg^{2+} or Zn^{2+} has also been unsuccessful (Fraser, 1957).

Choosing which patients with infantile HPP might be candidates for therapeutic trials is made difficult because some show progressive skeletal demineralization leading to a fatal outcome (Whyte *et al.*, 1982b), yet others demonstrate spontaneous, and sometimes quite significant, improvement (Ish-Shalom *et al.*, 1986; Whyte *et al.*, 1986a).

Hypercalcemia in infantile HPP can be treated by restriction of dietary calcium, but care must be taken not to exacerbate the progressive skeletal demineralization that may occur from HPP itself (Whyte *et al.*, 1982b, 1986a). If necessary, glucocorticoids may also be helpful (Whyte *et al.*, 1982b). Subcutaneous injections of salmon calcitonin could address the hypercalcemia and block skeletal demineralization in HPP (Barcia *et al.*, 1997), but experience with bisphosphonates that are derivatives of PP_i has not been reported and these antiresorptives could pose a problem for a rickets or osteomalacia (Whyte, 2001).

Hypothetically, drugs that stimulate TNSALP biosynthesis in the skeleton, such as teriparatide, could be beneficial for HPP (Whyte *et al.*, 2007). However, the hypophosphatasemia can be quite refractory. I know of hypophosphatasemia persisting despite viral hepatitis or end-stage kidney disease in affected adults (unpublished work).

Enzyme replacement therapy (EzRT) for HPP has been attempted by intravenous (i.v.) infusion of several types of ALP. In general, the results have been disappointing. In 1972, serum from a patient with Paget's bone disease, given by Macpherson and colleagues to an affected infant, was said to precede radiographic improvement (Macpherson *et al.*, 1972). In 1982, i.v. infusions weekly of fresh plasma from healthy subjects were followed by clinical and radiographic advances in one child (Albeggiani and Cataldo, 1982). However, subsequent infusions of Paget's bone disease plasma showed no significant clinical or radiographic benefit for four patients with infantile HPP that proved lethal (Whyte *et al.*, 1982b, 1984). After a brief trial of prednisone, bovine PTH 1–34, and then plasma pooled from several healthy individuals, transient correction of hypophosphatasemia and remarkable clinical, radiographic, and histological improvement occurred in a boy with infantile HPP who died soon after of pneumonia (Whyte *et al.*, 1986a). He was later found to be homozygous for a TNSALP missense mutation (Whyte *et al.*, 2006). However, subsequent trials of plasma infusions for other patients did not produce this response (Whyte *et al.*, 1988). In follow-up of a brief report in 1989 that suggested that i.v. administration of ALP purified from liver improved the histological appearance of bone and decreased urinary PEA levels (Weninger *et al.*, 1989), we gave i.v. infusions of purified placental ALP to an affected infant (Whyte *et al.*, 1992). Despite repeated doses that caused transient hyperphosphatasemia, there were only modest decrements of plasma PLP and urinary PEA concentrations with no change in urinary PP_i levels, and no clinical or radiographic improvement (Whyte *et al.*, 1992). This trial followed documentation that elevated concentrations of PLP in plasma and increased levels of PEA and PP_i in urine fell as placental ALP corrected the hypophosphatasemia of pregnant women who were carriers for HPP (Whyte *et al.*, 1995). Placental ALP seems to hydrolyze the same substrates as TNSALP. Perhaps pregnancy (with placental ALP *in situ*) might represent an "endogenous" EzRT. However, the symptoms, bone density, and so on, of affected women with HPP have not been assessed immediately before and after a pregnancy.

These discouraging cumulative observations from EzRT perhaps reflected much greater tissue needs for ALP compared with levels achieved in the circulation by such treatments. Alternatively, the findings suggested that ALP must be within the skeleton itself (and/or perhaps bound to a lipid bilayer) for physiological activity and therapeutic efficacy (Whyte *et al.*, 1995). In fact, treatment of TNSALP knockout mice with a bone-targeted, recombinant form of human TNSALP has essentially prevented the skeletal and dental disease and vitamin B_6-dependent epilepsy (Millán *et al.*, 2008). Extreme skeletal disease in perinatal HPP despite placental ALP circulating in the mother shows that the *in utero* environment is not clearly protective (Whyte, 2001).

Indeed, there are two reports of rescue and unequivocal clinical and radiographic improvement in infantile HPP following attempts to transplant cells of mesenchymal origin hoping that sufficient numbers of TNSALP-replete osteoblasts would form. Nevertheless, these changes occurred without significant biochemical alterations, and the mechanism for the disease amelioration remains unproven (Whyte *et al.*, 1998; Cahill *et al.*, 2007).

Because patients with HPP are often hyperphosphatemic (Whyte, 2001), restriction and/or pharmacological binding of dietary P_i could potentially be therapeutic for HPP (Wenkert *et al.*, 2002). Excess extracellular P_i may be acting as a competitive inhibitor of TNSALP (McComb *et al.*, 1979; Coburn *et al.*, 1998), or suppressing TNSALP gene expression (Goseki-Sone *et al.*, 1999).

In 2007, injections of teriparatide (recombinant PTH 1–34) were associated with clinical, biochemical, and radiographic improvement in a woman with adult HPP (Whyte *et al.*, 2007). Benefits may have resulted from increased TNSALP expression in her osteoblasts and/or PTH-induced phosphaturia enhancing TNSALP catalysis as shown by increases in ALP activity and decreases in P_i levels in her serum.

Supportive

Symptoms from calcium pyrophosphate crystal deposition, or perhaps periarticular calcium phosphate precipitation ("calcific periarthritis"), may respond to nonsteroidal anti-inflammatory agents.

Intramedullary rodding of femoral fractures or pseudofractures has become a mainstay of orthopedic management (Coe *et al.*, 1986).

Prenatal Diagnosis

Perinatal HPP has been diagnosed *in utero* during the second trimester by using ultrasonography with particular attention to the shape and mineralization of the skull and major long bones (van Dongen *et al.*, 1990). However, it is now well documented that relatively mild HPP, inherited either as autosomal dominant or autosomal recessive trait, can cause *in utero* bowing of major long bones suggestive of potentially lethal HPP, yet the deformities correct spontaneously after birth followed by the phenotype of childhood HPP (Moore *et al.*, 1999; Pauli *et al.*, 1999; Wenkert *et al.*, 2007; Stevenson *et al.*, 2008). Now, *TNSALP* mutation detection is possible (Henthorn and Whyte, 1995b), and offered by several fee-for-service laboratories. The great number of specific defects in *TNSALP* complicates the search, necessitating examination of *TNSALP* exons and splice sites (Mumm *et al.*, 2002).

PHYSIOLOGICAL ROLE OF ALP EXPLORED IN HYPOPHOSPHATASIA

As reviewed earlier, several roles for TNSALP in skeletal formation have been proposed (Table I). Investigation of HPP has been a "window of opportunity" to explore a number of them in humans.

Discovery by Weiss and coworkers two decades ago that homozygosity for a deactivating mutation within *TNSALP* caused severe HPP (Weiss *et al.*, 1988) proved Robison correct; i.e., ALP functions critically in skeletal mineralization (Robison, 1923). Subsequent characterization of the dental manifestations of HPP revealed that TNSALP is also important for formation of the primary teeth.

Electron microscopy of bone and cartilage obtained at autopsy from severely affected HPP patients demonstrated that deficient activity of TNSALP causes a fundamental disturbance in skeletal mineralization. In 1985, Ornoy and coworkers reported that the process of "primary mineralization" is impaired in HPP (Ornoy *et al.*, 1985). Hydroxyapatite crystals were found near, but not within HPP MVs. Later, however, Anderson and colleagues concluded that secondary (extravesicular), not primary (vesicular), mineralization is impeded in HPP (Anderson *et al.*, 1997). There appears to be failure of hydroxyapatite crystal growth after MVs rupture.

Defects in the cementum and dentin in HPP teeth have seemed to be analogous to those in the skeleton (Lundgren *et al.*, 1991). A prominent role for TNSALP during two critical phases of dental biomineralization, initiation and completion, has been proposed (Hotton *et al.*, 1999). In 2005, Van den Bos and coworkers showed that both cellular and acellular cementum formation is impaired, but not mineralization of dentin (Van den Bos *et al.*, 2005).

Although the kidneys, liver, and adrenal glands are rich in TNSALP in health (McComb *et al.*, 1979), their functions do not seem directly impaired in HPP (see later), except that reclamation of filtered P_i by the kidney is enhanced. This disturbance does not seem to be caused always by suppression of circulating PTH levels (Whyte and Rettinger, 1987).

It has been suggested that TNSALP deficiency in HPP might impair the biosynthesis of phospholipids causing pulmonary atelectasis, but the respiratory problems of severely affected patients are likely caused by rib cage fractures and deformities, which may also account for the hypoplastic lungs (Silver *et al.*, 1988).

It is clear from studies of HPP patients that TNSALP is critically important in humans, not only for mineralization of hard tissues, but also for the dephosphorylation of extracellular PLP (Whyte, 1994). Why patients with severe HPP experience idiopathic seizures is becoming understood. A recent case report delineates how the biosynthesis of select neurotransmitters can be impaired in severe HPP (Baumgartner-Sigl *et al.*, 2006). As discussed later, epilepsy in the murine model of infantile HPP is associated with low levels of γ-aminobutyric acid in the central nervous system (Waymire *et al.*, 1995). Pyridoxine supplementation is necessary to extend the lives of these mice (Fedde *et al.*, 1999).

TNSALP Substrates

As reviewed later, the discoveries that PEA, PLP, and PP_i accumulate endogenously in HPP were essential for elucidating the physiological role of TNSALP in humans. Each was inferred to be a natural substrate for TNSALP. However, a preliminary study using ^{31}P. Magnetic resonance spectroscopy of HPP urine indicates that there are at least several additional phosphorylated TNSALP substrates, but their identities are not known (Whyte *et al.*, 2000).

Phosphoethanolamine

The reports in 1955 by McCance and colleagues (1955) and Fraser and coworkers (1955) that PEA levels are elevated in the urine and plasma of HPP patients provided a second biochemical marker for this disorder and identified the first natural substrate for TNSALP. In 1968, Rasmussen showed that this phosphocompound appears in the urine when plasma levels are scarcely detectable; i.e., there is essentially no renal threshold for PEA excretion (Rasmussen, 1968).

Although the metabolic origin of PEA is uncertain, it is not considered to be a derivative of phosphatidylethanolamine, i.e., not from plasma membrane phospholipid breakdown. The principal source of circulating PEA has been reported to be the liver, which metabolizes PEA to ammonia, acetaldehyde, and P_i in a reaction catalyzed by *O*-phosphorylethanolamine phospholyase (Gron, 1978). Indeed, in one family with adult HPP, urine levels of PEA correlated inversely with the activity in serum of the liver (but not bone) isoform of TNSALP (Millán *et al.*, 1980). Now, PEA is understood to be a component of the phosphatidylinositol-glycan linkage apparatus (Low and Zilversmit, 1980). Hence, serum and urine PEA could be a degradation product from this tether for cell surface (ecto-) proteins (see later).

Pyridoxal 5′-Phosphate

Discovery in 1985 that plasma levels of PLP are elevated in HPP was a significant advance in our understanding of the physiological role of TNSALP (Whyte *et al.*, 1985).

As reviewed in Fig. 10, the dietary forms of vitamin B_6 (pyridoxine, pyridoxal, and pyridoxamine, and their phosphorylated derivatives) are all converted to PLP in the liver (Dolphin *et al.*, 1986). Organ ablation studies revealed that the liver is the principal source of plasma PLP in mammals. PLP is released from hepatocytes into the circulation where more than 95% is coupled to albumin (Dolphin *et al.*, 1986). Some additional PLP in plasma is bound to various enzymes; and only a small amount circulates freely.

Like many phosphorylated compounds, PLP cannot cross plasma membranes, but must be dephosphorylated to pyridoxal (PL) before it can enter tissues. Inside cells, PL is rephosphorylated to PLP or converted to pyridoxamine 5′-phosphate which then act as cofactors for many and varied enzymatic reactions. Ultimately, vitamin B_6 is degraded to 4-pyridoxic acid, primarily in the liver, which is then excreted in urine (Dolphin *et al.*, 1986; Coburn *et al.*, 2001).

In disorders featuring elevations in circulating bone and liver TNSALP activity, plasma PLP concentrations are decreased (Anderson *et al.*, 1980). The low PLP levels were once erroneously attributed to vitamin B_6 deficiency. Discovery of elevated plasma levels of PLP in HPP led to recognition of the reciprocal relationship between plasma PLP levels and serum TNSALP activity. However, such changes in circulating PLP levels are usually not physiologically important because TNSALP controls extracellular, not intracellular, dephosphorylation of PLP. Elevated

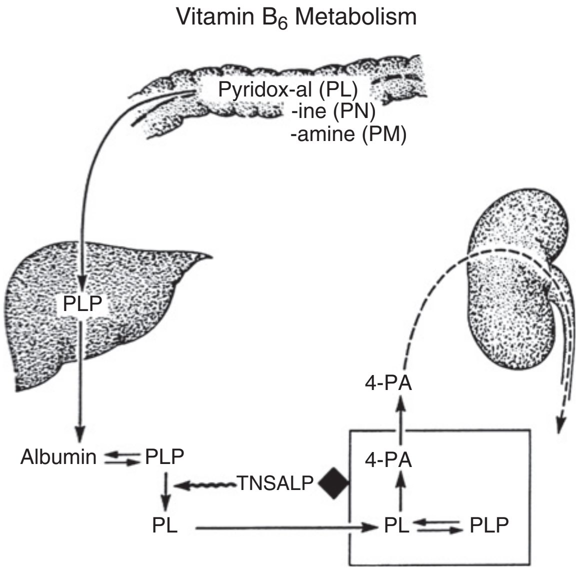

FIGURE 10 Role of TNSALP in vitamin B_6 metabolism. The various forms of vitamin B_6 are plentiful in the diet and are absorbed into the hepatic portal circulation (phosphorylated forms are first dephosphorylated in the gut). In the liver, each is converted to PLP, which is then secreted bound to albumin into the plasma. In order to enter tissues, plasma PLP must be dephosphorylated to PL which can traverse membranes. 4-Pyridoxic acid (4-PA), the major degradation product of vitamin B_6, is excreted in the urine. High plasma levels of PLP in hypophosphatasia, yet normal plasma concentrations of PL, are consistent with an ectoenzyme role for TNSALP in the extracellular dephosphorylatin of PLP to PL. [Reproduced with permission from Whyte (2001)].

plasma PLP levels in HPP do not reflect enhanced PLP synthesis, but instead a failure of extracellular hydrolysis of PLP (Whyte *et al.*, 1985). Investigation of HPP suggested this metabolic relationship because patients typically do not have symptoms of vitamin B_6 excess such as peripheral neuropathy (Dolphin *et al.*, 1986) (see later). Similarly, in all but the most severe cases of HPP, there are no signs or symptoms of deficiency of vitamin B_6 such as stomatitis, dermatitis, peripheral neuritis, anemia, or depression (Dolphin *et al.*, 1986). Furthermore, a variety of biochemical findings indicated that intracellular levels of vitamin B_6 are unremarkable in HPP. First, urine levels of 4-pyridoxic acid are normal in patients with childhood HPP (Whyte *et al.*, 1988). Second, these children respond normally to L-tryptophan loading—a test for vitamin B_6 deficiency (Whyte and Coburn, unpublished observation). Third, in homogenates of severely TNSALP-deficient HPP fibroblasts in culture, levels of PLP and the various other forms of vitamin B_6 are normal (Whyte *et al.*, 1986b). Finally, tissues obtained at autopsy from perinatal HPP (plasma PLP concentrations 50- to 900-times elevated) contain essentially normal levels of PLP, PL, and total vitameric B_6 (Whyte *et al.*, 1988). Accordingly, TNSALP seemed to function as an ectoenzyme (Whyte *et al.*, 1985; Fedde *et al.*, 1988). This was confirmed by others in studies of membrane attachment of placental ALP (Low and Saltiel, 1988).

Because TNSALP seems to condition the dephosphorylation of PLP to PL extracellularly, plasma levels of PL could be low in HPP. However, only patients with very severe HPP have low plasma PL concentrations—other forms of HPP show normal or sometimes elevated circulating PL levels (Whyte *et al.*, 1985).

Vitamin B_6 deficiency has been associated with renal stone disease and epilepsy. Nephrocalcinosis in infants with HPP is likely caused by hypercalciuria, but oxalate excess (a consequence of vitamin B_6 deficiency) has not been looked for in HPP (Dolphin *et al.*, 1986). Epilepsy in severe HPP occurs in patients who may have cranial deformity, intracranial hemorrhage, periodic apnea, etc. Nevertheless, the convulsions also have a metabolic explanation. PEA caused seizures when given i.v. to a severely affected infant during a study of PEA metabolism (Takahashi *et al.*, 1984). Furthermore, in two patients with perinatal HPP and epilepsy, both of whom had plasma PL levels below assay sensitivity, administration of vitamin B_6 as pyridoxine did not correct the seizure disorder (personal observation), perhaps because pyridoxine was converted to PLP rather than PL. Recently, it was shown that vitamin B_6-dependent seizures can be the presenting manifestation of infantile HPP. Additionally, it was noted that all reported HPP cases with such seizures proved fatal (Baumgartner-Sigl *et al.*, 2007). In fact, the *tnsalp* knockout mouse model for infantile HPP manifests epilepsy (see later) that requires pyridoxine administration to extend the life of the animal (Waymire *et al.*, 1995; Fedde *et al.*, 1999).

The clinical and biochemical observations concerning vitamin B_6 metabolism in HPP indicated an ectoenzyme role for TNSALP (Whyte *et al.*, 1985). In 1988, Fedde and coworkers used cultivated human osteosarcoma cells (Fedde *et al.*, 1988), and then dermal fibroblasts from patients with infantile HPP (Fedde *et al.*, 1990), exposed to PLP and PEA in the medium to confirm that TNSALP is primarily a plasma membrane-associated ectoenzyme (see later). In 1980, characterization of porcine kidney ALP as membrane-bound with phosphatidylinositol (Low and Zilversmit, 1980) had suggested the attachment mechanism for TNSALP (Low and Saltiel, 1988).

Inorganic Pyrophosphate

Discovery in 1965 and 1971 that PP_i levels are increased in HPP patient urine (Russell, 1965) and plasma (Russell *et al.*, 1971) provided a plausible explanation for the defective skeletal mineralization in HPP (Caswell *et al.*, 1991) (Fig. 11). By that time, PP_i was known to be a potent inhibitor of mineralization (Heinonen, 2001). Although at low concentrations PP_i can enhance calcium and P_i precipitation from solution to form amorphous calcium phosphate, at higher concentrations PP_i prevents the growth and dissolution of hydroxyapatite crystals (Caswell *et al.*, 1991).

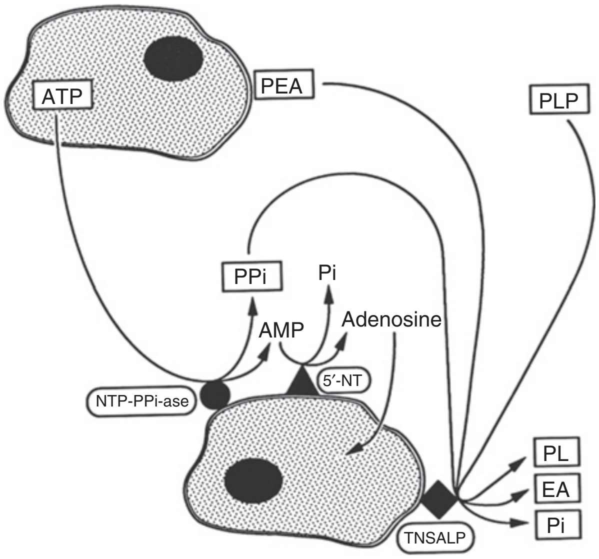

FIGURE 11 Metabolic basis for hypophosphatasia (hypothesis). Extracellular generation of PP_i, presumably by the action of nucleoside triphosphate pyrophosphatase (NTP-PP_i-ase), is normal. PP_i is also pumped extracellularly from within cells via an ANK channel (Millán, 2006). Extracellular degradation of PEA, PP_i, and PLP is diminished because of deficient ecto-TNSALP activity. Accumulation of PP_i extracellularly accounts for the CPPD precipitation and associated calcium phosphate crystal deposition. The inhibitory effect of PP_i on hydroxyapatite crystal growth accounts for the rickets/osteomalacia. [Reproduced with permission from Whyte (2001)].

Caswell and coworkers, demonstrated that nucleoside triphosphate pyrophosphatase (NTP-PP_i-ase, PC-1) activity is unremarkable in TNSALP-deficient fibroblasts from perinatal and infantile HPP patients. Generation of PP_i extracellularly from ATP by these cells is not hindered (Caswell *et al.*, 1986). Therefore, NTP-PP_i-ase seemed to be a distinctive enzyme from TNSALP. In 2000, this conclusion was supported by studies of osteoblasts from the *tnsalp* knockout mouse (see later) (Johnson *et al.*, 2000). From the studies of PLP metabolism in HPP revealing an ectoenzyme role for TNSALP, it was now understood how the clearance of $^{32}PP_i$ administered in the 1960s into the circulation of two adults with HPP was markedly delayed (R.G.G. Russell, personal communication). Endogenous accumulation of PP_i in HPP reflects defective extracellular hydrolysis of PP_i (Caswell *et al.*, 1991).

Consonant with the *in vitro* effects of PP_i, several disturbances of mineralization are observed in HPP that perhaps reflect local concentrations of PP_i together with other factors. Relatively minor excesses of PP_i may explain precipitation of amorphous calcium phosphate and the calcific periarthritis observed in some adults with HPP (Lassere and Jones, 1990). Furthermore, ALP has been shown to dissolve CPPD crystals *in vitro* (Xu *et al.*, 1991). This PP_i-ase activity seems to be unrelated to its capacity to hydrolyze phosphoesters. Thus, CPPD crystal deposition leading to chondrocalcinosis, pseudogout, and pyrophosphate arthropathy could be explained by failure of TNSALP not only to destroy PP_i, but also to hydrolyze CPPD crystals. Conversely, especially high concentrations of PP_i inhibit hydroxyapatite crystal formation and growth; hence, rickets or osteomalacia are expected characteristics of HPP. As discussed, electron microscopy of skeletal tissue from severely affected HPP patients, and in *tnsalp* knockout mice (see later), showed deficiency of secondary mineralization surrounding MVs (Anderson *et al.*, 1997).

Circulating TNSALP

Several observations suggest that ALP in the circulation is physiologically inactive. Infants with severe HPP who received i.v. infusions of plasma from patients with Paget's bone disease or purified placental ALP demonstrated no significant clinical or radiographic improvement despite transient increments in circulating ALP activity, sometimes to above normal levels. Such therapy failed to normalize urinary PEA or PP_i levels or plasma PLP concentrations (Whyte *et al.*, 1982b).

Deficiency of TNSALP activity intrinsic to the skeleton seems to account for the rickets and osteomalacia of HPP. In fact, in studies reminiscent of Robison's work (Robison, 1932), Fraser and Yendt reported in 1955 that rachitic rat cartilage would calcify in serum obtained from an infant with HPP, yet slices of the patient's costochondral junction would not mineralize in synthetic calcifying medium or in the pooled serum from healthy children (Fraser and Yendt, 1955). Subsequently, *in vitro* models showed that transfection of ALP cDNA conferred both catalysis against P_i esters and mineralization in a calcification system (Yoon *et al.*, 1989; Farley *et al.*, 1994). Nevertheless, there has been skepticism that this type of experiment reflects biomineralization, because increased P_i in metastable solutions is expected to precipitate calcium phosphate (Khouja *et al.*, 1990). However, it is probably necessary to augment ALP activity at the skeletal level to treat HPP. In fact, experience with marrow cell transplantation for two babies with infantile HPP, who then had clinical and radiographic improvement despite essentially unaltered biochemical abnormalities in blood and urine, suggests that even small increases in ALP activity within the skeleton can improve mineralization in HPP (Whyte *et al.*, 1998). Transient transfection studies of various *TNSALP* mutations causing HPP also suggested that small differences in the deficiency of ALP activity within the skeleton can account for lethal versus nonlethal patient outcomes (Mornet *et al.*, 1998).

Hypophosphatasia Fibroblast Studies

Much of our insight concerning the physiological role of TNSALP has come from investigation of people with HPP. Before the availability of *tnsalp* knockout mice for study (see later), dermal fibroblasts in culture proved useful (Fedde *et al.*, 1990; Whyte and Vrabel, 1985; Whyte *et al.*, 1986b). Fibroblasts express some TNSALP-like activity that peaks at confluence in culture (Whyte and Vrabel, 1987). Such cells obtained from severely affected HPP patients are profoundly deficient (less than 5% control) in ALP activity (Whyte *et al.*, 1983), but the residual activity seems to represent a form of TNSALP (Whyte *et al.*, 1987).

Preliminary studies using HPP fibroblasts indicated that their phospholipid composition and rates of $^{32}P_i$ accumulation were normal (Tsutsumi *et al.*, 1986; Whyte and Vrabel, 1983). In 1990, Fedde and coworkers demonstrated that TNSALP is present primarily on the surface of these cells (Fedde *et al.*, 1990). The ectophosphatase activity of TNSALP hydrolyzed extracellular PEA and PLP under physiological conditions. Although some reports suggested that ALP conditions cell growth and differentiation by influencing the phosphorylation of nucleotide pools, HPP fibroblasts proliferate at normal rates in culture (Whyte *et al.*, 1983). Furthermore, TNSALP did not seem to be a phosphoprotein phosphatase acting at the plasma membrane (Fedde *et al.*, 1993).

tnsalp Knockout Mice

Since 1995, *tnsalp* knockout mouse studies have complimented and expanded on insight gained from investigation of HPP patients concerning the pathogenesis of the skeletal, dental, and neurological disease resulting from

TNSALP deficiency (Millán, 2006). Now, *tnsalp* null mice are also being used as a preclinical model to test EzRT for HPP (Millán *et al.*, 2008). In 1995, Waymire and coworkers developed and studied a *tnsalp* null mouse that manifested the deranged vitamin B_6 metabolism of HPP causing lethal seizures from deficient γ-aminobutyric acid in the brain (Waymire *et al.*, 1995). With pyridoxal treatment, the epilepsy was controlled temporarily, and the animals survived long enough to develop dental disease. In 1997, Narisawa and colleagues developed a different *tnsalp* null mouse with vitamin B_6-dependent epilepsy that also manifested skeletal and dental disease (Narisawa *et al.*, 1997). In 1999, Fedde and coworkers demonstrated that both of these murine models recapitulate the infantile form of HPP remarkably well, including the acquired skeletal, dental, and neurological disease together with endogenous accumulation of PEA, PP_i, and PLP (Fedde *et al.*, 1999). In 2000, the mineralization defect of HPP was reproduced with osteoblasts in culture from these mice (Wennberg *et al.*, 2000). The disturbance did not seem to be related to the aberrations in vitamin B_6 metabolism (Narisawa *et al.*, 2001). Defects in secondary skeletal mineralization were also confirmed by electron microscopy (Anderson *et al.*, 2004). Subsequently, double-knockout mouse studies showed that skeletal formation is essentially normal in mice that lack both *tnsalp* and the inorganic pyrophosphate generating enzyme PC-1 (NTP-PP_i-ase), adding further support to experience with hypophosphatasia patients that PP_i accumulation owing to TNSALP deficiency causes the defective skeletal mineralization (Hessle *et al.*, 2002). Furthermore, *tnsalp* expressed under control of the APO-E promoter in the liver of *tnsalp* knockout mice prevented the skeletal disease of HPP. It is uncertain, however, if *tnsalp* in the liver itself, or liberated in especially high amounts into the circulation of these mice, explained the beneficial effects. In 2007, Hough and coworkers characterized a much milder, acquired, semi-dominant form of HPP in mice generated by *N*-ethyl-*N*-nitrosourea exposure. Adult mice manifested late-onset skeletal disease and arthropathy (Hough *et al.*, 2007).

SUMMARY

HPP features defective skeletal mineralization that manifests as rickets in newborns, infants, children, and adolescents, and as osteomalacia in adults. Clinical expressivity is, however, extremely variable and explained largely by autosomal dominant or autosomal recessive patterns of inheritance, and by the multitude of associated loss-of-function mutations in *TNSALP*. Perinatal HPP is apparent *in utero* and causes stillbirth from severe skeletal hypomineralization and deformity. Infantile HPP presents by age 6 months as a seemingly acquired form of rickets. Sometimes there is functional craniosynostosis and nephrocalcinosis from hypercalcemia and hypercalciuria. About 50% of these babies succumb to worsening rachitic disease compromising pulmonary function. Vitamin B_6-dependent epilepsy designates a lethal outcome. Childhood HPP features premature loss of deciduous teeth, rickets, and muscle weakness. Adult HPP causes recurrent metatarsal stress fractures, femoral pseudofractures, and occasionally arthritis from CPPD crystal deposition, and rarely calcific periarthritis from precipitation of calcium phosphate forming hydroxyapatite. Odonto-HPP refers to premature tooth loss from deficient dental cementum, but no skeletal manifestations.

Perinatal and infantile HPP are transmitted as autosomal recessive traits owing to homozygosity or compound heterozygosity involving $\approx$200 different loss-of-function mutations in *TNSALP*. The mutations compromise the activity and/or structure of this homodimeric or homotetrameric enzyme, and sometimes its intracellular processing. Most are missense defects. In some kindreds, relatively mild HPP is inherited as an autosomal dominant trait owing to a *TNSALP* mutation that seems to exert a dominant-negative effect. However, individuals with even the mildest forms of HPP can be compound heterozygotes for *TNSALP* mutations.

Prenatal diagnosis of HPP is possible. During the second trimester, fetal ultrasonography or radiography has proven helpful for perinatal HPP. From the first trimester, chorionic villus samples have been used successfully for *TNSALP* mutation detection. However, the considerable number and variety of *TNSALP* mutations, as well as the influence of other factors on the HPP phenotype, makes prognostication difficult.

Three phosphocompounds (PEA, PP_i, and PLP) accumulate endogenously in HPP. A variety of evidence from HPP studies shows that PLP, a cofactor form of vitamin B_6, collects extracellularly, whereas intracellular PLP levels are normal in all but the most severely affected patients. This explains the absence of symptoms of deficiency or toxicity from vitamin B_6 in HPP, and indicates that TNSALP functions as an ectoenzyme. Extracellular accumulation of PP_i at high concentrations acts as an inhibitor of hydroxyapatite crystal growth, and seems to explain the associated CPPD crystal deposition and defective mineralization of bones and teeth.

There is no established medical treatment for HPP. ALP replacement by intravenous infusion of ALP from various human tissues has been disappointing, suggesting that circulating TNSALP is physiologically inactive. Marrow cell transplantation has been followed by considerable clinical and radiographic improvement, but without significant biochemical changes, in two girls with infantile HPP. The mechanism, however, remains unproven. Teriparatide has stimulated bone ALP levels and appeared to heal fractures in adult HPP. TNSALP null mouse studies suggest that recombinant, bone-targeted TNSALP will be helpful for HPP.

CONCLUSIONS

Hypophosphatasia (HPP) is a rare but remarkably instructive inborn error of metabolism that confirms in humans a critical role for the tissue nonspecific ("liver/bone/kidney") isoenzyme of ALP (TNSALP) in skeletal mineralization and in the formation of dental cementum. Subnormal serum ALP activity (hypophosphatasemia), the biochemical hallmark of HPP, reflects a generalized deficiency of TNSALP activity. Three tissue-specific ALP isoenzymes in humans (intestinal, placental, and germ-cell ALP) are not compromised.

Insight from HPP has provided most of our understanding of the physiological role of TNSALP in humans. Discovery of increased endogenous levels of phosphoethanolamine (PEA), inorganic pyrophosphate (PP_i), and pyridoxal 5′-phosphate (PLP) in HPP demonstrated that TNSALP is catalytically active toward a variety of natural substrates; acting not only as a phosphomonoester phosphohydrolase, but also as an inorganic pyrophosphatase. Because in health these substrates occur at nanomolar or micromolar concentrations extracellularly, TNSALP acts physiologically toward them at much lower concentrations than for artificial substrates used in routine clinical assays of ALP activity. Clearly, TNSALP functions at physiological pH, and "alkaline phosphatase" is a misnomer (Whyte, 1994, 2001).

Clinical investigation of vitamin B_6 metabolism in HPP, supported by HPP fibroblast studies, confirmed that TNSALP acts as an ectoenzyme. Extracellular accumulation of PEA, PP_i, and PLP in HPP is caused by deficient ecto-TNSALP activity. Despite the increments of membrane-impermeable PLP in the plasma, normal levels of PL in plasma (and presumably in other extracellular fluids and in tissues) explain the absence of signs or symptoms of vitamin B_6 deficiency (or toxicity) in all but the most severely affected HPP patients.

The source of PLP in HPP appears to be in the liver. PEA may derive from degradation of the phosphatidylinositol-glycan moiety that anchors many proteins to cells surfaces. PP_i is formed extracellularly by cell surface NTP-PP_i-ase, and pumped from cells by a channel protein called ANK. Hyperphosphatemia owing to increased TmP/GFR in HPP suggests that TNSALP plays a direct role in renal P_i excretion.

In HPP, calcium phosphate crystal deposition rarely causes calcific periarthritis, but calcium pyrophosphate dihydrate crystal deposition frequently results in chondrocalcinosis, and sometimes in pseudogout, or PP_i arthropathy. Calcific periarthritis reflects the regional effect of PP_i at low concentrations to stimulate amorphous calcium phosphate formation. Rickets and osteomalacia in HPP reflect the effect of high extracellular concentrations of PP_i to inhibit hydroxyapatite crystal formation and growth at sites of skeletal mineralization. Trials of ALP replacement therapy for HPP suggest that TNSALP acts physiologically at the level of the skeleton, but not in the circulation.

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Chapter 74

Paget's Disease of Bone

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THE PATIENT

Paget's disease is a common disorder of the skeleton that is local in nature and extremely variable in its clinical manifestations (Singer and Krane, 1998). The majority of patients are without symptoms. When symptoms are present, skeletal deformity and pain are most common. Deformities are usually most apparent in the skull, face, and lower extremities. Pain is of several origins. Localized bone pain is surprisingly uncommon, but joint pain (hips and knees) is seen not infrequently owing to degenerative arthritis. Pain of neural or spinal origin is unusual but the most severe.

A variety of complications may first bring the patient to medical attention. The complications depend both on the affected skeletal sites and the overall extent of the disease (six bones are commonly involved). Patients with Paget's disease in the skull often develop a hearing deficit if the temporal bone is affected. Massive enlargement of the cranium is associated with basilar impression and neurological impairment. Vertebral involvement may produce compression fractures, spinal stenosis, neurological impairment, and degenerative arthritis. Paget's disease in the pelvis and femurs commonly is associated with degenerative arthritis of the hips. Involvement of the femur and tibia may lead to pathological fractures of these long bones. Nonunion of a femoral fracture is relatively common. Degenerative arthritis in the knees is also a common feature when lower extremity long bones are extensively involved by Paget's disease. The most serious complication is the development of a sarcoma that fortunately occurs in only less than 1% of patients. It always arises in a pagetic lesion and not in unaffected bone. Systemic complications of Paget's disease generally occur with more extensive disease. Hypercalcemia, preceded by hypercalcuria, usually is noted with total bed rest and is related to the accelerated bone resorption induced by immobilization. Increased cardiac output, and less commonly, congestive heart failure, is a consequence of the great vascularity of bones affected by Paget's disease. Hyperuricemia has been observed in males with extensive disease and may reflect increased purine turnover.

RADIOLOGY AND NUCLEAR MEDICINE

The diagnosis of Paget's disease is primarily accomplished by roentgenographic evaluation of the skeleton. Over the years it has become clear that the disease evolves through several stages as observed by serial roentgenograms.

The initial stage of the disease is represented by a localized area of reduced bone density often referred to as an osteolytic lesion. This is most readily detected in the skull where it is found as a discrete round or oval lesion in the frontal or occipital bones. It is called *osteoporosis circumscripta.* Paget's disease in the long bones almost always begins in the subchondral region of either epiphysis (uncommonly, both may be affected simultaneously). The osteolytic process has then been seen to advance proximally or distally at about 1 cm/year in the untreated patient. The advancing front usually has a V-shaped or arrowhead appearance.

In the most advanced stage of Paget's disease, the areas of previous osteolytic dominance now are characterized by a chaotic sclerotic appearance, a phase that is called osteoblastic or osteosclerotic. In long bones the osteolytic phase is commonly seen preceding the osteosclerotic region when much of the bone has been affected by the disease. Another feature of this phase is considerable thickening of the sclerotic bone, which can reach monumental proportions in the skull. Osteolytic activity of a secondary nature often can be observed as clefts in the thickened bone. It is

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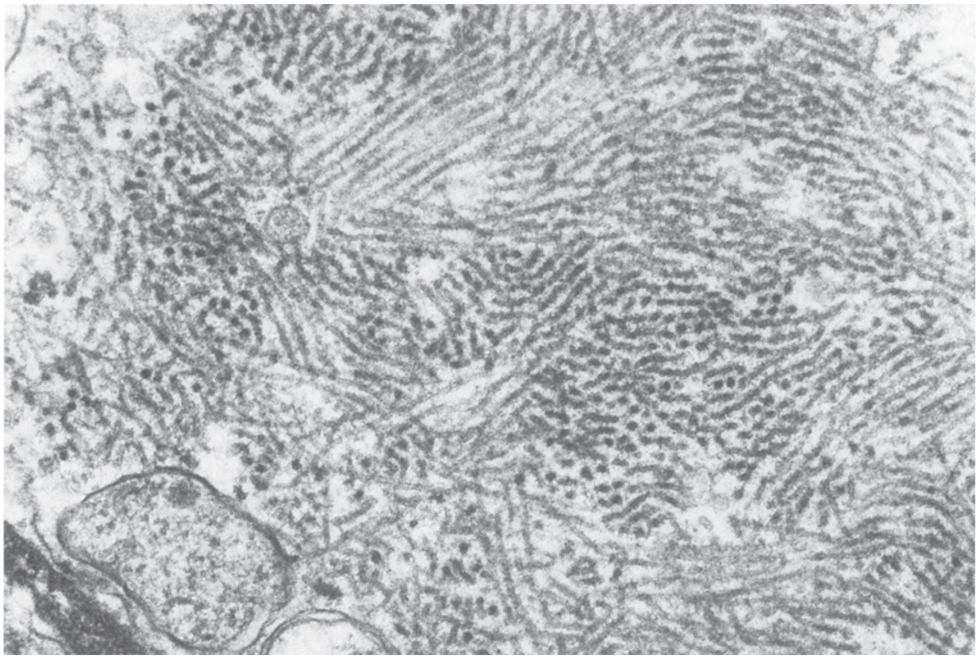

FIGURE 1 Electron micrograph of a nuclear inclusion in an osteoclast of a patient with Paget's disease. The microfilaments are seen both in longitudinal array and in paracrystalline array in cross section. Provided by Dr. Barbara G. Mills.

likely that the evolution of the disease into its most severe form occurs over much of the life span of the patient. Two other features of the disease have been noted through serial roentgenographic observations. Although the disease can slowly course through an entire bone, it does not cross a joint space to affect an adjacent bone. Also it is extremely rare for new lesions of Paget's disease to be detected at any site in the skeleton after the diagnosis and extent of the disease has been determined initially.

Bone scans are the most sensitive means of detecting pagetic lesions. Radiolabeled bisphosphonates accumulate in regions where blood flow and bone formation are increased and can outline early lesions that are not detectable on roentgenograms. Radioactive gallium can also define areas of Paget's disease activity because of uptake of gallium by osteoclasts.

HISTOPATHOLOGY

Beginning with the observations of Schmorl in 1932, it has become appreciated that the osteoclast is the dominant cell in the pathogenesis of Paget's disease. Osteoclasts are increased in number in the Haversian canals of the cortex in the absence of other abnormalities and are in great numbers at the leading edge of the osteolytic front. The osteoclasts in Paget's disease may be far greater in size than osteoclasts in normal bone and contain as many as 100 nuclei in a cross section compared with two or three nuclei in a normal osteoclast. Osteoclasts of Paget's disease have a characteristic ultrastructural abnormality (Gherardi *et al.*, 1980; Howatson and Fornasier, 1982; Mills and Singer, 1976; Rebel *et al.*, 1974). This consists of microfilaments, sometimes grouped in a paracrystalline array, located in the nucleus and sometimes in the cytoplasm of osteoclasts (Fig. 1). These microfilaments are not seen in nonpagetic bone or bone marrow cells. These inclusions closely resemble nucleocapsids of viruses of the Paramyxoviridae family, a group of RNA viruses responsible for some of the most common childhood diseases. Despite the finding of these inclusions in the vast majority of patients studied, the budding off of an infectious virus from the osteoclasts has rarely been observed (Abe *et al.*, 1995).

Osteoblasts are another prominent feature of the cellular pathology of Paget's disease. Large numbers of osteoblasts are often found near areas of resorbed bone and may even be prominent in a lesion that appears purely osteolytic by x-ray. The osteoblasts are usually prism-shaped or polyhedral and contain abundant rough endoplasmic reticulum, mitochondria, and a well-developed Golgi zone. These signs of cellular activity are consistent with the increased bone formation in active lesions established by the use of double labeling with tetracycline.

In addition to the increased numbers of osteoclasts and osteoblasts, the marrow of pagetic lesions tends to be grossly abnormal. The normal hematopoietic elements are usually absent and replaced by mononuclear cells of indeterminate origin intermixed with highly vascular connective tissue.

The bone matrix in Paget's disease is highly abnormal in structure and arises as a consequence of disordered bone resorption and formation. The matrix consists of a "mosaic" of irregularly shaped pieces of lamellar bone with an erratic pattern of cement lines. The matrix is interspersed with numerous foci of woven bone, which, in adults, is ordinarily found associated with fracture healing.

BIOCHEMISTRY

The biochemical findings in Paget's disease help to provide an integrated assessment of the cellular events occurring throughout the skeleton of affected patients.

Historically, the earliest index of bone matrix resorption was measurement of urinary hydroxyproline excretion while ingesting a low-gelatin diet. This index is well correlated with the extent of the disease despite the fact that hydroxyproline is a prominent component of extraskeletal connective tissue as well as skeletal collagen. Measurement of collagen cross-link degradation products in urine provides more specific measurements of skeletal matrix. Urinary N-telopeptide, C-telopeptide, pyridinoline, and deoxypyridinoline have all been reported to be more specific indices of skeletal matrix resorption and are not influenced by dietary gelatin (Alvarez *et al.*, 1995; Reid *et al.,* 2004; Shankar and Hosking, 2006).

Serum tartrate-resistant acid phosphatase, presumably released by osteoclasts, appears to be another index of bone resorption in Paget's disease but is not routinely available (Kraenzlin *et al.*, 1990).

Osteoblast activity can be assessed by measurement in the serum of total alkaline phosphatase activity, bone-specific alkaline phosphatase activity, osteocalcin concentration, and procollagen type 1 N-terminal propeptide (P1NP) concentration. The most useful of these serum markers are the total alkaline phosphatase, bone-specific alkaline phosphatase, and P1NP (Alvarez *et al.*, 1995; Reid *et al*, 2004; Shankar and Hosking, 2006). For reasons that are not understood.

TREATMENT OF PAGET'S DISEASE

Salmon calcitonin by injection and etidronate disodium by the oral route were the first effective medications introduced about 30 years ago. They generally suppress biochemical parameters of the disease by 50%. Intravenous pamidronate disodium, oral alendronate, and risedronate sodium are more recent, more potent bisphosphonates that can reduce bone turnover to normal in the majority of patients with Paget's disease (Singer and Krane, 1998). In some countries zoledronic acid has been approved for treatment of Paget's disease. A 15-minute intravenous infusion suppresses biochemical activity into the normal range even in patients with markedly elevated parameters of bone resorption and formation (Reid *et al.*, 2005). The response usually persists for at least two years (Hosking *et al.*, 2007).

Surgery is sometimes necessary to treat patients with associated degenerative arthritis of the hip (total hip replacement) and of the knee (high tibial osteotomy). Orthopedic and/or neurosurgical procedures may occasionally be necessary after fractures and when skull or vertebral complications are present.

EVIDENCE FOR THE PRESENCE OF PARAMYXOVIRUSES IN PAGET'S DISEASE

In early studies, Mills and colleagues attempted to rescue an infectious virus from cells cultured from surgical specimens of Paget's disease (B. G. Mills and F. R. Singer, unpublished observations; Mills *et al.*, 1979). After trypsinization or after growing cells from explants of pagetic bones, cells were cultivated for months and then cocultivated with cell lines used to isolate Paramyxoviridae viruses. These studies failed to demonstrate an infectious virus in pagetic bone. Perhaps this is not surprising because little anatomic evidence of an infectious virus has been found in any specimen of Paget's disease, and the mononuclear cells cultured from surgical specimens have rarely been found to exhibit nuclear inclusions. Mills and colleagues (1985) also attempted to develop an animal model of Paget's disease by injecting crushed bone extracts or lysates from cells cultured from pagetic bone into the tibiae of athymic nude mice. Nothing resembling Paget's disease was induced in the mouse bone, although one cell line did produce an osteosarcoma-like lesion reproducibly.

Despite the failure to find an infectious (mature) virus in specimens of Paget's disease, abundant evidence has been generated using immunohistological and molecular biological techniques that indicate the presence of viral antigens and mRNA in Paget's disease. Initially Rebel and colleagues (1980a) used a variety of antisera directed against measles virus to demonstrate measles antigen in the osteoclasts of 20 patients with Paget's disease, but not in one patient with fluorosis and another with a healing fracture. Positive results were obtained with both indirect immunofluorescence (Fig. 2) and immunoperoxidase techniques (Rebel *et al.*, 1980b). Mills and colleagues (1980, 1981) initially reported that cell cultures from pagetic bone, as well as bone sections from 12 patients, were positive when stained with an antiserum against respiratory syncytial virus but not with antisera against measles virus, parainfluenza viruses, influenza A and B, rubella, mumps, and herpes simplex. Subsequently, Mills and colleagues (1984) did observe that measles virus nucleocapsid protein antigens were present in the osteoclasts and/or cultured bone cells of most patients with Paget's disease. Of particular interest was their finding that in serial sections of pagetic bone, both measles virus and respiratory syncytial virus nucleocapsid antigens were demonstrable in the same osteoclasts. This observation could not be explained by cross-reactivity of the measles virus monoclonal antibodies used in this study with respiratory syncytial virus

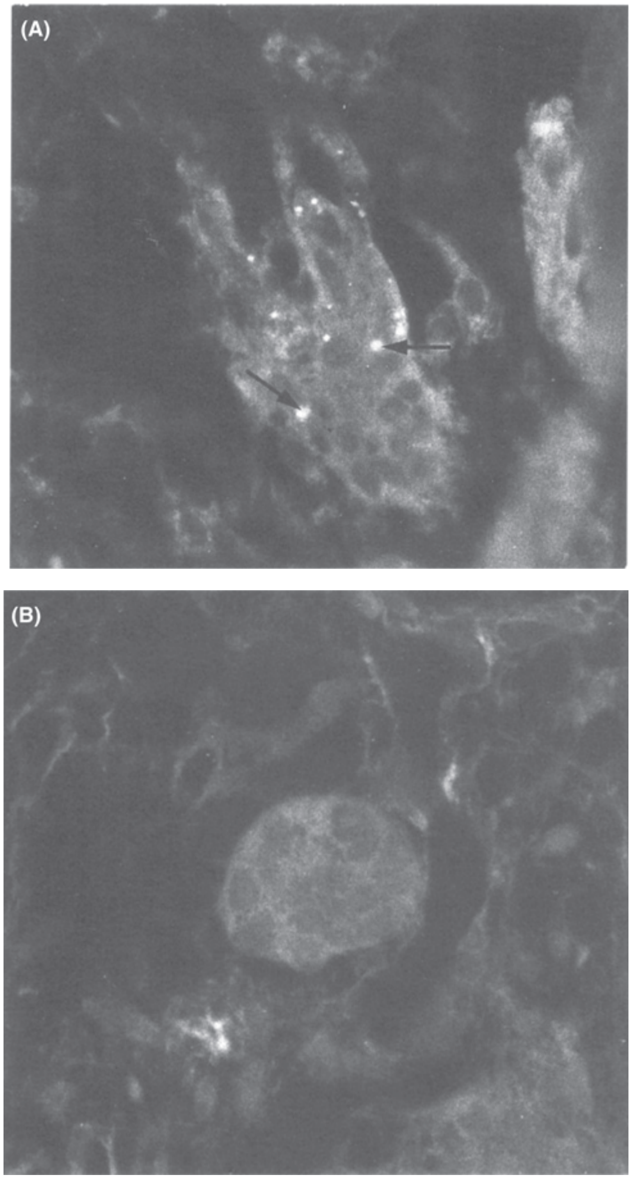

FIGURE 2 (**A**) A section of decalcified pagetic bone incubated with rabbit anti-LEC serum followed by goal antiserum coupled to fluorescein isothiocyanate. The LEC strain of measles virus was isolated from a patient with subacute sclerosing panencephalitis (SSPE). The osteoclast shows a strong positive punctate (*arrows*) fluorescent reaction. (**B**) A section of decalcified pagetic bone was treated as in **A**, except nonimmune rabbit serum was used instead of anti-LEC serum. There is no fluorescent reaction. Reproduced with permission from Rebel *et al*. (1980b).

antigens and vice versa. In other immunohistological studies, Basle and colleagues (1985) demonstrated not only measles virus antigens in 6 of 6 specimens of Paget's disease but also simian virus 5 in 6 of 6 and parainfluenza 3 in 3 of 6 specimens. No reactivity with respiratory syncytial virus, mumps virus, influenza virus, or adenovirus type 5 antibodies was found in this study. Subsequently, Basle did find a positive response with a monoclonal antibody against respiratory syncytial virus provided by Mills (personal communication). He also noted the presence of measles virus and respiratory syncytial virus antigens in the same osteoclasts on serial sections of pagetic bone. Mills and colleagues (1994) have also studied immunohistological staining of long-term cultures of marrow from patients with Paget's disease and from normal subjects with respect to viral antigens. In 12 of 12 patients, measles virus and/or respiratory syncytial virus nucleocapsid antigens were found in 40 to 50% of the mononuclear cells and/or multinucleated cells in culture. In the control subjects, less than 5% of cells were positive. Reddy and colleagues (1995) found measles virus transcripts in peripheral blood mononuclear cells in 4 of 5 patients and 0 of 10 normal subjects.

In the past 20 years, the techniques of molecular biology have increasingly been applied to the issue of the identity of the osteoclast inclusions of Paget's disease. In the first study, Basle and colleagues (1986) used *in situ* hybridization with a cloned measles virus cDNA probe specific for the nucleocapsid protein to search for measles virus RNA sequences in the bone of five patients. These sequences were detected in 80 to 90% of the osteoclasts in these specimens. Surprisingly, 30 to 40% of the mononuclear cells in these bone specimens also had detectable mRNA sequences of measles virus nucleocapsid protein. Evidence of the measles virus was found in osteoblasts, osteocytes, fibroblasts, lymphocytes, and monocytes. Negative results were obtained in three subjects who had fluorosis, fracture healing, and hyperparathyroidism, respectively.

Because an epidemiological study in England indicated that patients with Paget's disease were more likely to have had a pet dog in the past (O'Driscoll and Anderson, 1985), Gordon and colleagues (1991) used *in situ* hybridization to examine the possibility that canine distemper virus might be involved in Paget's disease. Canine distemper virus is a paramyxovirus with considerable structural homology to measles virus. Bone biopsies from 27 patients and 6 patients with other bone disorders (primary hyperparathyroidism, prostatic carcinoma, osteoporosis, osteomalacia, and fracture healing) were studied. Sense and antisense RNA probes to the nucleocapsid and fusion genes of the canine distemper virus were used as well as RNA probes to mRNA sequences of measles virus nucleocapsid protein. There was no demonstrable cross-reactivity of these probes with the heterologous virus under the experimental conditions used by the investigators. Eleven of 25 patients showed hybridization with the antisense but not the sense canine distemper virus fusion protein probe. Ten of 26 patients also showed hybridization with the antisense but not the sense probe for canine distemper virus nucleocapsid protein. The canine distemper virus probes produced hybridization in 80% of multinucleated osteoclasts, in 60% of osteoblasts, and in osteocytes as well as marrow mononuclear cells (monocytes, lymphocytes). No significant hybridization was found with the measles virus probe, and none of the control specimens reacted with any of the probes discussed earlier. In a subsequent study, Gordon and colleagues (1992) obtained pagetic bone for RNA extraction, reverse transcribed the RNA, and specifically amplified for canine distemper virus and measles virus sequences by using the polymerase chain reaction (PCR) technique. They found that 8 of 13 patients had canine distemper virus nucleic acid sequences, and 1 of 10 patients had measles virus nucleic acid sequences in the bone specimens. One patient had both. Dideoxy sequencing of the canine distemper virus PCR products revealed 2% base pair (bp) changes in a 187-bp fragment from within position 1231–1464 of the nucleocapsid gene compared with the Onderstpoort strain of canine distemper virus. Further support of the hypothesis that canine distemper virus could be involved in the pathogenesis of Paget's disease was sought by examining the bones of dogs with distemper infections (Mee *et al.*, 1992). In 2 of 4 dogs studied, *in situ* hybridization of metaphyseal specimens revealed strong signals with sense and antisense probes for the nucleocapsid and phosphoprotein genes. The osteoclasts were strongly positive but osteoblasts, osteocytes, and bone marrow cells were also positive. Mee and colleagues (1993) also have found canine distemper virus transcripts in the bone cells of dogs with metaphyseal osteopathy. This is an acute disorder affecting young rapidly growing dogs whose signs include fever, anorexia, and painful swollen metaphyses. The histology does not resemble Paget's disease. Although the presence of canine distemper virus transcripts in dogs with distemper or metaphyseal osteopathy does not relate directly to Paget's disease, it does indicate that a paramyxovirus can infect mammalian bone cells. This has also been demonstrated *in vitro* by incubating canine distemper virus with canine bone marrow (Mee *et al.*, 1995).

Additional evidence of paramyxovirus nucleocapsid transcripts in Paget's disease has come from the studies of Reddy and colleagues. They studied bone marrow mononuclear cells obtained from aspirations of the iliac crests of six patients with radiologically demonstrable Paget's disease and from the aspirations of 10 normal subjects (Reddy *et al.*, 1995). Using the reverse transcriptase-PCR techniques, they observed that 5 of 6 patients had measles virus nucleocapsid transcripts, whereas none of the 10 normal subjects had detectable transcripts. Dideoxy sequencing of the PCR fragments revealed several mutations within the position 1360–1371 bp of the nucleocapsid gene compared with the Edmonston strain of measles virus. These mutations are in the same region as the mutations reported in the canine distemper virus nucleocapsid gene. Because granulocyte macrophage colony-forming units (CFU-GM), the most likely osteoclast precursors, circulate in the peripheral blood, Reddy and colleagues (1996) examined peripheral blood samples for the presence of measles virus nucleocapsid transcripts by reverse transcriptase-PCR in Paget's disease and control subjects. In 9 of 13 patients, measles virus transcripts were detected. They were localized to peripheral blood monocytes (whose precursor is CFU-GM) by *in situ* hybridization. Studies were negative in 10 control subjects.

Recently, Kurihara and colleagues (2006) targeted the measles virus nucleocapsid gene to cells of the osteoclast lineage in transgenic mice. Osteoclast formation in these animals was markedly increased and displayed many of the characteristics of pagetic osteoclasts. Further, 40% of these animals developed bone lesions that were localized and were very similar to bone lesions found in Paget's patients (Fig. 3). None of these findings were present in littermate controls.

Three studies from the United Kingdom, one from the United States, and one from New Zealand have produced negative results with respect to detection of Paramyxoviridae mRNA in Paget's disease. In one study, RNA extracts of 10 bone specimens failed to exhibit measles virus, canine distemper virus, respiratory syncytial virus, or parainfluenza 3 virus transcripts after reverse transcriptase-PCR evaluation (Ralston *et al.*, 1991). In a second study, both bone cells cultured from pagetic explants and bone biopsies were studied similarly by reverse transcriptase-PCR techniques for the presence of measles virus and canine distemper virus transcripts (Birch *et al.*, 1994). Completely negative results were obtained. A second U.K. study using the same primers also failed to find measles virus or canine distemper virus transcripts in long-term bone marrow cultures from lesions of Paget's disease (Ooi *et al.*, 2000). In the U.S. study, Nuovo and colleagues (1992) also could not detect measles virus-specific cDNA in pagetic specimens by using the PCR and *in situ* hybridization in combination. The New Zealand study (Matthews, *et al.*, 2008) reported no evidence of measles virus ribonucleic acid in 13 osteoblast and 13 bone marrow cell cultures. The explanation for the disparate results is unclear because these investigators demonstrated that they can detect very low levels of measles virus transcripts by PCR (Ralston *et al.*, 2007).

Several studies have addressed the levels of circulating antibodies against various paramyxoviruses in patients with Paget's disease. Antibody titers have not been found to be greater in Paget's disease than in control subjects (Basle *et al.*, 1983; Gordon *et al.*, 1993; Pringle *et al.*, 1985).

CELLULAR AND MOLECULAR BIOLOGY OF PAGET'S DISEASE

The development of *in vitro* techniques for the study of the ontogeny of human osteoclasts has made it possible to gain new insights into the pathogenesis of Paget's disease. Kukita and colleagues (1990) first established long-term cultures of marrow from involved bones from patients with Paget's disease and noted that the multinucleated cells that formed shared many of the characteristics of pagetic osteoclasts. Compared with osteoclast-like cells formed in normal marrow cultures, the pagetic osteoclast-like cells

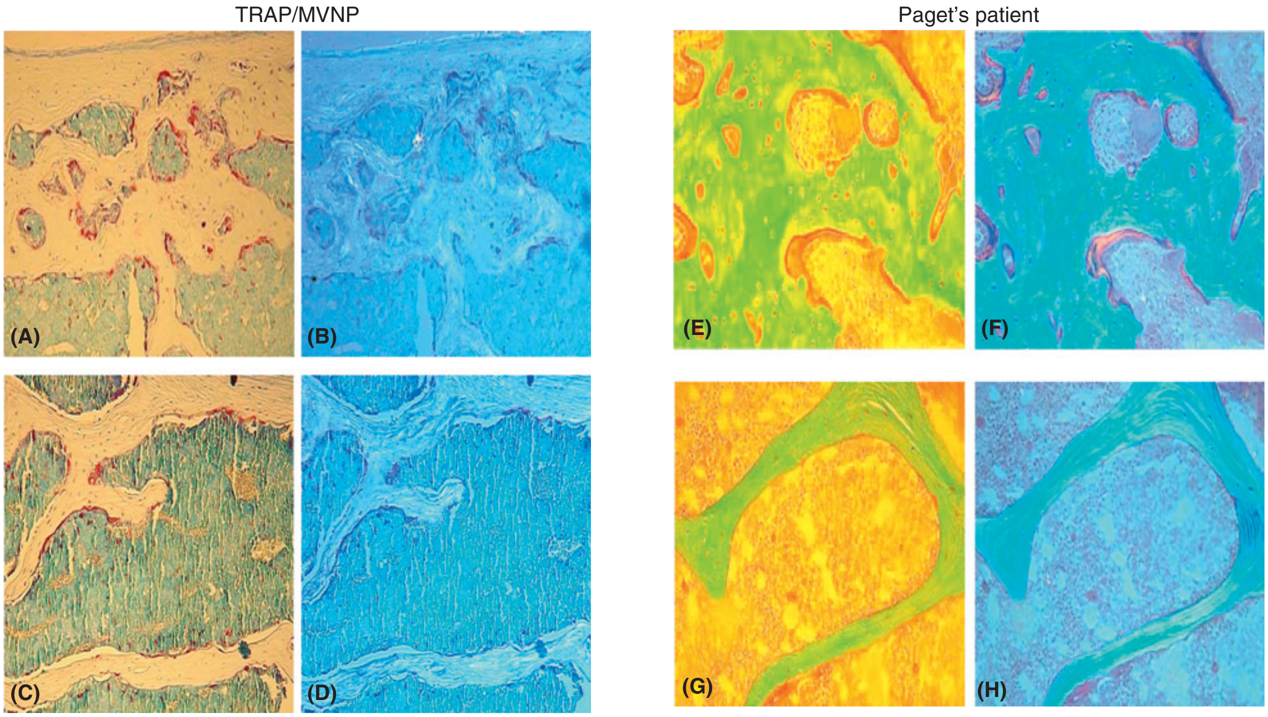

FIGURE 3 Histological features of a 12-month-old tartrate-resistant acid phosphatase/Edmonston strain measles virus nucleocapsid protein (TRACP/E-MVNP) transgenic mouse compared with wild-type (WT) controls. Histological features in a TRAPC/E-MVNP mouse (**A** and **B**) compared with a WT control (**C** and **D**) at 12 months of age. Note thickened, irregular trabeculae, increased osteoclast number (**A**), tunneling resorption, and increased amounts of woven bone in the TRAPC/E-MVNP mouse (**B**) compared with the WT control (**C** and **D**). Provided for comparison are sections from a 58-year-old woman with Paget's disease (**E** and **F**) and a 58-year-old normal subject (**G** and **H**). (**A** and **C**) TRACP stain, counterstained with methyl green-thionin. (**B** and **D**) Same sections viewed under polarized light to reveal woven bone. (**E** and **G**) Goldner's trichrome stain. (**F** and **H**) Same sections viewed under polarized light. Original magnification, ×100. From Kurihara *et al*. (2006). (See plate section)

formed more rapidly and in much greater numbers (10- to 100-fold greater), had increased numbers of nuclei, and had higher levels of tartrate-resistant acid phosphatase. Examination of these cells by electron microscopy did reveal many features of osteoclasts found in pagetic bone biopsies, but the characteristic nuclear and cytoplasmic inclusions were not observed. As mentioned previously, the antigens of measles virus and respiratory syncytial virus nucleocapsids were detectable in these cells (Mills *et al*., 1994); apparently the nucleocapsid structures do not form in this *in vitro* setting, perhaps because the formation of nucleocapsid structures requires more than the nucleocapsid gene alone.

Because the increased numbers of osteoclasts in pagetic lesions are of obvious importance in the pathogenesis of the disease, it seemed logical to examine osteoclast precursors in the marrow aspirates to determine whether they were abnormal or whether other cells in the marrow microenvironment were participants in the pathology. Demulder and colleagues (1993) examined CFU-GM in cultures of unfractionated marrow mononuclear cells and found that CFU-GM colony formation was significantly increased compared with that of normal cells. Using an antibody that recognizes the CD34 antigen present on most hematopoietic precursors, they also isolated enriched hematopoietic precursors and found similar numbers of osteoclast precursors in pagetic and normal marrow aspirations. Subsequent coculture experiments with highly purified hematopoietic precursors ($CD34^+$ cells) and nonhematopoietic marrow accessory cells ($CD34^-$ cells) demonstrated that the growth of pagetic precursors was significantly enhanced by both normal and pagetic $CD34^-$ cells. CFU-GM colony formation was also significantly increased when normal $CD34^+$ cells were cocultured with pagetic, but not normal, $CD34^-$ cells. CFU-GM colony-derived cells from pagetic patients also formed osteoclast-like multinucleated cells with 1,25-dihydroxyvitamin D (1,25-$(OH)_2D_3$) in concentrations 1/10th of that required for normal multinucleated cell formation. Thus, these experiments suggest that osteoclast precursors are abnormal in Paget's disease and that other cells in the pagetic marrow microenvironment may stimulate the growth and differentiation of these abnormal precursors. Menaa and coworkers (2000a) have extended the original studies of Demulder *et al.* (1993) to further understand the enhanced sensitivity of osteoclast precursors from patients with Paget's disease to the marrow microenvironment and the enhanced osteoclastogenic potential of the marrow microenvironment from Paget's patients.

Menaa and coworkers (2000a) showed that pagetic osteoclast precursors are hyperresponsive to RANK ligand, a member of the tumor necrosis factor (TNF) gene family, which is absolutely required for osteoclast formation. The increased sensitivity to RANK ligand was caused by the additive effects of interleukin-6 produced by the pagetic marrow and RANK ligand on osteoclast formation. Furthermore, Menaa and coworkers showed that marrow stromal cells from patients with Paget's disease expressed higher levels of RANK ligand than normal marrow stromal cells, although, recently, Naot and colleagues (2007) did not find increased RANKL expression by osteoblasts from pagetic lesions by using gene expression profiling. Thus, in pagetic lesions the osteoclast precursors are hyperresponsive to RANK ligand, and increased amounts of RANK ligand may be expressed in the marrow microenvironment, further enhancing the osteoclastogenic potential of the pagetic lesion. In areas of bone not affected by Paget's disease, enhanced expression of RANK ligand was not detected.

As noted earlier, a strong candidate for a significant autocrine/paracrine factor involved in the increased osteoclast formation in Paget's disease is interleukin-6. Roodman and colleagues (1992) found that conditioned media from long-term pagetic marrow cultures increased multinucleated cell formation in normal marrow cultures, and antibodies to interleukin-6 blocked the stimulatory activity. Antibodies to interleukin-1, GM-CSF, and TNF-α had no effect on the stimulatory activity. *In situ* hybridization studies demonstrated that the multinucleated cells in the pagetic marrow cultures were actively transcribing interleukin-6 mRNA. In addition, bone marrow plasma samples obtained from sites of Paget's disease had increased levels of interleukin-6 in 9 of 10 patients compared with samples from normal subjects. Peripheral plasma also had elevated interleukin-6 levels in 17 of 27 patients. In another study, basal plasma interleukin-6 activity was increased in 19 of 22 patients (Schweitzer *et al.*, 1995). The concept that interleukin-6 may be an important autocrine/paracrine factor in Paget's disease is also supported by the studies of Hoyland and colleagues (1994a), who used *in situ* hybridization to localize the expression of interleukin-6, interleukin-6 receptor, and interleukin-6 transcription factor in the bone of patients with Paget's disease in comparison with those with osteoarthritis. The osteoblasts in both disorders expressed all three mRNAs, but in Paget's disease interleukin-6 and its receptor mRNA showed higher levels of expression. In the osteoclasts of both disorders, the receptor and transcription factor were expressed, but only in Paget's disease was interleukin-6 mRNA expressed in osteoclasts. Hoyland and Sharpe (1994b) also examined the expression of c-*fos* proto-oncogene in the bone of six patients with Paget's disease by *in situ* hybridization. c-*fos* has been found to be important in the regulation of osteoclasts and was markedly upregulated in pagetic osteoclasts and, to a lesser extent, in the osteoblasts. It is possible that this is a consequence of interleukin-6 action (Korholz *et al.*, 1992).

Ralston and colleagues (1994) also studied cytokine and growth factor expression in bone explants of Paget's disease and in control subjects (postmenopausal women with and without osteoporosis and young bone graft patients) and could not find differences between pagetic and nonpagetic bone. Interleukin-6 mRNA was not detected in 40% of the pagetic specimens from severely affected individuals. There is no obvious explanation for their nonconfirmatory data.

Another abnormality of pagetic osteoclast precursors is that they are hyperresponsive to 1,25-$(OH)_2D_3$. Osteoclast precursors form osteoclasts *in vitro* with concentrations of 1,25-$(OH)_2D_3$ that are 1 to 2 logs less than that required to induce osteoclast formation by normal osteoclast precursors (Kukita *et al.*, 1990). Menaa and coworkers (2000b) have shown that this increased sensitivity to 1,25-$(OH)_2D_3$ is not caused by increased numbers of vitamin D receptors in pagetic osteoclast precursors or by mutations in the vitamin D receptor. Kurihara and colleagues (2000) have used a GST-vitamin D receptor fusion protein to further examine the increased sensitivity of pagetic osteoclast precursors to 1,25-$(OH)_2D_3$. These workers found that TAFII-20, a component of the TFIID transcription complex, was increased in osteoclast precursors from patients with Paget's disease compared with normal patients. This increase in expression of TAFII-20 did not require treatment of the cells with 1,25-$(OH)_2D_3$. These data suggest that pagetic osteoclast precursors express higher levels of TAFII-20 or have increased levels of a coactivator that can bind to TAFII-20 and the vitamin D receptor in the presence of lower concentrations of 1,25-$(OH)_2D_3$ to initiate transcription of vitamin D receptor (VDR) responsive genes.

GENETIC MUTATIONS LINKED TO PAGET'S DISEASE

As noted earlier, genetic factors are an important contributor to the etiology of Paget's disease, with 15 to 40% of affected patients having a first degree relative with Paget's disease. Several susceptibility loci for Paget's disease have been recently identified including 2q36, 5q31, 5q35, 10*p*13, 18q21–22, and 18q–23 (Cody *et al.*, 1997; Good *et al.*, 2002; Haslam *et al.*, 1998; Hocking *et al.*, 2001; Laurin *et al.*, 2001; Leach *et al.*, 2001). Mutations in the RANK gene on chromosome 18q21–22 have been linked to familial expansile osteolysis, a rare bone disorder that shares many clinical features of Paget's disease, but this mutation has only been found in a Korean family with Paget's disease. Laurin and coworkers (2002a) detected a point mutation, p392L, in the sequestosome-1 gene (p62). p62 is a ubiquitin-binding protein that is involved in the IL-1, TNF, and RANKL signaling pathways. Subsequently,

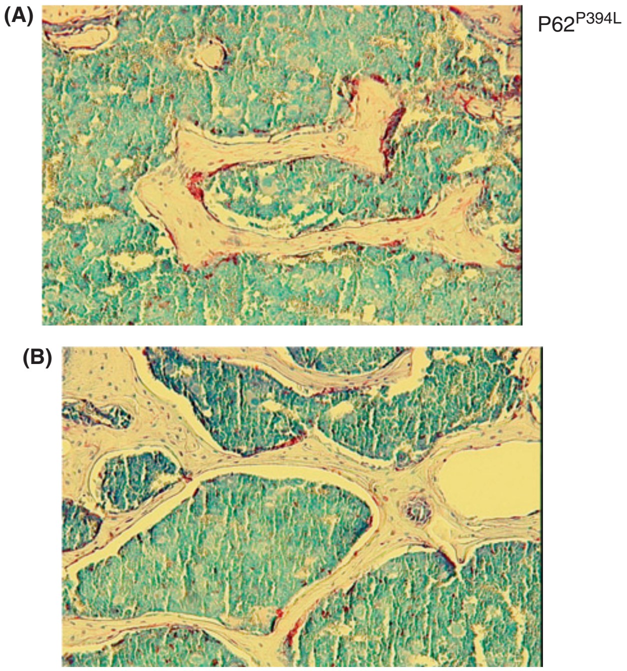

FIGURE 4 Histological studies of bone from TRAP-p62P392L mice. Vertebral cancellous bone from wild-type littermates (**A**) or TRAP-p62P392L mice (**B**). Note increased osteoclast perimeter (red stain), reduced cancellous bone volume, fewer and thinner trabeculae, and loss of trabecular connectivity in TRAP-p62P392L bone. From Kurihara *et al.* (2007). (See plate section)

other mutations in p62 have been found in both patients with familial and nonfamilial Paget's disease. However, the P392L mutation is the most frequent mutation in this gene and can be detected in up to 30% of patients with familial Paget's disease. Lucas and collegues (2008) have presented evidence that an unidentified gene on chromosome 10p13 may be present in most of the British families with Paget's disease who lack p62 mutations.

In recent studies, Kurihara and coworkers showed that transfection of normal human osteoclast precursors with a p62 construct containing the P392L mutation enhances the sensitivity of normal human osteoclast precursors for RANKL and results in increased osteoclast formation. However, these osteoclast precursors were not hyperresponsive to 1,25-$(OH)_2D_3$ nor did they contain increased numbers of nuclei per osteoclast, which are characteristics of pagetic osteoclasts (Kurihara *et al.*, 2007). Kurihara and coworkers have also targeted the P392L mutant p62 gene to cells in the osteoclast lineage in transgenic mice and found that these mice have increased osteoclast numbers but, in contrast to developing pagetic-like bone lesions, they developed severe osteopenia (Fig. 4). They also did not develop increased osteoblast activity that is characteristic of pagetic bone lesions. Osteoclasts from these transgene mice were not hypermultinucleated nor were osteoclast precursors from these animals hyperresponsive to 1,25-$(OH)_2D_3$. These *in vitro* and *in vivo* studies suggest that the P392L mutation in p62 increases osteoclast formation through increased RANK signaling.

Further, studies with families with Paget's disease linked to mutations in p62 also suggest that these mutations cannot completely account for the pathogenesis of Paget's disease, because the severity of the disease in family members carrying these mutations varies widely, and up to 83% of patients harboring the p62 mutation do not have Paget's disease (Laurin *et al.*, 2002b, Bolland *et al.*, 2007). These data suggest that additional factors may affect the pathogenesis of Paget's disease.

THE ETIOLOGY OF PAGET'S DISEASE

As reviewed earlier, Paget's disease is characterized by focal regions of highly exaggerated bone remodeling, with abnormalities in all phases of the bone remodeling process. In most affected individuals, it progresses slowly over many years without extending to new sites of involvement. The underlying pathophysiology appears to reflect a localized increase in the number of osteoclasts followed by a secondary increase in osteoblast activity. These osteoclasts have striking characteristics both by light and electron microscopy. In Paget's disease, the osteoclasts may be far greater in size than osteoclasts in normal individuals and patients with diseases in which osteoclasts are activated such as primary hyperparathyroidism. Genetic and nongenetic factors have been implicated in the pathogenesis of Paget's disease, and genetic factors are clearly an important component of the etiology of Paget's disease. However, microenvironmental factors appear to also play an important role. Two studies have shown that the prevalence of Paget's disease has decreased over the past 25 years in both the United Kingdom and New Zealand (Doyle *et al.*, 2002; van Staa *et al.*, 2002). Further, expression of the most common mutation linked to Paget's disease, the P392L and the p62, when expressed in normal osteoclast precursors or targeted to the osteoclast lineage in transgenic mice, does not induce pagetic lesions or pagetic-like osteoclasts. In contrast, expression of the measles virus nucleocapsid gene *in vitro* or *in vivo* results in formation of osteoclasts that express all the phenotypic characteristics of osteoclasts from patients with Paget's disease and form bone lesions that are very similar to those found in Paget's disease. The requirement for a nongenetic factor participating in the etiology of Paget's disease would explain why some individuals who have Paget's-associated mutations do not develop Paget's disease. One possibility is that such mutations predispose patients to Paget's disease by enhancing basal osteoclastogenesis, thereby creating a permissive environment for the development of Paget's disease. A second environmental factor, such as expression of certain viral proteins, may further alter signaling pathways or expression of specific transcription factors, which results in development of pagetic osteoclasts. These include changes in vitamin D receptor transcription as well as changes in the NF-κB signaling pathways and other signaling pathways involved in osteoclast formation. Further, the increased numbers of osteoclasts would then secrete high levels of IL-6, which would then further expand osteoclast formation. Because osteoclast and osteoblast activity remain coupled in Paget's disease, the increased osteoclast activity would result in increased osteoblast numbers and rapid formation of new bone. In addition, in preliminary studies Hiruma and colleagues (unpublished observations) have found that marrow stromal cells expressing the P392L mutation and p62 express high levels of RANKL when treated with low concentrations of $1,25\text{-}(OH)_2D_3$. These results suggest that genetic mutations linked to Paget's disease may increase osteoclast formation both by enhancing osteoclast activity per se as well as by creating a more osteoclastogenic environment in the pagetic lesions. However, these mutations by themselves are not sufficient to induce Paget's disease in animal models or pagetic-like osteoclasts in human marrow cultures.

Thus, there has been a tremendous output of new information on the etiology and pathophysiology of Paget's disease. Identification of genes involved in osteoclastogenesis that are mutated in Paget's disease and the characterization of nongenetic factors such as measles virus that may be involved have provided important insights in the control of bone remodeling in Paget's disease as well as normal bone. Studies of abnormal bone remodeling in Paget's disease could result in identification of coupling factors produced by osteoclasts, which enhance new bone formation. Understanding the pathophysiology of Paget's disease should provide important insights into the mechanisms that control normal osteoclast differentiation and bone formation and may lead to new therapies for both patients with Paget's disease and the identification of new anabolic factors for treating patients with severe bone loss.

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Genetic Determinants of Bone Mass and Osteoporotic Fracture

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INTRODUCTION

Genetic factors play an important role in the pathogenesis of osteoporosis. Although most interest has focused on uncovering the genetic determinants of bone mineral density (BMD), genetic factors also play a key role in regulating other phenotypes that predispose to osteoporotic fractures such as the ultrasound properties of bone, biochemical markers of bone turnover, and femoral neck geometry (Garnero *et al.,* 1996b; Bauer *et al.,* 1997; Faulkner *et al.,* 1993). In this chapter, I will review the strategies that have been used to identify and quantitate genetic influences on osteoporosis and then go on to discuss how these approaches have been applied to define candidate loci and genes that predispose to osteoporosis.

HERITABILITY OF OSTEOPOROSIS PHENOTYPES

Bone Mineral Density and Bone Loss

There is strong evidence for a genetic contribution to BMD and studies in twins and families have shown that between 50% and 85% of the variance in BMD is genetically determined depending on the skeletal site examined and characteristics of the population being studied (Christian *et al.,* 1989; Flicker *et al.,* 1995; Pocock *et al.,* 1987; Slemenda *et al.,* 1991; Smith *et al.,* 1973; Gueguen *et al.,* 1995; Krall and Dawson-Hughes, 1993). Although peak bone mass clearly has a strong genetic component, the data are conflicting with regard to the influence of genetic factors on age-related bone loss. In one study of wrist BMD in elderly male twins, no evidence for a genetic effect on bone loss was found (Christian *et al.,* 1989), whereas in another study of younger female twins, strong genetic effects on axial bone loss were observed (Kelly *et al.,* 1993). Further research is required to evaluate the role that genetic factors play in regulating bone loss.

Other Osteoporosis-Related Phenotypes

Other phenotypes such femoral neck geometry (Arden *et al.,* 1996; Flicker *et al.,* 1996), ultrasound properties of bone (Arden *et al.,* 1996; Knapp *et al.,* 2003; Hunter *et al.,* 2001b), and biochemical markers of bone turnover (Garnero *et al.,* 1996a; Hunter *et al.,* 2001a) have also been shown to have a strong heritable component. For example, heritability of biochemical markers of bone turnover has been estimated to range between 29% and 74% depending on the marker studied (Hunter *et al.,* 2001a), whereas heritability of quantitative ultrasound measurements have been reported to range between 53% and 62% (Arden *et al.,* 1996). The heritability of hip axis length and other aspects of femoral neck geometry has been estimated to range between 61% and 85% (Arden *et al.,* 1996; Koller *et al.,* 2001).

Fragility Fracture

Family history of fracture has been shown in several studies to be a risk factor for fracture independently of BMD (Cummings *et al.,* 1995; Torgerson *et al.,* 1996) and, in keeping with this, several investigators have reported that fracture has a significant heritable component. Studies of postmenopausal women and their first degree relatives from the United States showed that the heritability of wrist fracture was about 25% (Deng *et al.,* 2000), whereas similar studies in a cohort of female twins from the United Kingdom suggested that the heritability of wrist

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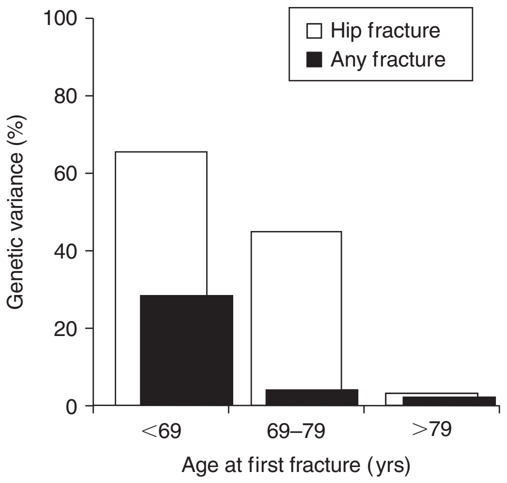

FIGURE 1 Changes in heritability of fracture with age. The genetic contribution to osteoporotic fracture (expressed as proportion of genetic variance) decreases with age. Data from Michaelsson *et al*. (2005).

fracture may be as much as 54% (Andrew *et al.,* 2005). Interestingly, the heritable component to wrist fracture in both of these studies seemed largely independent on BMD, suggesting that predisposition may have been mediated through genetic influences on bone turnover and bone geometry or nonskeletal risk factors such as cognitive function, neuromuscular control, or visual acuity. Contrasting with these observations, another heritability study of elderly twins from Finland showed little evidence to suggest that fractures were heritable (Kannus *et al.,* 1999). The largest and most comprehensive study of heritability of fracture was that of Michaelsson who elegantly demonstrated in a large study of Swedish twins that the heritability of fracture was high in those under the age of 69 years, but dropped off rapidly with age to reach a value of almost zero by the eighth decade (Michaelsson *et al.,* 2005) (Fig. 1).This illustrates that genetic factors play an important role in predisposing to fracture in younger people, but with increasing age, environmental factors come into play and eventually predominate.

PATTERNS OF INHERITANCE OF OSTEOPOROSIS

Segregation analysis in families has shown that regulation of BMD and other osteoporosis-related phenotypes is polygenic and determined by the effects of several genes each with relatively small effects rather than by a small number of genes with large effects (Gueguen *et al.,* 1995). Evidence has been presented to suggest that genes with larger effects may be involved at least in some populations (Liu *et al.,* 2004b; Deng *et al.,* 2002a). Irrespective of the contribution made by individual genetic variants, it is clear that these interact with environmental factors to regulate BMD, bone turnover, bone geometry, and susceptibility to fracture (Krall and Dawson-Hughes, 1993). Under some circumstances susceptibility to osteoporosis and fracture can be inherited in a Mendelian manner. Examples are osteogenesis imperfecta, owing to mutations in the type I collagen genes (Rowe, 1991) or the CRTAP gene (Barnes *et al.,* 2006), and osteoporosis-pseudoglioma syndrome owing to inactivating mutations in the *LRP5* gene (Gong *et al.,* 1998, 2001). Families have also been described with an inherited form of osteopetrosis linked to chromosome 11p12 where the causal gene has not been discovered (Vidal *et al.,* 2007). Osteoporosis can also form part of the phenotype in patients with inactivating mutations in the aromatase gene (Morishima *et al.,* 1995) and estrogen receptor alpha gene (Smith *et al.,* 1994). Other syndromes have been described in which affected individuals have high bone mass and are protected against osteoporotic fractures. Examples are the various autosomal dominant high-bone-mass syndromes associated with activating mutations in the *LRP5* gene (Johnson *et al.,* 1997; Van Wesenbeeck *et al.,* 2003; Little *et al.,* 2002; Boyden *et al.,* 2002) and the recessive syndromes of Sclerosteosis and Van Buchem disease that are caused by inactivating mutations in the Sclerostin (SOST) gene (Balemans *et al.,* 2001, 2002, 2005; Brunkow *et al.,* 2001). Interestingly, individuals who are heterozygous for disease-causing mutations in SOST also have high bone mass (Gardner *et al.,* 2005). In all of these examples, the consequences of the gene mutation on bone cell function are so profound as to overwhelm the effects of the many other genes that contribute to regulation of bone mass. However, even in such extreme cases, it is sometimes possible to identify polygenic effects on disease severity. The best examples of this are in osteogenesis imperfecta, where disease severity can vary markedly within and between families who have identical mutations in the collagen genes, presumably owing to the influence of other genes on bone mass and bone fragility (Willing, Cohn, and Byers, 1990).

IDENTIFYING OSTEOPOROSIS SUSCEPTIBILITY GENES

Several approaches have been used to identify the genes responsible for osteoporosis including linkage analysis in families, association studies in unrelated subjects, and animal studies (Fig. 2). Studies in experimental animals can also be of value in identifying the genes responsible for human disease, based on the assumption that key regulatory genes will be shared across species. Because these approaches are dealt with in more detail elsewhere (see Chapter 20) further discussion in this chapter will focus principally on the results of linkage and association studies of osteoporosis that have been performed in humans.

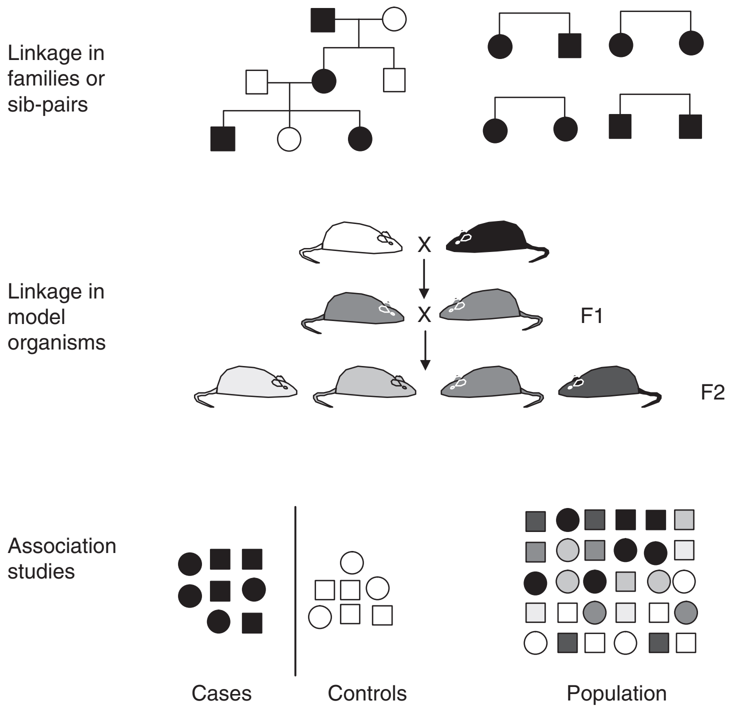

FIGURE 2 Techniques for detecting genes that predispose to osteoporosis. (**Top**) Human linkage studies are based on phenotyping members of families for the trait of interest (e.g., BMD) and relating inheritance of genetic markers in relation to inheritance of the phenotype. Phenotype can be dichotomized into affected (filled symbols) or unaffected (open symbols), or analyzed as a quantitative trait. (**Middle**) Linkage studies in animals typically involve setting up an experimental cross between two inbred mouse strains with opposing phenotypes (e.g., high and low BMD). The first generation of offspring (F_1) have intermediate BMD values owing to inheritance of high BMD alleles from one parent and low BMD alleles from the other. Brother and sister mating of the F1 generation results in a second generation of offspring (F_2) that have varying levels of BMD owing to recombination between parental chromosomes. By conducting a genome search in the F_2 generation, it is possible to relate inheritance of alleles from the parental strains to BMD levels, thereby localizing the genetic variants that regulate BMD. (**Bottom**) Association studies involve comparing frequency of alleles of candidate genes in unrelated subjects. This can either take the form of a case-control study (*bottom left*) in which patients with osteoporosis, or osteoporotic fractures (filled symbols) are compared with unaffected subjects (open symbols). Association studies can also be performed in population-based studies of individuals who have varying BMD (*bottom right*). Here, subjects are grouped according to carriage of alleles at the locus of interest and BMD values are compared in different genotype groups by analysis of variance.

LINKAGE ANALYSIS

Overview of Methodology

The classical approach for gene discovery is parametric linkage analysis that involves specifying a model of inheritance for the disease within a family (such as dominant or recessive) and looking for evidence of segregation of the disease in family members according to that model. Genotype data are then analyzed to look for evidence of segregation of alleles with the phenotype according to the specified disease model. Because the purpose of linkage analysis is to localize a disease gene, linkage studies are usually carried out on a genome-wide basis (Genome Wide Scan), which classically involves genotyping between 400 and 800 microsatellite markers spread at 5- to 10-cM intervals across the genome. In recent years however, higher-density panels of single nucleotide polymorphism (SNP) markers have also been used for genome-wide searches (Sawcer *et al.*, 2004). The related technique of nonparametric linkage uses a similar approach, but here no disease model is specified and instead the aim is to look for evidence of allele sharing in relation to sharing of the disease phenotype. For quantitative traits, variance component methods (Almasy and Blangero, 1998) or regression-based methods (Abecasis *et al.*, 2002) can be employed to estimate the proportion of genetic covariance between relatives as a function of identity by descent relationships at a marker, assuming that the marker is tightly linked to the disease-causing mutation. The results of linkage studies are typically expressed as lodscores that are defined as the logarithm of the odds that the disease locus and marker locus are linked. In parametric analysis, linkage is considered significant when the lodscore is greater than +3.3, whereas linkage is considered to be "suggestive" when the lodscore is greater than +1.9. Conversely, linkage can be excluded when the lodscore is less than −2.0. For nonparametric analysis, significant linkage is defined by a lodscore of more than about +3.6 and suggestive linkage by a lodscore greater than +2.2 (Nyholt, 2000). It is not possible to exclude linkage by nonparametric analysis.

Linkage studies in Osteoporosis

A few investigators have attempted to employ parametric linkage analysis to detect susceptibility loci for osteoporosis but so far this has not proved very fruitful (Devoto *et al.,* 1998; Spotila *et al.,* 1996; Vidal *et al.,* 2007). In view of this, most studies have been performed using non-parametric linkage analysis focusing principally on BMD as the phenotype of interest (Nui *et al.,* 1999; Devoto *et al.,* 2001; Karasik *et al.,* 2002a; Deng *et al.,* 2002b; Koller *et al.,* 2000; Ralston *et al.,* 2005; Streeten *et al.,* 2006; Kammerer *et al.,* 2003; Styrkarsdottir *et al.,* 2003; Hsu *et al.,* 2007). However the same approach has been successfully used to detect quantitative trait loci (QTL) for femoral neck geometry (Peacock *et al.,* 2005b; Koller *et al.,* 2003; Deng *et al.,* 2003; Demissie *et al.,* 2007) and ultrasound properties of bone (Wilson *et al.,* 2004; Karasik *et al.,* 2002b). Several genome-wide scans performed so far have identified QTL that meet the criteria for genome-wide significance for BMD but there has been limited replication of QTL between studies and a recent meta-analysis of nine genome-wide scans involving more than 11,842 subjects failed to detect evidence of genome-wide significance for any QTL (Ioannidis *et al.,* 2007). Some interesting findings have emerged from these studies, however. Most investigators who analyzed men and women together with adjustment for gender in the statistical model have identified different QTL than when gender-specific subgroup analysis has been performed (Ralston *et al.,* 2005; Streeten *et al.,* 2006; Karasik *et al.,* 2003; Hsu *et al.,* 2007; Kammerer *et al.,* 2003; Peacock *et al.,* 2005a; Koller *et al.,* 2000). This suggests that BMD is largely regulated in a gender-specific manner, as has been reported in mice (Orwoll *et al.,* 2001). Similar findings have been observed when data from old and young individuals has been analyzed separately rather than adjusting for age as a continuous variable in the statistical model (Karasik *et al.,* 2003; Ralston *et al.,* 2005; Kammerer *et al.,* 2003). This indicates that the genetic and environmental determinants of peak bone mass probably differ from those that regulate bone loss. Another consistent finding to emerge from genome-wide linkage studies is that the variants that regulate BMD largely do so in a site-specific manner because there has been very limited overlap between linkage peaks for BMD at different skeletal sites such as the spine and hip (Hsu *et al.,* 2007; Ralston *et al.,* 2005; Ioannidis *et al.,* 2007). This mirrors experience in other complex diseases (Altmuller *et al.,* 2001) and might reflect the fact that genes that regulate BMD differ in different populations or that genes that predispose to osteoporosis have modest effects that are difficult to detect reproducibly by conventional linkage analysis. To date, only one candidate gene for osteoporosis has been detected by genome-wide linkage scan. This is the *BMP2* gene that was discovered as the result of a positional cloning effort on a locus identified by a genome-wide scan in the population isolate of Iceland (Styrkarsdottir *et al.,* 2003). In this study, a coding variant of *BMP2* was implicated in the regulation of BMD in Icelandic and Danish subjects (Styrkarsdottir *et al.,* 2003). Nonetheless, this variant only accounted for part of the linkage signal and the association with BMD and fracture was not replicated in a study from Holland (Medici *et al.,* 2006).

There have been fewer genome-wide linkage scans for loci that regulate femoral neck geometry. Those that have been performed have detected evidence of significant linkage to some chromosomal regions, but there has been limited replication of peaks between studies (Peacock *et al.,* 2005b; Koller *et al.,* 2001; Demissie *et al.,* 2007). As in BMD, gender-specific effects in regulating bone geometry have been observed (Peacock *et al.,* 2005b). Two genome-wide scans have been carried out in relation to ultrasound properties of bone (Karasik *et al.,* 2002b; Wilson *et al.,* 2004), with differing results. Neither study detected QTL, which reached genome-wide significance, although several suggestive linkage peaks were detected.

ASSOCIATION STUDIES

Overview of Methodology

An association study typically involves studying polymorphisms in a candidate gene in a series of cases and controls and determining whether the allele distribution differs in the two groups by a chi-square test. For quantitative traits, such as BMD or ultrasound, the mean values are calculated according to genotype or allele at the chosen polymorphism and differences assessed by analysis of variance, usually with inclusion of confounding factors such as age, body weight and menopausal status in the statistical model. For many years, association studies were performed in relation to polymorphisms in candidate genes, but advances in genotyping technologies have now made it possible to perform association studies on a genome wide basis by analyzing large numbers (300,000 to 500,000) of closely spaced SNPs spread at regular intervals across the genome (Cardon and Abecasis, 2003). The rationale for this approach is that the SNP tested will be in linkage disequilibrium with causal variants in genes that predispose to the disease. Genome-wide association studies have been successfully applied to the study of many complex diseases and this approach is also now being applied to the study of osteoporosis and related phenotypes. Association studies are relatively easy to perform and can be powered to detect small effects of alleles, but they can give spurious results owing to population stratification, particularly when the sample sizes are limited and when insufficient care has been paid to matching cases and controls. These problems have probably been overestimated in the past, but can be circumvented in part by careful study design with statistical correction for confounding factors (Hoggart *et al.*, 2003). Another problem with candidate gene association studies is that they do not provide an opportunity for identifying new genes. This is a major advantage of genome-wide association studies that are "hypothesis free" and leave open the possibility of identifying novel genes and pathways that predispose to the trait under investigation. A disadvantage of genome-wide association approaches is that they involve performing a very large number of individual tests, which means that the statistical thresholds for significance have to be extremely stringent. In practice, most investigators use an approach whereby positive hits from first-round genome-wide association studies are analyzed in replication cohorts to confirm that the results are genuine.

The transmission disequilibrium test (TDT) is a special type of association study that is performed in related individuals and often used to confirm the results obtained from population-based association studies (Spielman *et al.*, 1993). The TDT tests the hypothesis that a polymorphism or allele contributes to disease by analyzing the frequency with which affected individuals inherit the allele from a heterozygous parent. If the allele contributes to the trait or disease of interest, then the probability that an affected person has inherited the allele from a heterozygous parent should vary from the expected Mendelian ratio of 50:50. Because the transmitted allele acts as the "case" and the nontransmitted allele acts as the "control," the TDT is unaffected by confounding owing to population stratification. Although TDT is a valuable technique, one important disadvantage is that only heterozygous individuals are informative, which can reduce the effective sample size available for study and limit statistical power.

When an association has been identified and replicated, defining a causative role for any individual polymorphism is difficult. This involves an exhaustive analysis of the region surrounding the (potentially) causal polymorphism to determine whether other polymorphisms nearby might be driving the association and functional studies to explore the biological effects of putative disease-causing polymorphisms on protein function and/or gene transcription *in vitro* and *in vivo*.

CANDIDATE GENES FOR OSTEOPOROSIS SUSCEPTIBILITY

Many candidate gene associations in the osteoporosis field are based on single studies with relatively small sample sizes that have not been replicated in different populations. In view of this, the following discussion will mainly focus on candidate genes that have been studied across several populations.

COLLAGEN TYPE I ALPHA 1 (*COL1A1*)

Type I collagen is a major structural protein of bone, and the genes encoding this protein (*COL1A1* and *COL1A2*) are strong candidates for the genetic regulation of bone mass. Mutations affecting the *COL1A1* and *COL1A2* genes are responsible for the vast majority of cases of osteogenesis imperfecta, a hereditary disease characterized by premature osteoporosis and bone fragility (Rowe, 1991). Polymorphisms affecting the coding regions of the collagen type I genes are rare and do not appear to be associated with osteoporosis (Spotila *et al.*, 1994), but there is accumulating evidence that polymorphisms in the regulatory regions of *COL1A1* regulate bone mass and susceptibility to osteoporotic fracture. In 1996 a polymorphisms was identified within intron 1 of the *COL1A1* gene that affected a binding site for the transcription factor Sp1, which was more prevalent in osteoporotic vertebral fracture patients than with controls and was found to be associated with BMD (Grant *et al.*, 1996). Subsequent work showed positive associations between the *COL1A1* Sp1 polymorphism bone mass and/or osteoporotic fractures in many populations (Garnero *et al.*, 1998; Langdahl *et al.*, 1998; Uitterlinden *et al.*, 1998; Roux *et al.*, 1998; Alvarez *et al.*, 1999; Weichetova *et al.*, 2000; McGuigan *et al.*, 2000; Braga *et al.*, 2000; Keen *et al.*, 1999; Langdahl *et al.*, 1998; Liu *et al.*, 2004a; Efstathiadou *et al.*, 2001), although not all studies have yielded positive results (Pluijm *et al.*, 2004; Ashford *et al.*, 2001; Berg *et al.*, 2000; Heegaard *et al.*, 2000). Three independent meta-analyses concluded that the *COL1A1* Sp1 polymorphism was associated with osteoporotic fractures (Efstathiadou *et al.*, 2001) and bone density (Mann and Ralston, 2003; Lohmueller *et al.*, 2003). In one study the increased risk of fracture was found to be greater than expected for the allele-specific difference in BMD (Mann and Ralston, 2003). The largest individual study of the *COL1A1* Sp1 polymorphism in relation to BMD and fracture performed to date is the GENOMOS study where a participant-level meta-analysis of data was performed in 20,786 subjects. In this study *COL1A1* Sp1 alleles were found to be associated with spine and hip BMD with a recessive model of inheritance, which contrasts with the codominant model reported in other studies and meta-analyses. The Sp1 polymorphism was also associated with incident vertebral fractures in women from this study with an effect size similar to that reported in previous studies (Ralston *et al.*, 2006).

The relationship between *COL1A1* alleles and bone loss has been studied by two groups, following on from the observations made by Uitterlinden who found that genotype-related differences in BMD increased with age (Uitterlinden *et al.*, 1998). In the study by Harris and colleagues, the *COL1A1* "TT" genotype was strongly associated increased bone loss over a 5-year period in 243 elderly men and women (Harris *et al.,* 2000), and similar findings were reported by MacDonald in a large-scale study involving more than 3000 perimenopausal women (MacDonald *et al.,* 2001). However, in another study of 133 postmenopausal women monitored for more than 18 years no association between the *COL1A1* Sp1 polymorphism and bone loss was reported (Heegaard *et al.,* 2000). The *COL1A1* Sp1 polymorphism has been studied in relation to BMD in children and adolescents but the results have been contradictory. In one study, *COL1A1* alleles were strongly related to volumetric BMD measured by quantitative computed tomography in Mexican-American girls (Sainz *et al.,*1999). In another study, an association was reported with BMD in Finnish girls during early puberty (Suuriniemi *et al.,* 2006). However, no association was found between *COL1A1* alleles and BMD as measured by DEXA in another study of prepubertal Caucasian girls (Tao *et al.,* 1999).

The *COL1A1* Sp1 polymorphism has also been associated with bone size and femoral neck geometry. In one study, the Sp1 "T" allele was associated with an increased femoral neck-shaft angle (Qureshi *et al.,* 2001), and another study showed an association with the "T" allele and reduced wrist size, although no effects on bone size were found at the hip (Long *et al.,* 2004).

The population prevalence of the *COL1A1* Sp1 polymorphism differs markedly in different populations. The osteoporosis associated "T" allele is relatively common in Caucasian populations, but is rare in the African subcontinent and seems to be virtually absent from Asian populations (Beavan *et al.,* 1998; Nakajima *et al.,* 1999; Lau *et al.,*2004). This raises the possibility that differences in population prevalence of *COL1A1* Sp1 alleles may contribute to ethnic differences in fracture risk (Beavan *et al.,* 1998).

Extensive studies have been performed on the molecular mechanism by which the Sp1 polymorphism predisposes to osteoporosis. The osteoporosis-associated *COL1A1* "T" allele has higher affinity for Sp1 protein binding than the wild-type "G" allele and the allele-specific transcription from the "T" allele has been found to be 3-fold higher than the "G" allele in heterozygotes. In keeping with this, cultured osteoblasts from subjects who are heterozygous for the G/T polymorphism produce increased amounts of collagen alpha 1 protein relative to alpha 2 *in vitro* compared with "GG" homozygotes, and also express increased amounts of *COL1A1* mRNA relative to *COL1A2* mRNA. These differences in *COL1A1* transcription are accompanied by differences in the mechanical strength of bone and bone mineralization. Biomechanical studies have shown that bone cores from G/T heterozygotes have significantly reduced bone strength *ex vivo* than those from GG homozygotes and also are less well mineralized (Stewart *et al.,* 2005; Mann *et al.,* 2001). Corresponding with this, studies *in vitro* have also shown evidence of defective mineralization in bone cores cultured from Sp1 G/T heterozygotes compared with G/G homozygotes. Overall, the data are consistent with a model whereby the "T" allele of the *COL1A1* Sp1 polymorphism increases *COL1A1* gene transcription, which leads to increased collagen alpha 1 protein production, an abnormal ratio of alpha 1 to alpha 2 protein chains, a subtle defect in bone mineralization, and reduced bone strength, leading to an increased risk of fracture (Fig. 3).

Polymorphisms have also been described in the promoter region of the *COL1A1* gene that are in linkage disequilibrium with the Sp1 polymorphism, including an insertion/deletion polymorphism in a polythymidine tract at position −1663 (−1663indelT) and a G/T polymorphism at position −1997 (−1997G/T). The −1997G/T polymorphism was found to be associated with BMD in Spanish postmenopausal women and to interact with the Sp1 polymorphism in regulating BMD (Garcia-Giralt *et al.,* 2002). Similar findings were reported in another group of women from the United States (Liu *et al.,* 2004a). The largest study of these polymorphisms is that of Stewart who reported that haplotypes defined by all three polymorphisms regulated spine and hip BMD in women from the United Kingdom with effects that were stronger than those of the individual SNP (Stewart *et al.,* 2006). There is evidence that the promoter polymorphisms are functional. The −1663indelT polymorphism is situated at a binding site for the transcription factor NMP4 and promoter-reporter assays show that different promoter haplotypes differed in their ability to regulate reporter gene expression with high levels of transcription associated with the −1997G −1663delT haplotype (Garcia-Giralt *et al.,* 2005).

In summary, current evidence suggests that common allelic variants in the 5′ flank of the *COL1A1* gene are associated with bone mass and susceptibility to osteoporotic fracture, and in particular, vertebral fractures. Three polymorphisms have been identified in the promoter at positions −1997 and −1663 and one in the intron at position +1245 affecting a Sp1 binding site. There is evidence to suggest that at least two of these polymorphisms have functional effects on DNA binding and *COL1A1* transcription, and it appears that this affects collagen protein production and bone strength. It remains unclear, however, to what extent the individual polymorphisms are responsible for the clinical associations that have been reported.

ESTROGEN RECEPTOR ALPHA (*ESR1*)

Estrogen, by interacting with its receptors in bone and other tissues, plays an important role in regulating skeletal growth and maintenance of bone mass. Knockout mice for ERα and ERβ have reduced BMD compared with wild-type controls (Windahl *et al.,* 1999; Korach, 1994) and osteoporosis has also been observed in a man with an inactivating mutation of the ERα gene (Smith *et al.,* 1994). These data indicate that the estrogen receptor genes *ESR1* and *ESR2* genes are strong candidates for genetic regulation of bone

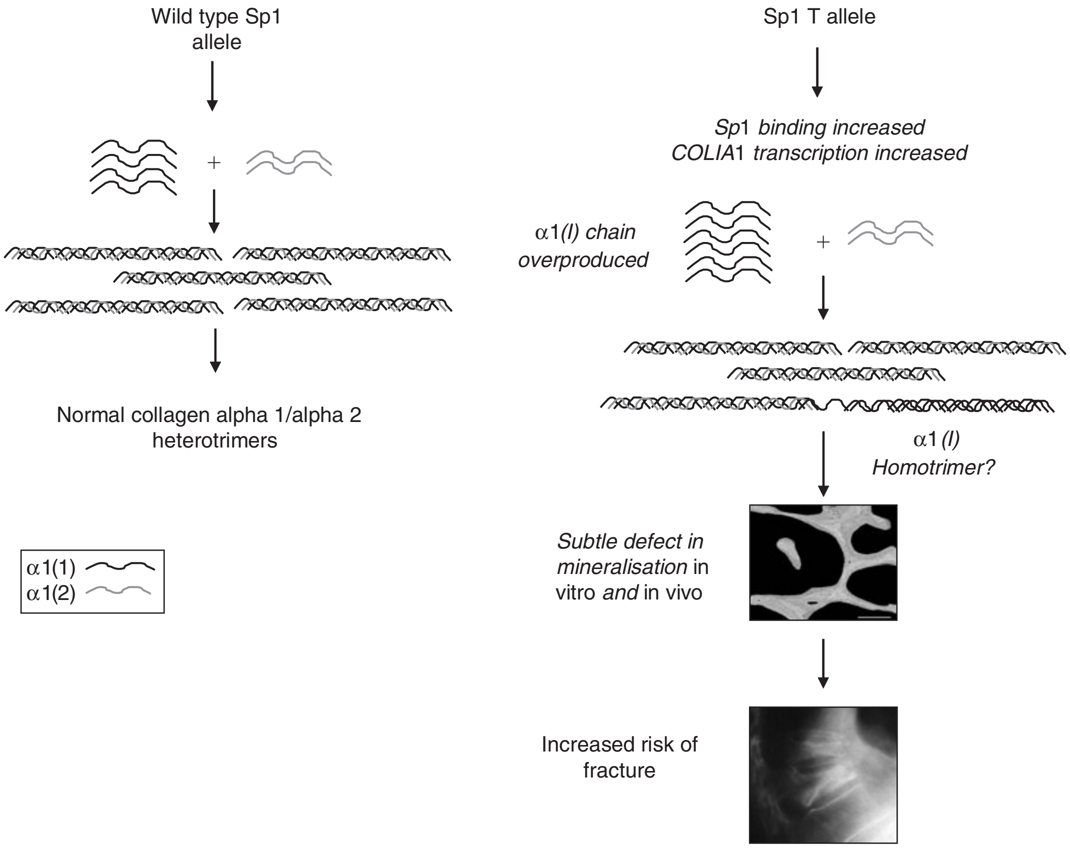

FIGURE 3 Mechanisms of osteoporosis mediated by *COL1A1* Sp1 polymorphism. The *COL1A1* Sp1 T allele has increased affinity for Sp1 binding and *COL1A1* mRNA abundance is increased in G/T heterozygotes. This causes an imbalance in the ratio of alpha 1 to alpha 2 chains, probably resulting in accumulation of collagen 1 alpha 1 homotrimer. This adversely affects mineralization, reduces bone strength, and predisposes to osteoporotic fracture.

mass. Sano *et al.* (1995) reported a positive association between a TA repeat polymorphism in the *ESR1* gene promoter and bone mass in a small Japanese study. Similar results were reported by groups in the United States and Italy (Sowers *et al.,* 1999; Becherini *et al.,* 2000). Other investigators have reported positive associations between haplotypes defined by *PvuII* and/or *XbaI* polymorphisms in the first intron of the *ESR1* gene and bone mass (Kobayashi *et al.,* 1996; Sowers *et al.,* 1999; Mizunuma *et al.,* 1997; Ongphiphadhanakul *et al.,* 1998; Albagha *et al.,* 2001b) as well as age at menopause (Weel *et al.,* 1999). In contrast, other studies in Korean (Han *et al.,* 1997), Belgian (Vandevyer *et al.,* 1999), and Italian (Gennari *et al.,*1998) women found no association between *PvuII* polymorphisms and bone mass.

Polymorphisms of *ESR1* have also been studied in relation to postmenopausal bone loss. In a longitudinal study of 322 Finnish women, increased rates of early postmenopausal bone loss were observed in women who carried the "P" allele at the *ESR1 PvuII* polymorphism (Salmen *et al.,* 2000), but this was not confirmed by another study in the United States (Willing *et al.,* 1998). In contrast, a relatively large-scale study involving 3054 women in the United Kingdom showed higher rates of bone loss in women who carried the px haplotype, lower femoral neck BMD in postmenopausal women, and reduced calcaneal broadband ultrasound attenuation (Albagha *et al.,* 2005). A meta-analysis of association studies performed up until 2002 involving 5834 participants showed no evidence of an association between BMD and fracture for the *PvuII* polymorphism but a positive association between BMD and fracture for the *XbaI* polymorphism, with a protective effect of the XX genotype (Ioannidis *et al.,* 2002). Another large-scale individual level meta-analysis from the GENOMOS study involving 18,917 individuals showed no association between the TA repeat, *PvuII*, or *XbaI* polymorphism with BMD, but a significant association between the *XbaI* polymorphism and fracture, which was independent of BMD (Ioannidis *et al.,* 2004).

The molecular mechanism by which *ESR1* polymorphisms influence osteoporosis and fracture are unclear, but there is evidence that the intron polymorphisms may affect gene transcription. For example, the *PvuII* polymorphism lies within consensus recognition sites for the AP4 and Myb transcription factors (Albagha *et al.,* 2001a; Herrington *et al.,* 2002b) and promoter-reporter assays have shown that the *PvuII* polymorphism influences Myb-driven transcription *in vitro* (Herrington *et al.,* 2002a). Other studies have suggested that the *XbaI* and *PvuII* polymorphisms influence reporter gene transcription *in vitro* (Maruyama *et al.,* 2000). In this regard, it is of interest that the *PvuII* and *XbaI* polymorphisms are located within a region that is 70 to 80% conserved in the human, mouse, and rat genomes, whereas the TA repeat polymorphism is not conserved to any significant extent across species, suggesting that the intron plays an important role in regulating *ESR1* function.

In summary, there is evidence that the *PvuII* and *XbaI* polymorphisms within intron 1 of *ESR1* are functional variants. Effects on BMD, bone loss, and ultrasound properties of bone have been reported but the data remain conflicting. The association between the *XbaI* polymorphism and fractures noted in the GENOMOS study is an intriguing finding and raises the possibility that *ESR1* alleles may predispose to fracture by a mechanism independent of BMD.

LIPOPROTEIN RECEPTOR-RELATED PROTEIN 5 (*LRP5*)

The Lipoprotein Receptor-Related Proteins encoded by the *LRP5* and *LRP6* genes are transmembrane proteins that function as coreceptors for canonical Wnt signaling. Wnt signaling plays an important role in several key developmental processes including cell fate decisions, limb patterning, osteoblast and chondrocyte differentiation, and development of the central nervous system and of other organs (Johnson *et al.,* 2004).

The *LRP5* pathway was discovered to be a key regulator of bone mass following linkage studies in two rare human diseases: the osteoporosis-pseudoglioma syndrome (OPPS), which is a recessively inherited condition characterized by severe, early-onset osteoporosis and congenital blindness owing to vitreous opacity (Gong *et al.,* 1998), and the high-bone-mass (HBM) syndrome, which is an asymptomatic autosomal dominant disorder characterized by increased bone mineral density (Johnson *et al.,* 1997). Both of these conditions were mapped to the same region of chromosome 11*q*12 in the late 1990s and different mutations in the *LRP5* gene were identified as the cause of both disorders by positional cloning studies (Gong *et al.,* 2001; Little *et al.,* 2002; Boyden *et al.,* 2002). The high-bone-mass syndrome was found to be caused by a heterozygous missense mutation causing a substitution of valine for glycine at codon 171 (G171V) of *LRP5* within the first beta-propeller motif of the molecule (Little *et al.,* 2002). The OPPS syndrome was found to be caused by different homozygous missense, nonsense, and frameshift mutations throughout the gene (Gong *et al.,* 2001). Since these original reports, several additional missense mutations of *LRP5* have been identified as a cause of high bone mass and all of these cluster in or around the first beta-propeller motif of *LRP5* (Van Wesenbeeck *et al.,* 2003).

There is evidence that subtle variations in *LRP5* underlie variation of BMD in the general population. Several association studies and family-based studies have been performed in which various common polymorphisms of *LRP5* have been related to BMD and/or osteoporotic fracture. Most of these studies have shown evidence of associations between *LRP5* alleles and BMD, and interestingly, these associations have been particularly strong in men (Urano *et al.,* 2004; Koh *et al.,* 2004; Ferrari *et al.,* 2004; van Meurs *et al.,* 2006). Although many variants have been studied, the most likely functional candidates are an alanine to valine amino acid substitution at position 1330 (A1330V) and a valine to methionine variant at codon 667 (V667M). Evidence of an interaction between the *LRP5* A1330V variant and a coding polymorphism of LRP6 (1062V) has also been gained in the Rotterdam study, where polymorphisms of both genes were found to have an additive effect on fracture susceptibility (van Meurs *et al.,* 2006). Interactions between *LRP5* and *LRP6* as regulators of BMD have also been observed in preclinical studies where skeletal phenotyping of mice with targeted inactivation of both receptors revealed allele dose-dependent deficits in BMD and limb formation, suggesting that there is functional redundancy between these two genes in regulating bone and limb development (Holmen *et al.,* 2004).

Functional studies have shown that the mutations that cause OPPS produce a truncated or nonfunctional *LRP5* protein (Gong *et al.,* 2001) and inactivation of *LRP5* by gene targeting in mice causes a low-bone-mass phenotype, which provides a phenocopy for the human disease syndrome (Kato *et al.,* 2002). Analysis of bone histomorphometry from these mice has shown that the low bone mass is a consequence of decreased osteoblast proliferation and reduced bone matrix deposition rather than an increased bone resorption (Kato *et al.,* 2002). The G171V mutation that was associated with high bone mass in the family studied by Johnson and colleagues (Little *et al.,* 2002; Johnson *et al.,* 1997) was found to cause increased bone mass when expressed in transgenic mice (Babij *et al.,* 2003). In these studies, the mineral apposition rate was increased and the rate of osteoblast apoptosis was reduced, whereas eroded surface (reflecting bone resorption) was unaffected. The mutations of *LRP5* that cause high bone mass seem to upregulate β-catenin signaling by inhibiting interactions between LRP5 and Dkk1 an inhibitor of Wnt signaling. Studies of Boyden and colleagues (Boyden *et al.,* 2002) showed that the G171V mutation did not result in constitutive activation of *LRP5* signaling *in vitro* but instead that the mutation impaired Dkk1-mediated inhibition of Wnt-stimulated *LRP5* signaling. Another study reached the same conclusion in showing that several HBM-associated mutants (G171V, G171R, A214T, A214V, A242T, T253I, and D111Y) were resistant to Dkk1 inhibition compared with wild-type *LRP5* and had lower affinity for Dkk1 binding (Ai *et al.,* 2005). Less work has been done on the *LRP5* polymorphisms, but promoter-reporter assays have indicated that different haplotypes for the V667M and A1330V polymorphisms differ in their ability to activate reporter gene transcription, indicating that they are also functional (Kiel *et al.,* 2007).

In conclusion, the data so far indicate that genetic variation in *LRP5* and possibly *LRP6* plays an important role in regulation of bone mass and susceptibility to osteoporotic fractures in humans. Not only do rare mutations in the *LRP5* gene play a major role in regulating BMD, but more subtle polymorphisms seem also to regulate BMD in the normal population.

TRANSFORMING GROWTH FACTOR BETA 1 (*TGFB1*)

Transforming growth factor beta 1 (TGFβ1) encoded by the *TGFB1* gene is a cytokine with pleiotropic effects on cell growth and differentiation. TGFβ1 plays an important role in bone metabolism where it is thought to act as a coupling factor between bone resorption and bone formation. Mutations in *TGFB1* have been described in the syndrome of Camurati–Engelmann disease (CED), a rare disorder characterized by increased bone turnover, bone pain, and osteosclerosis, mainly affecting the diaphysis of long bones (Janssens *et al.,* 2000; Kinoshita *et al.,* 2000). The mutations that cause CED mainly cluster in the latency-associated peptide (LAP) region of TGFβ1 and prevent or inhibit binding of LAP to the mature TGFβ1 molecule (Janssens *et al.,* 2003). The effect of this is to increase levels of bioactive TGFβ1 that presumably is the cause of the increased bone turnover in the disease (McGowan *et al.,* 2003). A large number of studies have been performed on possible associations between polymorphisms in *TGFB1* and osteoporosis-related phenotypes. A rare C-deletion polymorphism in intron 4 of *TGFB1* has been associated with low BMD, increased bone turnover, and osteoporotic fracture in one study from Denmark (Langdahl *et al.,* 1997) and very similar results were recently reported in another study from Italy (Bertoldo *et al.,* 2000). Although this polymorphism is close to the splice junction, it does not affect the splice acceptor site and the functional effects on TGFβ1 function (if any) are unknown. Another polymorphism of the *TGFB1*-coding region has been described that causes a leucine-proline substitution in the signal peptide region of TGFβ1 at amino acid 10. The C allele of the codon 10 polymorphism has been associated with high BMD and a reduced frequency of osteoporotic fractures in two Japanese populations (Yamada *et al.,* 1998), with BMD in Japanese adolescents (Yamada *et al.,* 1999), and with reduced rates of bone loss and improved response to treatment with alphacalcidol, an active metabolite of vitamin D (Yamada *et al.,* 2000). This polymorphism is associated with raised circulating levels of TGFβ1 suggesting that it may influence protein secretion or stability. However, two promoter polymorphisms of TGFβ1 have been described that are also associated with circulating TGFβ1 levels (Grainger *et al.,* 1999). The largest study of *TGFB1* polymorphisms in relation to osteoporosis phenotypes was that of McGuigan who performed a comprehensive analysis of common polymorphisms in relation to BMD, bone loss, biochemical markers of bone turnover, and fracture (McGuigan *et al.,* 2007). This study showed strong linkage disequilibrium between the polymorphisms, but no convincing association between BMD, bone loss, or fracture. In view of this, the data do not support the view that common polymorphisms of *TGFB1* contribute significantly to the genetic regulation of BMD or fracture, at least in Caucasian populations.

VITAMIN D RECEPTOR (VDR)

The active metabolites of vitamin D play an important role in regulating bone cell function and maintenance of serum calcium homeostasis by binding to the vitamin D receptor and regulating the expression of a number of response genes. Polymorphisms affecting both the 3′ and 5′ regions of the VDR gene have been studied in relation to BMD and other phenotypes relevant to the pathogenesis of osteoporosis. The first study of VDR genotypes in relation to calcium metabolism were those of Morrison who found an association between polymorphisms affecting the 3′ region of the gene and circulating osteocalcin levels (Morrison *et al.,* 1992). In a subsequent study, the same group reported a significant association between *BsmI* polymorphisms in intron 8 of VDR and BMD in a twin study and a population-based study, but this association was later found to be much weaker than originally reported because of genotyping errors (Morrison *et al.,* 1997). A large number of studies have now been carried out looking at the association between BMD and other aspects of calcium metabolism in relation to *BsmI* and other polymorphisms at the VDR locus. Several studies have supported the original findings, whereas others have found no significant association and still others have reported an inverse association to those originally reported (Houston *et al.,* 1996; Uitterlinden *et al.,* 1996). Gong and colleagues reviewed the results of 75 articles and abstracts published between 1994 and 1998 that related BMD and associated skeletal phenotypes to VDR polymorphisms. The main conclusions to emerge from this study were that there was a highly significant association between VDR polymorphisms and BMD overall and that positive results were significantly more common in studies that included premenopausal rather than postmenopausal women (Gong *et al.,* 1999). Several investigators have looked for possible associations between the VDR *BsmI* alleles and fracture. In one, a positive association was found but this was only significant in a subgroup of women aged 75 years and older (Feskanich *et al.,* 1998). Three other studies found no association between VDR 3' alleles and osteoporotic fracture (Houston *et al.,* 1996; Looney *et al.,* 1995; Ensrud *et al.,* 1999). Another common polymorphism has been described in exon 2 of the VDR gene which is a T-C transition, within exon 2 recognized by the *FokI* restriction enzyme (Arai *et al.,* 1997; Gross *et al.,* 1998a). This transition introduces an alternative translational start codon that results in a shorter isoform of the VDR gene. The *FokI* polymorphism has been associated with BMD in some studies (Harris *et al.,* 1997; Gross *et al.,* 1997; Arai *et al.,* 1997) but not in others (Eccleshall *et al.,* 1998; Langdahl *et al.,* 2000; Sowers *et al.,* 1999). The *FokI* polymorphism has also been studied in relation to osteoporotic fractures. In one study of Italian women *FokI* alleles were associated with fracture (Gennari *et al.,* 1999) but no association was found in Danish women (Langdahl *et al.,* 2000). Another common G/A polymorphism affecting a binding site for the transcription factor Cdx2 in the VDR promoter was described by Arai and found to be associated with BMD in a cohort of 261 Japanese women, with lower bone mass in carriers of the "A" allele (Arai *et al.,* 2001).

The most comprehensive single study of VDR alleles in relation to osteoporosis-related phenotypes was that of Fang and colleagues who conducted a large-scale study of haplotype tagging SNP of VDR in 6418 participants of the Rotterdam study (Fang *et al.,* 2005). As the result of this analysis, the authors identified haplotypes in the promoter and 3'-untranslated region that were associated with an increased risk of fracture. For a subgroup of individuals who carried risk alleles at both sites, the fracture risk was significantly increased by 48% when compared with control subjects. Surprisingly, the risk alleles for fracture identified in this study were not associated with differences in BMD. Although this was a large and well-conducted study, the risk estimates were modest, and if correction had been applied for all the combinations of haplotypes tested (and their interactions), the association would not have been significant. In the largest study reported to date, Uitterlinden and colleagues performed an individual level meta-analysis of common VDR polymorphisms in relation to BMD and fracture in the GENOMOS consortium. This study, which involved 26,242 subjects, found no association between VDR alleles and BMD or fracture with the exception of the Cdx2 polymorphism where subgroup analysis revealed a modest association with vertebral fracture.

Many workers have presented evidence to suggest that the relationship between VDR polymorphisms and BMD may be modified by environmental factors such as dietary calcium intake (Krall *et al.,* 1995; Ferrari *et al.,* 1995) and vitamin D status (Graafmans *et al.,* 1997). In keeping with this view, intestinal calcium absorption has been associated with the *Bsm1* VDR polymorphism in some studies (Dawson-Hughes *et al.,* 1995; Gennari *et al.,* 1997). The mechanism by which this occurs is unclear, however, and no association has been found between genotype and mucosal VDR density (Barger-Lux *et al.,* 1995; Gross *et al.,* 1998a). A positive association between the *FokI* polymorphism and intestinal calcium absorption was reported in one study (Ames *et al.,* 1999), but two other studies yielded negative results (Cauley *et al.,* 1999a; Zmuda *et al.,* 1999). The largest study of VDR alleles in relation to dietary calcium intake was that of MacDonald who, in a population study of about 3000 British women, found no association between VDR alleles and BMD. In this study no evidence of an interaction between VDR alleles, dietary calcium intake, serum 25-hydroxyvitamin D levels, and BMD was observed (MacDonald *et al.,* 2006). The only positive finding in this study was a weak association between the Cdx2 polymorphisms and bone loss, although this was not significant after correction for multiple testing.

Many investigators have conducted functional analysis of individual VDR polymorphisms and haplotypes. Reporter gene constructs prepared from the 3′ region of the VDR gene in different individuals have shown evidence of haplotype-specific differences in gene transcription, raising the possibility that polymorphisms in this region may be involved in regulating RNA stability (Morrison *et al.,* 1994). In support of this view, cell lines that were heterozygous for the TaqI polymorphism showed differences in allele-specific transcription of the VDR gene (Verbeek *et al.,* 1997). In this study, however, transcripts from the "t" allele were 30% more abundant than the "T," which is the opposite from the result expected on the basis of Morrison's results (Morrison *et al.,* 1994). In another study, evidence of differences in allele-specific transcription were observed in relation to 3′ VDR haplotypes in bone samples from male subjects in the MrOs study (Grundberg *et al.,* 2007). Specifically, carriage of haplotype 1 (baT) was associated with increased VDR mRNA abundance and this haplotype was also associated with an increased risk of fracture in men. Other *in vitro* studies have shown no differences in allele-specific transcription, mRNA stability, or ligand binding in relation to the *BsmI* polymorphism (Mocharla *et al.,* 1997; Gross *et al.,* 1998b; Durrin *et al.,* 1999). Studies *in vitro* have shown that different VDR *FokI* alleles differ in their ability to drive reporter gene expression (Arai *et al.,* 1997; Jurutka *et al.,* 2000) and the polymorphic variant lacking three amino acids ("F") has also been found to interact with human basal transcription factor IIB more efficiently than the longer isoform ("f"). Finally, peripheral blood mononuclear cells (PBMCs) from "FF" individuals were also been found to be more sensitive to the growth-inhibitory effects of calcitriol than PBMC from "Ff" and "ff" individuals (Colin *et al.,* 2000). Contrasting with these results, however, Gross and colleagues found no evidence of functional differences between *FokI* alleles in terms of ligand binding, DNA binding, or transactivation activity. There is good evidence that the Cdx2 polymorphism within the promoter of the VDR gene is functional. Arai and colleagues noted that the G allele had reduced affinity for CDx2 protein binding and also had a 70% reduced ability to drive reporter gene expression compared with the A allele (Arai *et al.*, 2001).

In summary, the studies that have been performed to date do not support the hypothesis that allelic variation in the VDR gene plays a major role in regulating bone mass or osteoporotic fracture, although there is evidence that some of the polymorphisms described have functional effects at least *in vitro*. Some evidence has been put forward to suggest that the effects of VDR alleles may be modified by dietary calcium and vitamin D intake, but the data are conflicting and large-scale studies have failed to convincingly show such interactions.

OTHER CANDIDATE GENES

A large number of other candidate genes have been studied in relation to BMD and susceptibility to osteoporotic fracture and these are summarized in Tables I to V. Further information on the potential role of these genes in regulating susceptibility to osteoporosis can be found in recent review articles (Liu *et al.*, 2003; Albagha and Ralston, 2006).

GENE–GENE INTERACTIONS

Several investigators have studied the relationship between combinations of candidate gene polymorphisms and BMD. Willing and colleagues (1998) looked at the interaction between VDR and *ESR1* polymorphisms in predicting BMD in a series of 171 postmenopausal women and found that individuals with a combination of *ESR1* PvuII "PP" and VDR "bb" genotypes had very-high-average BMD values at all skeletal sites examined. Another study by Gennari *et al.* (1998) in a population of postmenopausal Italian women showed that the combination of VDR and *ESR1* genotypes identified subgroups of individuals with very high and very low BMD. However, Vandevyer found no significant interaction between VDR and *ESR1* genotypes in predicting BMD in Belgian postmenopausal women (Vandevyer *et al.*, 1999). The best studies of gene–gene interactions were performed in the Rotterdam study.

TABLE I Candidate genes for Osteoporosis—Hormones, Receptors, and Related Factors

Function	Protein	Gene	Reference
Converts adrenal androgens to estrogen	Aromatase	CYP19	Tofteng *et al.* (2003)
Receptor for testosterone	Androgen receptor	AR	Sowers *et al.* (1999)
Receptor for extracellular calcium	Calcium-sensing receptor	CASR	Bollerslev *et al.* (2004)
Inhibits osteoclast activity	Calcitonin	CT	Miyao *et al.* (2000)
Regulates osteoclast activity	Calcitonin receptor	CTR	Masi *et al.* (1998)
Receptor for estrogen	Estrogen receptor alpha	ESR1	Ioannidis *et al.* (2002)
Receptor for estrogen	Estrogen receptor beta	ESR2	Ogawa *et al.* (2000)
Involved in estrogen metabolism	17 alpha hydroxylase	CYP17	Somner *et al.* (2004)
Receptor for gonadotrophin-releasing hormone	Gonadotrophin-releasing hormone 1	GNRH1	Iwasaki *et al.* (2003)
Receptor for glucocorticoids	Glucocorticoid receptor	GCCR	Huizenga *et al.* (1998)
Stimulates growth	Growth hormone	GH1	Dennison *et al.* (2004)
Stimulates growth	Insulin-like growth factor 1	IGF1	Rivadeneira *et al.* (2003)
Receptor for Leptin	Leptin receptor	LEPR	Koh *et al.* (2002)
Coactivator of steroid receptor action	Nuclear receptor coactivator 3	NCOA3	Sheu *et al.* (2006)
Activates bone turnover	PTH	PTH	Hosoi *et al.* (1999)
Receptor for PTH	PTHr1	PTHR1	Scillitani *et al.* (2006)
Coactivator of steroid receptor	Retinoblastoma protein-binding zinc finger protein (RIZ)	PRDM2	Grundberg *et al.* (2004)
Transport of vitamin D	Vitamin D-binding protein	DBP	Papiha *et al.* (1999)
Receptor for vitamin D	Vitamin D receptor	VDR	Uitterlinden *et al.* (2006)

TABLE II Candidate Genes for Osteoporosis—Cytokines, Growth Factors, and Receptors

Function	Protein	Gene	Reference
Stimulates osteoblast differentiation	Bone morphogenic protein 2	BMP2	Styrkarsdottir *et al.* (2003)
Stimulates osteoblast differentiation	Bone morphogenic protein 4 Chemokine CC motif receptor 2	BMP4 CCR2	Ramesh *et al.* (2005) Yamada *et al.* (2002a)
Stimulates osteoclast activity	Interleukin-1	IL1	Knudsen *et al.* (2007)
Inhibits osteoclast activity	Interleukin 1 receptor antagonist	IL1RN	Keen *et al.* (1998)
Regulates IL-1 signaling	Interleukin-1 receptor-associated kinase	IRAK	
Osteoblast differentiation	Lipoprotein receptor-related protein 5	LRP5	van Meurs *et al.* (2006)
Osteoblast differentiation	Lipoprotein receptor-related protein 6	LRP6	van Meurs *et al.* (2006)
Inhibits osteoclast activity	Osteoprotegerin	TNFRS11B	Ueland *et al.* (2007)
Inhibits osteoclast differentiation	Osteoclast-associated receptor	OSCAR	Kim *et al.* (2005)
Regulates osteoclast differentiation	Receptor activator of nuclear factor kappa b	TNFRSF11A	Koh *et al.* (2007b)
Stimulates osteoclast differentiation and function	Receptor activator of nuclear factor kappa b ligand	TNFSF11	Xiong *et al.* (2007)
Regulates TNF and IL-1 signaling	Tumor necrosis factor receptor factor 6	TRAF6	Vidal *et al.* (2007)
Regulates TNF signaling	TRAF family member-associated NFkB activator	TANK	Ishida *et al.* (2003)
Inhibits LRP5 signaling	Sclerostin	SOST	Uitterlinden *et al.* (2004)
Regulates osteoclast and osteoblast function	Transforming growth factor beta 1	TGFB1	McGuigan *et al.* (2007)
Regulates osteoclast and osteoblast function	Tumor necrosis factor receptor 1	TNFRSF1B	Albagha *et al.* (2002)

TABLE III Candidate genes for Osteoporosis—Bone Matrix Components and Modifying enzymes

Function	Protein	Gene	Reference
Component of bone matrix	Alpha-2-HS glycoprotein	AHSG	Zmuda *et al.* (1998)
Degrades bone matrix	Cathepsin K	CATK	Giraudeau *et al.* (2004)
Component of bone matrix	Collagen type 1 alpha 1	COL1A1	Ralston *et al.* (2006)
Component of bone matrix	Collagen type 1 alpha 2	COL1A2	Suuriniemi *et al.* (2003)
Component of bone matrix	Matrix Gla protein	MGP	Tsukamoto *et al.* (2000)
Degrades collagen	Matrix metalloproteinase 1	MMP1	Yamada *et al.* (2002b)
Degrades collagen	Matrix metalloproteinase 9	MMP9	Yamada *et al.* (2004)
Component of bone matrix	Osteocalcin	BGP	Dohi *et al.* (1998)
Hydroxylates lysine residues in collagen	Procollagen-lysine, 2-oxoglutarate-5-dioxegenase	PLOD1	Tasker *et al.* (2004)

TABLE IV Candidate Genes for Osteoporosis—Lipid Metabolism and Other Metabolic Pathways

Function	Protein	Gene	Reference
Involved in lipid transport	Apolipoprotein E	APOE	Cauley *et al.* (1999b)
Transcription factor involved in adipocyte differentiation	Forkhead box C2	FOXC2	Yamada *et al.* (2006)
Transmembrane protein with multiple functions involved in phosphate and calcium metabolism	Klotho	KL	Ogata *et al.* (2002)
Lactose-metabolizing enzyme	Lactase	LCT	Obermayer-Pietsch *et al.* (2004)
Enzyme involved in homocysteine metabolism	Methionine synthase reductase	MTRR	Kim *et al.* (2006)
Enzyme involved in homocysteine metabolism	Methylene tetrahydrofolate reductase	MTHFR	Abrahamsen *et al.* (2003)
Adipocyte protein involved in lipid metabolism	Perilipin	PLIN	Yamada *et al.* (2006)
Transcription factor involved in osteoblast and adipocyte differentiation	Peroxisome proliferator-activated receptor gamma	PPARG	Ogawa *et al.* (1999)

TABLE V Candidate Genes for Osteoporosis—Miscellaneous

Function	Protein	Gene	Reference
Cell cycle regulator	Cyclin-dependent kinase inhibitor 1c	CDK1	Urano *et al.* (2000)
Osteoclast chloride channel	Chloride channel 7	CLCN7	Pettersson *et al.* (2005)
Lactose-metabolizing enzyme	Lactase	LCT	Obermayer-Pietsch *et al.* (2004)
Transcription factor expressed in bone marrow	LIM domain protein RIL	PDLIM4	Omasu *et al.* (2003)
Glycoprotein involved in immune regulation	Major histocompatibility complex, class 1 A	HLA-A	Tsuji *et al.* (1998)
Transcription factor involved in regulating osteoclast differentiation	Microphthalmia-associated transcription factor	MITF	Koh *et al.* (2007a)
Receptor for semaphorin 3A; implicated in neural control of bone remodeling	Plexin A2	PLXNA2	Hwang *et al.* (2006)
Esterases involved in metabolizing toxins and oxidized lipids	Paranoxase 1 and 2	PON1/ PNO2	Yamada *et al.* (2003)
Component of osteoclast proton pump	T-cell immune regulator 1	TCIRG1	Sobacchi *et al.* (2004)
Trancription factor that regulates osteoblast differentiation	Runt-related transcription factor 2	RUNX2	Vaughan *et al.* (2004)
Regulates monocyte / macrophage activity	Semaphorin 7A	SEMA7A	Koh *et al.* (2006)
Involved in DNA replication and genomic stability	Werner helicase	WRN	Ogata *et al.* (2001)
Regulates bone formation in mice	Brain natriuretic peptide	BNP	Kajita *et al.* (2003)

Uitterlinden and colleagues reported that VDR haplotypes and the *COL1A1* Sp1 polymorphism interacted to regulate susceptibility to fracture in 1004 women from this study. Carriers of the highest-risk alleles for both genes had a 4.4 fold increase in fracture risk compared with the reference group, raising the possibility that this approach could be used to identify patients and low and high risk of osteoporotic fractures. In another analysis of the Rotterdam population, Rivadeneira reported that alleles of *ESR1*, *ESR2,* and *IGF-1* all interacted to regulate susceptibility to osteoporotic fracture and other phenotypes including BMD and aspects of femoral neck structure in 6363 subjects (Rivadeneira *et al.,* 2006). The authors reported a significant interaction between these three genes and the phenotypes studied in women, which persisted after correction for multiple testing, although interestingly no effects were observed in men.

GENETIC DETERMINANTS OF TREATMENT RESPONSE

Uncovering the genetic determinants of response to therapeutic agents is a subject of increasing interest because it raises the prospect of being able to predict individual responses to drug treatment on the basis of genetic profiling (Roses, 2000). Several investigators have now looked at associations between candidate gene polymorphisms and the response of BMD to antiosteoporotic treatments.

Calcium and Vitamin D

Krall and colleagues (1995) studied the relationship between VDR alleles and bone loss in 229 women who had participated in a controlled trial of calcium supplements in the prevention of postmenopausal bone loss. The calcium-supplemented group showed no relationship between VDR genotype and bone loss, whereas in the placebo group, bone loss was significantly greater in the BB group when compared with the other genotype groups. Graafmans *et al.* (1997) studied the response to vitamin D supplementation in a series of 81 postmenopausal Dutch women who had taken part in a placebo-controlled trial of vitamin D supplementation on BMD and fracture incidence. These workers observed that the 2-year change of BMD values in the vitamin D group relative to the placebo group was significantly higher in the "BB" and "Bb" genotypes when compared with the "bb" genotype group. This study is of interest in relation to another study by the same group that showed that the "bb" genotype also had low BMD in a population-based study. Yamada and colleagues (2000) studied the relationship between response of BMD to one-alpha-hydroxyvitamin D in relation to a signal peptide polymorphism of the *TGFB1* gene. This study comprised 363 postmenopausal women who were treated with one-alpha-hydroxyvitamin D ($n = 117$) or with HRT ($n = 116$), or who were untreated ($n = 130$). Individuals with the high BMD-associated "CC" genotype responded significantly better to vitamin D treatment than the other genotype groups. The same trend was observed in the HRT group, but the differences were not significant.

Hormone Replacement Therapy

Some information is available on the relationship between candidate gene polymorphisms and response to HRT. Ongphiphadhanakul and colleagues (2000) studied the relationship between *ESR1* polymorphisms and the 1-year response to HRT treatment in 124 postmenopausal Thai women. Individuals with the "pp" genotype at the *ESR1* PvuII site were found to respond less well to HRT (+2.3% increase in BMD) than the other genotype groups (+6 to 7% increase in BMD). In a similar but larger study of 248 Korean women, however, Han and colleagues found no association between *XbaI* or *PvuII* polymorphisms and the 1-year response of BMD to HRT (Han *et al.,* 1999). Salmen *et al.* (2000) similarly found no association between the *ESR1* genotype and response to HRT in a study of 145 Finnish women. Taken together, these data do not support the view that *ESR1* polymorphisms consistently predict response to HRT. Other candidate genes have been also studied in relation to HRT response. They include TGFβ1 (discussed earlier) and ApoE, which was analyzed by Heikkinen and colleagues in a study of 232 women who were treated with HRT and followed-up after a 5-year period (Heikkinen *et al.,* 2000). No association was observed in this study between ApoE genotype and HRT responsiveness.

Bisphosphonates

Marc and colleagues (1999) looked at the relationship between VDR genotype and response to etidronate therapy in a small series of 24 postmenopausal women undergoing treatment with etidronate. The mean change in BMD over a 2-year period was significantly greater in the BB vs. the bb group with intermediate values in the heterozygotes. In another study, Qureshi *et al.* (2002) looked at the association between *COL1A1* genotype and the response to etidronate treatment in a series of 48 early-postmenopausal women who took part in a randomized, controlled trial of etidronate in the prevention of postmenopausal bone loss. Although no difference was observed in response of spine BMD to etidronate treatment, those with the "s" allele responded significantly less well at the femoral neck when compared with "SS" homozygotes. These preliminary data are of interest in raising the possibility that genetic markers can be used to predict therapeutic response to bisphosphonates, but further work in larger groups of patients will be required to confirm and extend these observations.

IMPLICATIONS FOR CLINICAL PRACTICE

Studies on the genetic basis of osteoporosis have potentially important implications for clinical practice. Mapping and identification of genes that regulate BMD offer the prospect of identifying novel molecules that can serve as targets for drug design in the search for new treatments for bone diseases. Already, studies on the genetics of rare bone diseases have identified several molecules and pathways such as Sclerostin and the Wnt-*LRP5* pathway that now form the focus for the design of new anti-osteoporosis drugs. In addition, genetic markers that are associated with BMD or osteoporotic fracture could be used in the identification of patients at risk for fracture. The most promising candidate genes identified so far in this respect are *COL1A1*, *ESR1,* and *LRP5* although much work is needed to explore whether these and other candidate gene polymorphisms can be successfully used along with other clinical variables such as BMD and biochemical markers to predict patients at risk of fracture.

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Chapter 76

Pathophysiology of Osteoporosis

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INTRODUCTION

Osteoporosis was a relatively rare disorder until recent times when increased longevity and changes in lifestyle produced a large population of older individuals with fragile skeletons. Osteoporosis has been defined as a condition in which a low bone mass and changes in microarchitecture that compromise bone strength lead to increased fracture risk (NIH, 2001). This definition would include both primary and secondary osteoporosis. Primary osteoporosis, which has been variously termed postmenopausal, involutional, senile, and idiopathic, involves multiple pathogenetic mechanisms, many of which have not yet been adequately defined. In addition to primary osteoporosis, there are many disorders that can lead to skeletal fragility including (1) genetic disorders such as osteogenesis imperfecta and the osteoporosis-pseudoglioma syndrome; (2) endocrine disorders including Cushing syndrome, hyperparathyroidism, hyperthyroidism, and hypogonadism; and (3) inflammatory and nutritional disorders such as rheumatoid arthritis, Crohn's disease, and celiac disease. The pathogenetic mechanisms for many of these disorders are covered in other chapters. This chapter will summarize the known key elements in the pathophysiology of primary osteoporosis and describe a number of additional potential mechanisms based on our increasing understanding of the complexity of bone biology and its regulation.

BASIC PATHOPHYSIOLOGY

There are three critical factors that can lead to the development of skeletal fragility (Raisz, 2005). (1) *Failure to achieve optimal peak bone mass and strength*. This is largely determined by genetic background (Albagha *et al.*, 2006; Ralston *et al.*, 2007; Nguyen *et al.*, 2003), but can also be substantially affected by lifestyle (Lock *et al.*, 2006; Matkovic *et al.*, 1979). (2) *Accelerated bone loss owing to resorption*. This appears to be less dependent on genetic background (Pocock *et al.*, 1987; Michaelsson *et al.*, 2005). Estrogen deficiency, in particular, at the menopause, but also in older males, plays a substantial role in increasing bone loss (Amin *et al.*, 2006; Falahati-Nini *et al*, 2000). Calcium and vitamin D deficiency leading to secondary hyperparathyroidism are also important (Lips, 2001). (3) *An impaired bone formation response during remodeling*. A gradual decrease in the capacity to form adequate amounts of new bone to maintain bone mass during remodeling may begin shortly after peak bone mass has been achieved (Lips *et al.*, 1978) Its mechanisms are not clear, but changes in local and systemic growth factor production probably are important (Charatcharoenwitthaya *et al.*, 2007; Zhou *et al.*, 2006). Cytokines have been implicated in both accelerated resorption and in impaired formation.

In the past, osteoporosis was regarded as a disorder in which both the microarchitecture and macroarchitecture of bone were abnormal but the chemical composition was normal. However, there are genetic, environmental, and disease-related factors that could produce subtle alternations in the crystal structure and matrix composition of bone, and thus increase its fragility (Herrmann *et al.*, 2006; McLean *et al.*, 2004; Saito *et al.*, 2006; Tang *et al.*, 2007; Tinetti *et al.*, 2006; van Meurs *et al.*, 2004). Finally, a key element in the risk for nonvertebral fractures is the likelihood of falling, which may involve calcium-regulating hormones, particularly vitamin D (Nguyen *et al.*, 2007; Bischoff-Ferrari *et al.*, 2004). Although this nonskeletal feature (namely, risk of falling) is beyond the scope of this chapter, fall prevention should be a critical component of fracture prevention regimens in the elderly (Oliver *et al.*, 2007; Tinetti, 2003).

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DETERMINANTS OF PEAK BONE MASS AND STRENGTH

Many genes have been identified as affecting peak bone mass and strength (Ralston, 2007). Genome-wide analyses comparing humans and animals with greater or less skeletal fragility have produced a large number of quantitative trait loci for which the genes have still not been identified. The power of genetic influences on the skeleton is strikingly illustrated by the examples of high-bone-mass phenotypes owing to activating mutations of LRP-5, a coreceptor of the Wnt signaling pathway or loss of the Wnt inhibitor sclerostin (Balemans *et al*., 2001; Little *et al*., 2002) and the severe, early-onset osteoporosis that can occur when the LRP5 gene is inactivated (Gong *et al*., 2001). Polymorphisms of the components of this pathway (Bollerslev *et al*., 2005; Grundberg *et al*., 2007; Hartikka *et al*., 2005; Xiong *et al*., 2007), as well as systemic hormones and local factors and their receptors, have been shown to affect bone mass and fracture risk (Ioannidis *et al*., 2004). In some cases, fracture risk appears to be increased independent of effects on bone density.

Nutrition and lifestyle can also determine whether or not optimal peak bone mass and strength are achieved. There is ample evidence that adequate amounts of calcium, vitamin D, and physical activity as well as good general nutrition are necessary to achieve the optimal peak bone mass that has been genetically programmed to be reached (Chevalley *et al*., 2008). Moreover, environmental effects may also influence genetically programmed acquisition of peak bone mass (Ferrari *et al*., 1999). In addition, smoking (Wong *et al*., 2007), excess alcohol intake, and a number of drugs that are used in children and young adults can adversely affect the acquisition of peak bone mass.

BONE RESORPTION

Even in individuals with relatively low peak bone mass, fragility fractures are uncommon before the age of 50. This is in part owing to the likelihood that microarchitecture has not yet begun to deteriorate. Increased bone resorption that results not only in loss of bone mass, but also in disruption of microarchitecture is critical to development of skeletal fragility. Increased bone resorption is a function of aging and accelerated by age-related events such as the menopause. Osteoclastic bone resorption is described in detail elsewhere in this book. The increase in bone resorption in osteoporosis is probably the result of many factors that affect the formation and function of osteoclasts. These effects may be mediated through the interaction of receptor activator of NFκB ligand (RANKL) from osteoblasts, and possibly other marrow cells, with its receptor RANK on osteoclast precursors (Kearns *et al.* 2007). This interaction can be blocked by the natural RANKL inhibitor, osteoprotegerin (OPG). Studies in osteoporosis are consistent with an increase in RANKL activity. RANKL can be affected by hormones (Eghbali-Fatourechi *et al*., 2003), with increases seen in the context of estrogen deficiency and parathyroid hormone administration. Increases in RANKL are often associated with a decrease in OPG (Falahati-Nini *et al*., 2000; Huang, *et al*., 2007; Hofbauer *et al*., 1999; Syed *et al*., 2005; Taxel *et al*., 2007). OPG deficiency can cause osteoporosis in animals (Bucay *et al*., 1998) but it does not appear to be critically important in the pathophysiology of primary osteoporosis in humans. In addition to the RANKL-OPG system, many cytokines have been implicated as stimulators of osteoclastic activity. In animal models, there is evidence that IL-1, TNFα, IL-6, prostaglandin E_2 (PGE_2), and IL-11 are all key regulators. The amount of bone lost is influenced when specific cytokines are deleted or inhibited against a backdrop of the ovariectomized animal. While of interest, these studies do not necessarily indicate a pathophysiological role for that specific cytokine (Ammann *et al*., 1997; Horwitz *et al*., 2002; Kimble *et al*., 1995; Lorenzo *et al*., 1998; Pacifici, 2007; Weitzmann *et al*., 2001). There is likely to be a complex interplay between and among local factors that influence osteoclastic bone resorption. Some act in concert with, and others antagonistic of, responses that might otherwise be seen when a specific factor is studied alone (Kawaguchi, 1995). It is also important to appreciate the point that cytokine production may be regulated by oxidative damage, which has been implicated in estrogen deficiency and in age-related bone loss (Jagger *et al*., 2005; Lean *et al*., 2005).

The end result of excessive osteoclast activity, not compensated by a concomitant increase in osteoblast activity, is skeletal fragility. The microarchitectural deterioration probably reflects the intensity and duration of osteoclast activity as well as the number of osteoclasts formed. RANKL can prolong osteoclast survival whereas estrogen can shorten survival by increasing apoptosis (Hughes *et al*., 1996). In addition to OPG, other naturally occurring antiresorptive factors include calcitonin and cytokines such as IL-4 (Palmqvist *et al*., 2006; Silfversward *et al*., 2007). TGFβ, interferon-γ, and granulocyte-macrophage colony-stimulating factor have also been shown to inhibit osteoclast formation or activity, but they have not yet been implicated in the pathophysiology of osteoporosis. Deficiency of calcitonin does not appear to be a major mechanism for bone loss in primary osteoporosis (Tiegs *et al*., 1985), although there is little information on inhibitory cytokines.

Osteoporotic women are usually estrogen deficient. Evidence also points to the importance of estrogen in bone loss in the aging male as well. There is a graded effect of estrogen on bone in both men and women, such that the lowest levels are associated with the highest fracture risk (Cummings *et al*., 1998; Amin *et al*., 2006). This is consistent with the finding that low doses of estradiol, which have little effect on the breast and uterus, can prevent bone loss (Prestwood *et al.,* 2003). Although estrogen may affect systemic hormones and also play a direct role in the activity of differentiated bone cells, there is also evidence that estrogen can affect the production or activity of local factors, including cytokines, prostaglandins, and growth factors. There are many local factors that respond to estrogen, and these factors can modulate each other, so that decreasing or increasing a single factor may not give an accurate picture of its physiological or pathological role. Data from animal models and cell and tissue cultures suggest that estrogen deficiency increases and estrogen replacement decreases the activities of IL-1, TNF-*a*, and IL-6, as well as prostaglandins (Kimble *et al.,* 1995; Manolagas and Jilka, 1995). Estrogen can increase TGFβ, leading to osteoclast apoptosis (Hughes *et al.,* 1996), and increase OPG, which would block both formation and activity of osteoclasts (Hofbauer *et al.,* 1999). Estrogen may also affect TGFβ signaling in T cells (Gao *et al.,* 2004). A significant study of the role of osteoclast regulation by estrogen is one carried out in women who were premenopausal, or postmenopausal with and without estrogen treatment (Eghbali-Fatourechi *et al.,* 2003). Serum levels of RANKL or OPG did not differ among the groups, but analyses of bone marrow mononuclear cells in these subjects showed that the production of RANKL per cell was increased up to 3-fold in untreated postmenopausal women, and furthermore correlated negatively with serum estradiol-17β levels and positively with bone resorption markers. The study draws attention to the close involvement of cells of the immune system in estrogen action on the skeleton, but without the increases in T- or B-cell numbers that occur in the mouse. Importantly, it highlights the fact that studies of cytokine-mediated processes in bone need to focus on the bone microenvironment.

Although any or all of these mechanisms have been put forward to explain the increase osteoclast production and bone resorption that follows estrogen withdrawal, there still remains the possibility that estrogen acts directly on the osteoclast. Evidence in support of this has been obtained in mice in which a direct effect of estrogen inhibiting osteoclast apoptosis via increased Fas ligand has been discovered through experiments in which the ERα was specifically deleted (Nakamura *et al.,* 2007).

Osteoclastic activation through mechanisms already described initiate the bone remodeling cycle. What limits this activity in time is not understood, but *in vitro* it is known that osteoclasts resorb bone at a single site for only a few hours before moving on to another site. *In vivo* the resorptive phase of the bone remodeling cycle probably lasts 2 weeks or less, followed by a brief reversal phase (Bonewald, 2007) and a much longer phase of bone formation that lasts several months. Because of this difference in the duration of removal and formation of bone at remodeling sites, any increase in the overall activation frequency of remodeling sites will result in at least a transient decrease in bone mass and perhaps also microarchitectural weakness.

COUPLING OF BONE RESORPTION TO FORMATION

If the acceleration of bone resorption were matched by an equal or greater increase in bone formation there might be transient bone loss, but ultimately the removed bone would be replaced by new bone. Hence, the continuous loss of bone that occurs in osteoporosis implies a defect in the coupling process. Thus, it is of great importance to determine what factors regulate coupling. The initiating of formation following resorption could be influenced by factors derived from osteoclasts (see later). The signal could also come from osteocytes that are uncovered during resorption and release matrix components or factors from the mononuclear cells that are present during the reversal phase. These factors could stimulate the migration, replication, and differentiation of osteoblast precursors. Current candidates for such a role include insulin-like growth factor (IGF), TGFβ, and the components of the Wnt signaling pathway (Krishnan *et al.,* 2006). The Wnt signaling pathway might involve release of regulators that prevent this pathway from fully expressing its anabolic potential. For example, osteocytes express sclerostin, an inhibitor of Wnt signaling. The coupling process by which bone formation follows resorptive activity could involve a reduction in sclerostin expression. Signals in this regard could be related to mechanical forces, to parathyroid hormone, and to other factors (Bellido *et al.,* 2005; Keller, 2005; Sawakami *et al.,* 2006). Estrogen deficiency may play a role in the impaired formation response during remodeling, because animals lacking ERα showed decreased formation responses to mechanical loading by a mechanism that involves the Wnt signaling pathway (Armstrong *et al.,* 2007).

BONE FORMATION

An inadequate bone formation response during remodeling is not only critical for the development of skeletal fragility, but may also be at least as important as increased resorption in some cases (Eriksen *et al.,* 1990; Parfitt *et al.,* 1995). A progressive age-related decrease in the size of the packets of new bone formed at remodeling sites (mean wall thickness) probably begins soon after peak bone mass is achieved (Khosla *et al.,* 2006; Lips *et al.,* 1978). It appears to be similar in both men and women and does not show a clear change in rate at the menopause. Many mechanisms have been implicated to account for this imbalance of bone remodeling. A progressive decrease in either production of IGF-1, its local release, or its receptor responses have been postulated. Osteoporosis in middle-aged men, without clear etiology, is associated with decreased IGF-1 production (Rosen, 2004). Mechanical loading may enhance IGF-1 receptor signaling (Triplett *et al.,* 2007), and can also increase local prostaglandin and nitric oxide production (Kapur *et al.,* 2003; Wadhwa *et al.,* 2002). Mechanical stimulation can also decrease sclerostin expression (Robling *et al.,* 2007). Skeletal blood flow decreases with aging and this may decrease nitric oxide production (Prisby *et al.,* 2007).

A progressive change in the differentiation of marrow stromal precursor cells so that fewer progenitor cells become osteoblasts and more become adipocytes might also play a role. Increased apoptosis of osteoblasts and osteocytes has been found in response to glucocorticoids and may be an important component of this form of secondary osteoporosis (Grassi *et al.,* 2007; Jilka *et al.,* 2007). Oxidative stress could not only enhance resorption but also impair formation (Lean *et al.,* 2005). Impaired vascular supply to bone is another possibility. Finally, a number of the cytokines that have been implicated in increasing bone resorption, particularly TNFα, can also inhibit bone formation. Other growth factors including TGFβ, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF), can all be produced by bone cells as well as adjacent hematopoietic and vascular cells in the marrow.

Although the bone formation response during remodeling may be impaired in osteoporosis, it is certainly not abrogated. In postmenopausal women with rapid rates of bone resorption, the biochemical markers for bone formation also increase. This largely reflects the increase in the number of remodeling sites. Thus, although many new formation sites may be initiated, the amount of bone formed at these sites is decreased. The so-called high-turnover state, then, is still associated with more resorption than formation, reflecting the essential imbalance in bone remodeling that characterizes the osteoporotic process.

OSTEOCLAST PRODUCTS IN THE COUPLING PROCESS

Observations made in genetically manipulated mice suggest that the osteoclast itself could also be the source of an activity that contributes to the fine control of coupling of bone formation to resorption. Generation of coupling activity was suggested by increased bone formation in OPG$^{-/-}$ mice (Nakamura *et al.,* 2007), which are severely osteoporotic because of excessive osteoclast formation presumably owing to excessive RANKL activity. In bone sections from mice obtained in this high-turnover state, active bone resorption sites commonly were seen in association with active osteoblasts nearby, suggesting that coupling activity in this high-turnover state could be caused by the osteoclasts themselves. Cytokines that signal through gp130 play an important role in intercellular communication processes in bone, with evidence indicating that they can be involved in regulating mice in which each of the two gp130-dependent signaling pathways was specifically attenuated (Sims *et al.,* 2004, 2005). Inactivation of the SHP2/ras/MAPK signaling pathway (gp130$^{Y757F/Y757F}$ mice) yielded mice with greater osteoclast numbers and bone resorption, as well as greater bone formation than wild-type mice. This increased bone remodeling resulted in less bone because the increase in resorption was relatively greater than that in formation. In other words, the coupling process was imprecise in a way that resembles the result of estrogen withdrawal, as in ovariectomy gp130$^{Y757F/Y757F}$ mice crossed with IL-6 null mice had similarly high osteoclast numbers and increased bone resorption; however, these mice showed no corresponding increase in bone formation and thus had extremely low bone mass. Thus resorption alone is insufficient to promote the coupled bone formation, but the active osteoclasts are the likely source. Furthermore, this indicated that stimulation of bone formation coupled to the high level of bone resorption in gp130$^{Y757F/Y757F}$ mice is an IL-6-dependent process, though it does not necessarily show that it is mediated by IL-6 itself (Sims *et al.,* 2004).

Some indication of an osteoclast role in coupling comes also from human genetics. In individuals with the osteopetrotic syndrome, autosomal dominant osteopetrosis II (ADOII), owing to inactivating mutations in the chloride-7 channel (ClC-7), bone resorption is deficient because of failure of the osteoclast acidification process. Bone formation in these patients is nevertheless normal, rather than diminished as might be expected because of the greatly impaired resorption (Cleiren *et al.,* 2001). Furthermore, in mice deficient in either c-*src* (Lowe *et al.,* 1993), ClC-7 (Kasper *et al.,* 2005), or tyrosine phosphatase epsilon (Aoki *et al.,* 1999), bone resorption is inhibited without inhibition of formation. In these three knockout mouse lines, osteoclast resorption is greatly reduced by the mutation, although osteoclast numbers are not reduced. Indeed, osteoclast numbers are actually increased because of reduced osteoclast apoptosis. A possibility is that these osteoclasts, although unable to resorb bone, are nevertheless capable of generating a factor (or factors) contributing to bone formation. On the other hand, mice lacking c-*fos,* which are unable to generate osteoclasts, have reduced bone formation as well as resorption (Grigoriadis *et al.,* 1994).

Current focus on the osteoclast as a contributor to bone formation within the BMU might provide information that helps in understanding how remodeling spaces are filled, a process very relevant to the pathogenesis of bone loss.

NEURAL PATHWAYS AND ENERGY METABOLISM

This new and rapidly evolving area of research is likely to shed further light on the pathogenesis of osteoporosis (Reid, 2007; Martin, 2007; Karsenty, 2006). There are three components of this topic that may act in quite different ways. (1) Effects of body weight on bone. Low body weight is clearly a risk factor for osteoporosis and increased body weight may have positive effects on bone mass, although the effects on fractures are less clear. The relative roles of lean versus fat mass in determining BMD in postmenopausal women is not clearly established, both probably play a role. One definite mechanism by which increased body mass could increase bone mass is by increased mechanical loading. Increased fat mass could also increase estrogen production because of the high levels of aromatase in adipose tissue. (2) Adipogenesis in marrow. There is a continuous increase in proportion of adipocytes in marrow with age and this appears to be exaggerated in osteoporotic patients (Moerman *et al.,* 2004). Factors that increase adipogenesis, such as thiazolidinediones (TZDs) that activate peroxizome proliferator-activated receptor (PPAR)-γ, can decrease osteoblastogenesis and cause bone loss (Lecka-Czernik *et al.,* 2006; Schwartz *et al.,* 2006). The anabolic effects of increased Wnt signaling may be caused not only by increased osteoblastogenesis, but also by inhibition of adipogenesis (Krishnan *et al.,* 2006). (3) Hormonal and neural interactions. The presence of nerve endings, particularly of sympathetic nerves, in bone has long been recognized. A number of neuropeptides have been shown to act on bone, largely by increasing resorption. However, the discovery that mice with leptin deficiency had high bone mass, despite hypogonadism, and that this depended on leptin action in the brain, pointed to a role for the central nervous system (Ducy *et al.,* 2000; Takeda *et al.,* 2002). The central action of leptin is most likely through activation of sympathetic tone that can result in decreased bone mass. Epidemiological studies suggest that blockade of the β-adrenergic pathway can increase bone mass and decrease fracture risk (Pasco *et al.,* 2004; Schlienger *et al.,* 2004; Reid *et al.,* 2005). Epidemiological studies also support a role for leptin as well as polymorphisms of the leptin receptor as determinants of bone mass and fracture risk (Fairbrother *et al.,* 2007; Lorentzon *et al.,* 2006). In addition to the β-adrenergic pathway, the cannabinoid receptor and cocaine-amphetamine-regulated transcript (Cart) and neuromedin U may play a positive role in skeletal regulation, possibly by inhibiting β-adrenergic signaling (Bab *et al.,* 2008; Elefteriou *et al.,* 2005; Ofek *et al.,* 2006).

Most recently, another potential form of crosstalk between the skeleton and energy metabolism has been described (Lee *et al.,* 2007). Mice lacking the bone-specific protein tyrosine phosphatase OST-PTP were found to be hypoglycemic with increased insulin secretion. Deletion of one allele of the osteocalcin gene corrected this metabolic phenotype. Moreover, osteocalcin could stimulate insulin and adiponectin expression in B cells. Interestingly the effect of undercarboxylated osteocalcin was greater than that of the fully carboxylated form that is active in the skeleton as a regulator of mineralization.

Because the factors discussed here may have both direct and indirect effects on skeletal tissue and may be mediated through the nervous system or through hormonal pathways, it will require much work to establish their specific roles in the maintenance of skeletal integrity as well as in the pathogenesis of osteoporosis. Nevertheless, these new findings point to new approaches that may help us to understand the enormous variation in skeletal fragility and fractures among older individuals.

SYSTEMIC FACTORS IN THE PATHOPHYSIOLOGY OF OSTEOPOROSIS

In addition to local factors that are ultimately and mechanistically of pivotal importance in the cellular pathophysiology of osteoporosis, systemic regulators of mineral metabolism are believed to play an important role. Clearly, a reduction in circulating concentrations of estrogens is the single most important reason why postmenopausal women are at greater risk for bone loss than premenopausal women (Lindsay, 1998). Observations from the Study of Osteoporotic Fractures have established further that the low "residual" estrogen levels in postmenopausal women not taking estrogens are determinants in bone mineral density and in fracture risk (Ettinger *et al.*, 1998; Cummings *et al.,* 1998). The importance of estrogen sufficiency in men also has been elucidated in two male models of estrogen deficiency involving the estrogen receptor (Smith *et al.*, 1994) and the aromatase enzyme that converts androgens to estrogens (Morishima *et al.*, 1995; Carani *et al.*, 1997; Bilezikian *et al.*, 1998; Grumbach and Auchus, 1999). Animal knockout experiments of the alpha-estrogen receptor and of the aromatase gene provide further support for the key role of estrogens in male development (Lubahn *et al.*, 1993; Couse and Korach, 1999; Windahl *et al.*, 1999; Oz *et al.*, 2000).

The importance of estrogen in male skeletal health is amplified further in epidemiological studies correlating declining estrogen levels, but not declining testosterone levels, in age-related bone loss in men (Khosla *et al.*, 2001). Falahati-Nini *et al.* (2000), Khosla and Bilezikian (2003), Fink *et al.* (2006), and Gennari and Bilezikian (2007a, 2007b) have shown recently that when men are rendered temporarily but acutely hypogonadal, estrogen is the prime regulator of the subsequent increase in markers of bone resorption.

The importance of androgens in establishing and maintaining bone mass is also well recognized (Kearns and Khosla, 2004; Mellstrom *et al,* 2006). In addition to the sex steroids, glucocorticosteroids are key systemic factors in bone metabolism. We recognize the glucocorticosteroids not so much for any major role they may play in normal mineral homeostasis but rather for their devastating impact on bone metabolism when present in excess (Canalis *et al.,* 2007a).

In Cushing's disease or when glucocorticosteroids are used therapeutically, bone loss is a major complication. Excessive thyroid hormone may also have a deleterious effect on bone metabolism (Stern and Lakatos, 1999). Elsewhere in this book, these points are considered in depth. This section deals primarily with evidence that casts a potential role for the calciotropic hormones in the pathophysiology of osteoporosis.

VITAMIN D

The potential role of vitamin D in the pathophysiology of osteoporosis should be distinguished from overt vitamin D deficiency that is associated with the clinical syndromes of rickets (children) or osteomalacia (adults). This discussion highlights, instead, the concept that subclinical vitamin D inadequacy occurring by any of the mechanisms described earlier leads to suboptimal peak bone mass or to impaired maintenance of calcium balance. The clinical endpoint, osteoporosis, is not believed to be different from any other presentation of age-related osteoporosis.

1,25-Dihydroxyvitamin D, the active metabolite of vitamin D, enhances the absorption of calcium from the gastrointestinal tract, helps to regulate calcium handling in the kidney, serves to maintain normal bone remodeling, and is an important regulator of parathyroid hormone (Deluca, 2004). Any abnormality in vitamin D formation, metabolism, or action could lead, therefore, to profound changes in calcium balance and result in bone loss. Many studies report a fall in the circulating concentration of 1,25-dihydroxyvitamin D with advancing age (Eastell *et al.*, 1991; Fujisawa *et al.*, 1998; Lanske and Razzaque, 2007). More consistently, levels of the substrate for 1,25-dihydroxyvitamin D, namely 25-hydroxyvitamin D, fall as a function of age (Blumsohn and Eastell, 1995). Reduced concentrations of these vitamin D metabolites are thus considered to be one reason why vitamin D deficiency is implicated in age-associated reductions in bone mass.

Inadequate sources of vitamin D from either diet or sunlight are the most obvious possibilities for reduced levels of vitamin D in older individuals. In the skin, ultraviolet B, at 290 to 315 nm, converts 7-dehydrocholesterol to previtamin D (Holick and Garabedian, 2006). 7-Dehydrocholesterol levels in the skin fall by about 50% between 20 and 80 years of age (McKenna, 1992). The process by which skin produces previtamin D is also a function of sunlight itself. In northern latitudes, sunlight may contribute very little to vitamin D stores during the long winter months (Bouillon *et al.*, 1987; Webb *et al.*, 1990). Even in Maine and Massachusetts, no synthesis of previtamin D occurs in the skin during the late fall and winter (Rosen *et al.*, 1994). Other factors such as avoidance of sunlight and the use of highly protective UV-blocking creams may contribute to the diminishing importance of sunlight as a source of vitamin D.

The other source of vitamin D, the diet, is also a factor contributing to the potential for inadequate stores with age. Among adult Americans, vitamin D intake is well below the recommended daily allowance of 400 to 600 IU (Holick, 2006). In addition, mechanisms associated with absorption of vitamin D from the gastrointestinal tract begin to decline with age (Barragry *et al.*, 1978).

Subclinical vitamin D deficiency has been shown in osteoporotic women (Holick *et al.*, 2005; Lips *et al.*, 2006; Holick, 2007). A number of studies indicate subclinical vitamin D deficiency among home-bound subjects or those living in extended care facilities (Chapuy *et al.*, 1992; Komar *et al.*, 1993, Gloth *et al.*, 1995; Lips, 2001).

Adequate vitamin D depends not only on sufficient sources and absorption but also on normal metabolism of the parent vitamin. Alteration of hepatic or renal hydroxylation of vitamin D or 25-hydroxyvitamin D, respectively, can lead to deficiency (Siegel and Bilezikian, 1995; Holick, 2007). The kidney becomes a pivotal organ in this discussion because it is the source of the active metabolite, 1,25-dihydroxyvitamin D. Factors that help to regulate the renal formation of 1,25-dihydroxyvitamin D are parathyroid hormone, phosphorus, calcium, and 1,25-dihydroxyvitamin D itself. A decline in the ability of the kidney to form 1,25-dihydroxyvitamin D develops with age. Older individuals respond to the stimulating effects of parathyroid hormone on 1,25-dihydroxyvitamin D production less well than young, healthy individuals (Riggs *et al.*, 1981; Tsai *et al.*, 1984). Despite attempts to demonstrate a specific defect in renal hydroxylating capacity in osteoporosis (Slovik *et al.*, 1981; Tsai *et al.*, 1984), most studies have equated the defect with declining renal function *per se* (Halloran *et al.*, 1990, 1996).

Other hypotheses related to the role of vitamin D in the pathogenesis of osteoporosis focus on reduced sensitivity of the small intestine to 1,25-dihydroxyvitamin D. Intestinal resistance to 1,25-dihydroxyvitamin D is a proposed primary alteration (Eastell *et al.*, 1991; Pattanaungkul *et al.*, 2000) owing to an acquired alteration in binding of 1,25-dihydroxyvitamin D to its receptors in the small intestine (Francis *et al.*, 1984), or to an age-related reduction in intestinal vitamin D receptor concentration (Ebeling *et al.*, 1992a; Gennari *et al.*, 1990; Horst *et al.*, 1990). An additional role may be the effect of vitamin D to reduce the risk of falls (Bischoff-Ferrari, 2004). Finally, controversial observations implicate genetic polymorphisms of the vitamin D receptor point to possible underlying genetic bases for osteoporosis. Such genetic alterations could have as a functional counterpart altered physiological action of vitamin D on calcium absorption. This subject, reviewed elsewhere, does not provide the kind of certainty that one requires to establish the vitamin D receptor in mechanisms associated with the pathophysiology of osteoporosis (Albagha *et al.*, 2006; Uitterlinden *et al.*, 2006; Williams and Spector, 2007).

PARATHYROID HORMONE

Because one of the basic pathophysiological features of osteoporosis is altered bone remodeling, a hormone that is critically important for normal bone remodeling has to be a suspect in the pathogenesis of the disease. Parathyroid hormone (PTH) is one such hormone in which its normal actions are focused at the bone-remodeling unit. Normal bone remodeling depends on normal dynamics and actions of PTH. With aging, not only is bone remodeling abnormal, but also there are changes in the synthesis, metabolism, and responsivity of parathyroid hormone. One hypothesis is that the age-associated changes in PTH are causally related to the age-associated changes in bone mass. On the other hand, some of the age-related changes in PTH may be adaptive, serving to protect the aging skeleton rather than weakening it. A contrary hypothesis would state that PTH has the potential to conserve the skeleton because it is known in specific therapeutic contexts to be anabolic for bone (Kurland *et al*., 2000; Canalis *et al*., 2007b; Girotra *et al*., 2006; Neer *et al*., 2001).

Even in primary hyperparathyroidism, a chronic state of excess PTH secretion, cancellous bone mass is often preserved not only with regard to BMD but also with regard to other structural, microarchitectural, and material properties of bone (Silverberg *et al.,* 1989a; Dempster *et al*., 2007; Roschger *et al*., 2007; Bilezikian *et al*., 2007). There are, thus, two fundamentally different views on PTH and the aging skeleton: that it contributes to bone loss and that it helps to protect from bone loss. These two concepts will be explored in this section.

Parathyroid Gland Function with Aging

The view that PTH increases with age was controversial, in part, because age-related declines in renal function lead to an expected secondary increase in circulating PTH. The compensatory increase in PTH could be caused by several factors. The aging kidney does not convert 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D efficiently because of impaired renal 1-alpha-hydroxylating ability. With reduced levels of 1,25-dihydroxyvitamin, its inhibitory effects on PTH synthesis would be reduced and PTH synthesis would be enhanced. In addition, the reduced 1,25-dihydroxyvitamin D level could be associated with reduced calcium absorption, lower levels of the serum calcium concentration (but not to an extent that it would be measurably lowered), and a further stimulus to PTH secretion. Although this concept is entirely feasible, and undoubtedly occurs when renal insufficiency ensues, it was important to establish that the species of circulating PTH, in the context of renal insufficiency, is biologically active and not owing simply to the accumulation of inactive hormone fragments. Assays for PTH that detect active hormone exclusively have helped to settle the point (Deftos, 2001). In renal insufficiency, both active and inactive fragments can accumulate. In the setting of renal insufficiency, then, one can see aspects of PTH that are deleterious to bone.

If PTH is going to be implicated as a causative agent in osteoporosis, however, the increase has to be largely independent of declining renal function, because a universal decline in renal function that would be associated with an increase in PTH (i.e., below 60 cc/min) is not characteristic of osteoporosis. Therefore, it must be shown that PTH levels rise with aging even when renal function is normal. To this point, Halloran *et al.* (1996) have shown that biologically active concentrations of PTH do increase normally with age, even in older individuals who have no apparent decline in renal function. Comparing young and elderly men with normal renal function, they showed that, despite normal serum ionized calcium activity, normal serum 1,25-dihydroxyvitamin D, and normal urinary calcium excretion, basal PTH levels were higher by 1.5-fold in older men than in younger men. The age-related increase in PTH may be associated with a similar rise in bone turnover as assessed both by bone markers (Delmas *et al*., 1983; Duda *et al*., 1988; Eastell *et al*., 1988; Vedi *et al*., 1982) and by histomorphometric indices (Kotowicz *et al*., 1990). Histomorphometric data obtained by Kotowicz *et al.* (1990) suggest that, in postmenopausal osteoporosis, for each picomole per liter rise in circulating PTH, osteoporotic women showed higher activation frequency, bone resorption rate, and cancellous bone loss.

Most studies, in addition to those of Halloran *et al.* (1996), have demonstrated increases in the serum PTH with age (Khosla *et al*., 2001). A few caveats are in order though. In studies that have shown age-related increases in PTH, the major increments appear to occur in individuals who are over 70 years old (Koh *et al*., 1997; Prince *et al*., 1995). Most studies attribute the age-related process of osteoporosis to be fully established well before the age of 70. Further, not all studies have confirmed the PTH levels rise with age (Gallagher *et al*., 1988).

If it is assumed that PTH levels rise with age, independent of declining renal function or other secondary causes, there are two fundamental hypotheses to be addressed. One links the increase in PTH to age-related bone loss; the other hypothesis links the increase in PTH to protection against age-related bone loss.

Parathyroid Hormone as a Contributing Factor to Osteoporosis

The accelerated rate of bone loss in the early postmenopausal years has been explained by the local release of bone-resorbing cytokines (Jilka *et al*., 2002). Riggs and Melton (1983) proposed that such local factors and the ensuing rapid loss of skeletal calcium could actually lead to suppression of PTH. Reduced PTH concentrations in osteoporotic women in the face of higher bone turnover could reflect enhanced skeletal sensitivity (Ebeling *et al.*, 1982; Kotowicz *et al.*, 1990) to these local bone-resorbing factors. Although some studies have shown PTH suppression in the early postmenopausal years, more often, PTH concentrations have not been shown to differ from those of age-matched controls. Nevertheless, normal circulating concentrations of PTH could still induce bone loss if sensitivity is heightened in the postmenopausal state. Observations in primary hyperparathyroidism could help to advance this argument. Primary hyperparathyroidism becomes clinically apparent most often in the early postmenopausal years when estrogen deficiency has ensued. Reduced estrogen levels could enhance skeletal sensitivity to PTH. The recent observations in individuals with primary hyperparathyroidism and normal serum calcium concentrations but elevated PTH levels (normocalcemic primary hyperparathyroidism) may be an opportunity to study earlier forms of primary hyperparathyroidism with regard to estrogen loss in the early menopausal state (Silverberg *et al.*, 2003; Maruani *et al.*, 2003; Tordjman *et al.*, 2004; Lowe *et al.*, 2007; Lundgren *et al.*, 1997, 2002). Will these individuals demonstrate more clinically apparent primary hyperparathyroidism with hypercalcemia when the menopause occurs? The demonstration in certain postmenopausal women with primary hyperparathyroidism that estrogen replacement therapy is associated with a reduction in calcium levels, but no changes in the circulating PTH, adds support to this idea (Marcus, 2002). The concept implicating PTH in osteoporosis would not so much depend on increases in PTH levels, but rather on increased sensitivity to PTH, particularly in the context of estrogen deficiency.

The notion of enhanced skeletal sensitivity to PTH is not new nor is the idea that this mechanism could account for the development of postmenopausal osteoporosis (Koh *et al.*, 1997; Prince *et al.*, 1995; Jilka *et al.*, 2002). However, enhanced sensitivity to PTH has not been universally demonstrated. Tsai *et al.* (1989) reported no difference between osteoporotic and healthy women in urinary excretion of calcium or hydroxyproline concentrations in response to bovine PTH. Ebeling *et al.* (1992) reported similar results when calcium deprivation was used to stimulate endogenous PTH secretion.

Circumstantial evidence does implicate PTH in the pathogenesis of age-related osteoporosis with regard both to estrogen deficiency, changes in bone turnover, and, to a limited extent, changes in BMD (Riggs *et al.*, 1988a, 1998b; Heshmati *et al.*, 1998; Khosla *et al.*, 1997; McKane *et al.*, 1997; Ledger *et al.*, 1995). Using peripheral quantitative computed tomography to distinguish cancellous from cortical elements, Boonen *et al.* (1997*)* showed a negative correlation between cortical bone loss and rising PTH levels. Ledger *et al.* (1994) showed that elevated PTH concentrations in the elderly can be reduced to levels seen in young normals by administration of 1,25-dihydroxyvitamin D.

Although these data argue for a role for PTH in the pathogenesis of age-related osteoporosis, their indirect nature argues for caution in establishing a causal link. Moreover, our new knowledge of the salutary effects of PTH on other properties of bone such as bone size, microarchitecture, bone mineral density distribution, and collagen maturity, along with its therapeutic potential in osteoporosis, have directed attention away from the deleterious aspects of PTH on the aging skeleton, and focused instead on its positive features.

Parathyroid Hormone as a Protective influence on the Aging Skeleton

Although there is little doubt that PTH levels normally increase with aging, these observations could be interpreted to suggest that these changes are important to maintain skeletal health and that in osteoporosis this compensatory mechanism is lost. In this view, reduced responsiveness of the parathyroid glands contributes to the development of osteoporosis. Altered responsiveness could be caused by changes in PTH's circadian rhythmicity. Daily PTH secretion follows a biphasic profile with peaks at approximately hours 2 and 18 (2 AM and 6 PM) (Tohme *et al.*, 1995; Calvo *et al.*, 1991; Markowitz *et al.*, 1992). A larger nocturnal peak may represent compensation for mild hypocalcemia induced by night-time fasting. Calvo *et al* (1991) reported that women exhibited a blunted PTH peak relative to that of men and, subsequently, a less dramatic decline in night-time urinary calcium excretion. Night-time urinary calcium excretion declined in men by 34%, whereas, in women, it decreased by only 17%. Postmenopausal osteoporotic women showed a further blunting of their nocturnal PTH peak, with no change in nocturnal fractional excretion of calcium (Eastell *et al.*, 1992). Inefficient renal calcium conservation, thus shown, could contribute to the osteoporotic process.

Another dynamic that could be abnormal in osteoporosis relates to the pulsatility, not the circadian rhymicity, of PTH. Sophisticated pulsatility studies by Prank *et al.* (1995) have shown that osteoporotic women demonstrate poorly predictable pulses and patterns of PTH. Discriminating statistic fitting could distinguish the pulsatility pattern in the osteoporotic subjects from normal subjects (Prank *et al.*, 1995; Deftos *et al.*, 1995). These observations were not confirmed by Samuels *et al.* (1997).

Further evidence that argues for an important protective role for PTH in osteoporosis comes from the work of Silverberg *et al.* (1986, 1989b). These studies were based on the premise that a mild hypocalcemic challenge should lead to increases in PTH concentration that differ among age groups and between normal and osteoporotic subjects. Oral phosphate was used to induce the hypocalcemic challenge. The first studies were conducted with normal subjects who differed only by age: a younger and an older group. Neither group had osteoporosis. In each case, when administered oral phosphate, the serum phosphorus concentration rose and the serum calcium level fell to the same extent. In response to the hypocalcemic stimulus young subjects showed a 43% increase in PTH concentration over baseline values, whereas older women showed a much more exuberant response to the same hypocalcemic stimulus, with a 2.5-fold increase over baseline levels. This protocol set up two opposing stimuli with respect to 1,25-dihydroxyvitamin D: phosphorus as an inhibitor and parathyroid hormone as a stimulus. In both cases, the opposing regulators were neutralized and 1,25-dihydroxyvitamin D concentration did not change. These data were interpreted to suggest that older, normal subjects require more PTH for a given hypocalcemic challenge to maintain 1,25-dihydroxyvitamin D levels.

The same protocol was utilized in a group of postmenopausal women with osteoporosis (Silverberg *et al.*, 1989b). After phosphate administration, these women experienced the same increase in serum phosphorus concentration and the same reduction in serum calcium concentration that was observed for the young subjects and the age-matched older women. In contrast to the marked increase in PTH their age-matched counterparts, the osteoporotic women demonstrated only a modest 43% increase. Although this increase was sufficient in younger individuals to prevent the inhibitory effects of phosphorus on 1,25-dihydroxyvitamin D production, it did not suffice in these osteoporotic women, and 1,25-dihydroxyvitamin D concentrations fell by 50%. These observations are consistent with the presence of an abnormality in parathyroid secretory function in osteoporosis, at least to a hypocalcemic stimulus. It also is consistent with the hypothesis that older subjects need a more responsive parathyroid system than younger subjects.

The need for more PTH with age could be achieved by altering the calcium set point. For any given serum calcium concentration, the PTH concentration is higher in the elderly. This could account for the age-related increase in PTH without any change in the circulating calcium concentration. When Ledger *et al.* (1994) studied this point with a provocative challenge, no age-related increase in the set point for PTH secretion could be demonstrated. When postmenopausal women with osteoporosis were studied, however, differences did emerge. Cosman *et al.* (1991) used infusions of the foreshortened but fully biologically active human PTH(1–34) to test suppressability of endogenous PTH secretion. It was possible to distinguish between exogenous PTH(1–34) and endogenous PTH(1–84) by using an immunoradiometric assay that measures only PTH(1–84). The results were consistent with a higher calcium set point in osteoporotic women. Similarly, Portale *et al.* (1997) showed in elderly men that the set point of PTH responsiveness to calcium is "shifted" to the right. Such results are consistent with a protective effect of PTH in the pathogenesis of osteoporosis.

Parathyroid Hormone: A Positive or Negative Factor in Age-Related Bone Loss?

The data argue that PTH can be viewed as either a negative or a positive factor in preservation of the postmenopausal skeleton. The age-related increase in PTH may be adaptive or maladaptive. More information will be needed to sort out these different views. However, we already have abundant information about the skeleton in the classic condition of PTH excess in primary hyperparathyroidism. In this disorder, excess parathyroid hormone leads to relative protection against bone loss in the lumbar spine (Silverberg *et al.*, 1989a). The data in primary hyperparathyroidism differ so strikingly from those in osteoporosis that one must conclude that the bone diseases of hyperparathyroidism and osteoporosis reflect completely different disorders. The protection accorded the very site that is at early risk for postmenopausal bone loss, namely the cancellous bone of the vertebral spine, suggests that the effects of PTH on the aging skeleton are best viewed as protective, not deleterious. Finally, the clear actions of PTH to improve bone strength when used as a therapeutic agent in osteoporosis further argues for its beneficial actions on the skeleton (Neer *et al.*, 2001; Lindsay *et al.*, 1997; Kurland *et al.*, 2000; Bilezikian *et al.*, 2005; Rubin *et al.*, 2005).

CONCLUSION

From this review, it is apparent that the pathophysiology of osteoporosis is not only complex, but potentially quite heterogeneous. Thus, an important goal of future laboratory and translational clinical research will be to find clinical, biochemical, genetic, and other markers that can indicate which of many pathogenetic mechanisms predominate in specific subsets of patients. Achieving this goal could result in substantial advances in diagnosis and therapy.

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Chapter 77

Evaluation of Risk for Osteoporotic Fracture

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INTRODUCTION

Osteoporosis is a systemic disease characterized by decreased bone strength with a consequent increase in skeletal fragility and susceptibility to fracture. This decreased bone strength depends on both the quantity of bone as measured by bone mineral density (BMD) using dual x-ray absorptiometry or DXA and the quality of bone, which depends on microarchitecture, macroarchitecture, and microdamage. In addition the risk of fracture depends on the risk of falling and the ability to adapt to such falls. As clinicians we try to identify patients at risk of fracture, hopefully before the fracture occurs, so that we may treat them and reduce their risk of fracture.

In this chapter I will briefly review the diagnostic value of BMD by DXA, of clinical risk factors, and of the potential utility of combining BMD measurement and clinical risk factors using the new WHO algorithm in predicting fracture risk.

CLINICAL RISK FACTORS FOR FRACTURES

Clinical risk factors for fracture may be used in one of four ways:

1. Risk factors may be used to determine patients at intermediate to high risk of fracture who may be appropriate for BMD testing.
2. Risk factors may be used in those situations where BMD is not available to determine patients who are intermediate or high risk of fracture and in whom treatment should be considered.
3. Clinical risk factors may be used in combination with BMD to determine the probability of fracture.
4. Clinical risk factors in combination with BMD have also been used in guidelines to help us choose which patients to treat.

Historically risk factors were divided into those factors that were risk factors for low BMD and those that were risk factors for fracture. Several clinical risk factors are associated with low BMD, but none is powerful enough to predict BMD level in individuals (Aloia *et al.*, 1985; Kleerekoper *et al.*, 1989; Dawson-Hughes *et al.*, 1993; Kanis *et al.*, 1994).

Clinical risk factors were further divided historically into those that were genetic or nonmodifiable versus those that were modifiable with intervention. These risk factors became long laundry lists for clinicians (Espallargues *et al.*, 2001) (Table I). However, these were not separated from risk factors that predicted fracture risk independent of BMD versus those that predicted fracture risk by predicting BMD.

TABLE I BMD-Related Risk Factors for Fracture

High Fracture Risk	Moderate Fracture Risk
Age greater than 70	Female sex
Low body weight	Current smoking
Weight loss	Low sunlight exposure
Physical activity	Family history of osteoporotic fracture
Corticosteroid use	Surgical menopause
Anticonvulsant drugs	Short fertile period of less than 30 years
Primary hyperparathyroidism	Absence of lactation
Diabetes type 1	Low calcium intake
Anorexia nervosa	Hyperparathyroidism
Gastrectomy	Hypothyroidism
Pernicious anemia	Diabetes type 2
Prior osteoporotic fracture	Rheumatoid arthritis

From Table 5 in Espallargues et al. (2001).

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Risk factors became important to the clinician when guidelines used them as a means of deciding which patients should get BMD testing, e.g., National Osteoporosis Foundation (NOF) website, or which patients should be treated. For example, the NOF suggests treatment for postmenopausal women with T scores less than -2 or less than -1.5 with clinical risk factors. However, the guidelines did not tell us how to use these risk factors when no BMD measurement was available or how to integrate them if more than one risk factor was available.

In the past few years, our focus as clinicians has switched from identifying patients with osteoporosis to identifying patients at risk of fracture. This has come from two sources: one, the recognition that many of the osteoporotic fractures in the community occur in individuals who do not have osteoporosis. The work of Siris in NORA and Wainwright in the Study of Osteoporotic Fracture (SOF) cohort, among others, has shown us that the majority of fractures are in postmenopausal women who do not have osteoporosis by T score. Wainwright, for example, showed that of those individuals in SOF who sustained a hip fracture, 54% had a T score in the hip greater than -2.5, ***% had a T score in lumbar spine greater than -2.5, and 42% had a T score in both hip and lumbar spine greater than -2.5. These data have helped us understand that BMD testing is specific but not sensitive for identification of patients at risk of fracture. Other risk factors must be playing a role. Furthermore, we recognize that globally access to BMD measurement is limited or not available, requiring that we develop means of identifying patients at risk independent of BMD.

We now separate risk factors into those which are independent of bone mineral density and those that are dependent on bone mineral density. Risk factors may also be separated further into those that impair bone strength, those that lead to excessive loads on weakened bone from falls, or in some cases ordinary activities of daily living. Risk factors that impair bone strength include such factors as bone density, age, hormonal insufficiency, nutritional deficiency, or a specific pathological process. Furthermore, bone strengthening may also be impaired by bone turnover. Risk factors related to excessive loading of the bone include falls and predisposition to falls and fall mechanics.

Some clinical risk factors predict some fractures better than others. For example, prevalent fracture and age predict risk of fracture better than smoking or alcohol use.

As Kanis *et al.* (2005a) have pointed out, there are a number of considerations in choosing risk factors for case finding. First, clinical risk factors should be easily ascertainable. For example, calcium intake is a risk factor for fracture in the elderly, but ascertaining calcium intake without use of a food frequency questionnaire, which is difficult for patients to complete. Second, clinical risk factors need to be intuitive. We recognize that dementia is associated with increased risk of fracture, yet osteoporosis and fracture risk are often not high on the health care agenda for these patients. Risk factors need to be useful in a global setting with similar predictions of fracture risk. Third, to be valuable, a risk factor needs to be amenable to therapeutic intervention. For example, falling is associated with increased fracture risk but we have no pharmacological intervention to reduce the risk of falling. Finally, risk factors should be based on evidence based medicine

HOW DO WE EXPRESS FRACTURE RISK PREDICTABILITY?

Historically predictability was expressed as increased relative risk when we compared prevalence of fracture in two cohorts, one with the risk factor and one without. With the availability of data from long-term prospective cohorts, epidemiologists have used the concept of gradient of risk, the increased relative risk of fracture per standard deviation change in a risk factor.

Use of Clinical Risk Factors for Fracture Prediction

In the absence of other risk predictors such as BMD, clinical risk factors can be used to assess fracture risk (Kanis *et al.*, 2005a). Risk factors can also be combined with BMD to improve fracture risk detection. Most clinical risk factors have effective similar magnitude, increasing the likelihood of fracture by 1.5 to 3 times more than that seen in unaffected individuals. Although there is good evidence that risk factors may increase fracture risk, it is less clear if different factors are independent of BMD, which is necessary if clinical risk factors are to be combined with BMD in predicting fractures. However, it is also important that we only include risk factors that are responsive to treatment. Although many of these risk factors are rare, they are common. A third of women with osteoporosis have at least one of them (Tannenbaum *et al.*, 2002), as do most men with osteoporosis (Orwoll, 1998).

Clinical risk factors are additive. Cummings has shown that hip fractures are 17 times greater among older women with five or more clinical risk factors, exclusive of bone density than among women with two risk factors or fewer (Cummings *et al.*, 1995). Clinical risk factors are additive to BMD in identifying women at risk; Cummings found that women with multiple risk factors were at even greater risk if their BMD was in the lowest third. Garnero *et al.* (1996) found that increased bone turnover was additive to low BMD in identifying women at high risk of fracture.

It is important to note that some risk factors, which have a devastating effect on an individual patient and which are uncommon, may rarely appear along with independent risk factors that are identified in the population studies, for example, Cushing's syndrome.

Bone Density

BMD is a strong surrogate measure of bone strength and a strong predictor of fracture risk. A large number of clinical risk factors may exert their effects through BMD. In the 1994 WHO criteria, osteoporosis was defined as a BMD *T* score of less than −2.5 at the femoral neck (Kanis *et al.*, 1994). Bone strength is determined by size of bone, shape, microarchitecture including number and connectivity of trabeculae, mineralization, and microdamage. Measurement of these other attributes is not currently available to the clinician although research is in progress.

Age

Almost all studies indicate increasing age is a strong determinant for fracture risk. Greater age is associated with lower BMD. Age predicts fracture risk independently of BMD (Kanis *et al.*, 2002). The instances of both vertebral and nonvertebral fracture rises rapidly after age 50 in women and men. The rise is most dramatic for fractures of the spine, beginning at age 50, and the hip, beginning at age 60. Part of the increased risk of aging is because of the loss of bone and quality of bone with age, part is because of increased risk of falling owing to changes in gait and loss of balance, part is because of reductions in physical activity, calcium, and/or vitamin D deficiency, as well as unmeasured risk factors (Table II). Consequently, two women who have the exact same bone density, a woman age 50 and a woman age 80, have different risks of most types of fractures. At a *T* score of −2.5 in the femoral neck, an 80-year-old woman has a 12.5% 10-year absolute risk of hip fracture, whereas a 50-year-old woman only has a risk of 2.5%. Not all fractures increase with increasing age. For example, wrist fractures rise rapidly with menopause in women but plateau and even decline after age 65, perhaps related to decreased activity and perhaps related to changes in the pattern of falls in elderly women.

Where BMD measurement is not available, body mass index (BMI) may be used. BMI predicts fracture risk similarly to BMD, except at low BMI where there is excess risk(De Laet *et al.*, 2005b). Leanness increases risk, and obesity is not protective.

TABLE II Intrinsic Risk Factors for Falls among the Elderly

Strong Evidence	Moderate Evidence
Older age	Arthritis
Reduced functional level	Acute illness
Cane/walker use	Anti-Parkinson's drugs
History of falls	Cardiac and antihypertension drugs
Low walking speed	Alcohol use
Reduced lower extremity strength	
Impaired postural sway	
Impaired reflexes	
Impaired vision	***
Reduced lower extremity sensory perception	
Neuromuscular diseases such as stroke	
Urinary incontinence	
Hypnotic and sedative drugs	
Antipsychotic drugs	
Cognitive impairment	
Depression and antidepressant drugs	

Nutritional Deficiencies

Deficits in calcium or vitamin D can impair peak bone mass as well as withdraw calcium from the skeleton (Heaney *et al.*, 2000). Poor dietary intake may occur with aging or with younger patients with eating disorders. However, even if dietary intake is adequate, absorption of calcium from the gut may be impaired in patients with gastrectomy, gastric bypass, or pernicious anemia , all of which have been associated with increased fracture risk (von Terpitz *et al.*, 2003). Calcium intake may also be reduced with inadequate vitamin D intake. Low vitamin D intake may result from low sunlight exposure, use of sunscreens, especially in institutionalized elderly, but also in ambulatory individuals with increased skin pigmentation or liberal use of sunscreens, liver disease, or use of drugs that interfere with vitamin D such as anticonvulsants (Pack *et al.*, 2004).

Hormonal Abnormalities

Estrogen deficiency is associated with rapid bone loss at menopause and also plays a role in later age-related bone loss in men and women (Riggs *et al.*, 2002). Estrogen deficiency is reflected in multiple risk factors that share decreased lifetime exposure to estrogen, such as surgical menopause, shortened time between menarche and menopause, premature ovarian failure, and athletic amenorrhea (Johnell *et al.,* 1995). Because testosterone is converted to estrogen, hypogonadism in men may also be related to low estrogen levels. Furthermore, iatrogenic hypogonadism may occur with use of gonadotropin-releasing hormone agonists in prostate cancer (Miyaji *et al.,* 2004) and of aromatase inhibitors in breast cancer (Eastell, 2007).

Diseases Associated with Structural Changes in Bone

Paget's disease is associated with fractures, most likely owing to disorganized bone (Pahlavan, 2000). Primary bone tumors such as osteosarcoma, metastases to bone from other areas, infiltration of bone by multiple myeloma that infiltrates bone marrow, or deposition diseases such as Gaucher's disease are associated with increased fracture risk (Whyte, 2003).

Prevalent Fracture

A history of prior fragility fracture indicates increased risk of future fractures regardless of bone mass (Kanis *et al*., 2004s). In two equivalent 60-year-old women, one who had fractured her wrist and one who has no history of fracture; the woman with a history of fracture followed over time has about a 1.5% to 2% greater risk of hip and other types of fracture than a woman without any history (Cummings *et al.,* 1995). This observation is confirmed in the placebo groups of randomized controlled trials. In the Multiple Outcomes of Raloxifene (MORE) trial, if we look at the placebo group over three years, there is a fivefold increased risk of clinical vertebral fracture in those women who had prevalent fracture at baseline in the placebo group versus those who did not (Ettinger *et al.,* 1999). Similar data have been shown in the placebo group of Fracture Intervention Trial (FIT) (Black *et al.,* 2000). Prevalent fracture may be a surrogate marker of bone quality. Genant *et al.* (2007) have shown in an analysis of the MORE trial that patients with fracture had microarchitectural changes.

Family History

Family history of osteoporotic fracture, more specifically hip fracture, predicts future fracture independent of BMD (Kanis *et al*., 2004b). Family history most likely reflects multiple genes as well as environmental influences such as behaviors or nutrition. Family history may be mediated by genetic differences in bone size, shape, or patterns of bone loss with aging or menopause.

Alcohol and Tobacco Use

Alcohol intake is a BMD-independent risk factor for fracture (Kanis *et al*., 2005c). Smoking is an BMD-independent risk factor for fracture (Kanis *et al*., 2005a).

Glucocorticoid Use

One of the most important complications of glucocorticoid therapy is loss of bone mass and increased risk of fracture. Glucocorticoid-induced osteoporosis is the most frequent cause of secondary osteoporosis(Devogelaer, 2006). In a recent meta-analysis of 42,000 men and women, the relative risk for osteoporotic fracture ranged from 2.63 to 1.71 and risk for hip fracture ranged from 4.42 to 2.48 from ages 50 to 85 (Kanis *et al*., 2004a). The fracture risk was similar in men and women and was only partially explained by BMD. In a retrospective cohort of 244,325 oral glucocorticoid users compared to population controls, a relative risk of 1.61 for hip fracture and 2.6 for vertebral fracture was observed (van Staa *et al.*, 2000). The risk was related to daily dose and there was no safe low dose. All fracture risks decreased rapidly after stopping oral glucocorticoid therapy, suggesting the risk is reversible.

The pathophysiology of glucocorticoid-induced osteoporosis is not yet completely understood with several different pathophysiological mechanisms. Glucocorticoid use is associated with a dramatic decrease in bone formation and an increase in bone resorption. Bone loss is most marked in the first months of therapy and slows down thereafter (Devogelaer, 2006).

Osteoporosis Secondary to Specific Diseases

Fracture risk may be increased in individuals who have specific diseases, for instance, bone loss may be increased in patients with serious inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus (SLE), and Crohn's, although this may be partially owing to the need for corticosteroid therapy, an important risk factor. Whether the increase in fracture risk is independent of corticosteroid use is unclear in inflammatory bowel disease, asthma, and chronic obstructive pulmonary disease.

Fracture Risk in Rheumatoid Arthritis

The first population-based study that estimated fracture risks among patients who had rheumatoid arthritis (RA) was published 20 years ago, reporting a relative risk estimate for pelvic fractures of 2.56 and for proximal femur fractures of 1.51 (Hooyman *et al.,* 1984). Ten years later, a morphometric study of spine radiographs in 76 postmenopausal women who had steroid-treated RA was compared with a sample of age-matched women. Vertebral fracture risk was increased considerably in RA, particularly in women age 50 to 59 with an odds ratio for the whole population as high as 6.2 (Peel *et al.,* 1995). A recent study showed that patients with RA have more vertebral deformities than do population-based controls with an odds ratio of 2.0 with moderate to severe deformities and 2.6 when multiple deformities are included (Orstavik *et al.,* 2004). In a population-based control study of 300 consecutive patients with hip fracture compared with age- and sex-matched community controls, crude risk for hip fracture was approximately doubled in patients who had RA; increasing markedly with increasing functional impairment (Cooper *et al*., 1995). More recently, a study in Finland reported an age- and sex-adjusted risk of hip fracture of 3.26 in 29 patients who had RA (Huusko *et al*., 2001). It is unclear what specific factors are associated with fractures in RA. Data from recent cross-sectional studies indicate that the presence of vertebral deformity was associated independently with age, long-term corticosteroid use, history of nonvertebral fracture, and low bone mass at total hip (Orstavik, 1993). Similarly, a worsening of function measured by a one-point increase in HAQ was associated with a 70% increase in the risk of vertebral fracture in 461 cases of RA (Sinigaglia, 2000).

Systemic Lupus

Data on fractures in systemic lupus are scanty. The most extensive retrospective population-based study in a self-report of fracture of 702 women with lupus followed for almost 6000 person-years stated that the fracture risk was increased in those who had a lupus with a standardized morbidity ratio of 4.7 as compared with a control group of similar age (Ramsey-Goldman *et al*., 1999). Associated with fractures were older age, longer disease, longer corticosteroid exposure, less use of oral contraceptives, and menopause. However, in the multivariate model, only older age at time of lupus diagnosis and a longer duration of corticosteroid use were independent determinants of fractures.

Ankylosing Spondylitis

Radiographic bone loss in ankylosing spondylitis (AS) has been documented for a long time. As a consequence of osteoporosis, vertebral compression fractures are reported frequently in ankylosing spondylitis because the pain associated with them is attributed to exacerbation of spondylitis rather than to fracture. There is an early increase in fracture (Cooper *et al*., 1995), consistent with the reports of significant bone loss in early disease. Cooper *et al*. (1994), using a retrospective population-based study, showed an increase in vertebral fracture risk as great as 7.6 in comparison with expected fracture incidence in the same community. In general, vertebral fractures in patients with AS occur with increasing age and severity of disease (Donnelly *et al*., 1994; Mitra *et al.,* 2000; Cooper *et al*., 1994).

Bone Turnover as a Predictor of Fracture Risk

High levels of bone turnover markers are associated with high rates of bone loss in postmenopausal women. This relationship is stronger in a woman in the first 5 years after menopause. In older women, however, the relationship is not strong enough to be useful in an individual patient (Bauer *et al*., 1999; Gennaro *et al*., 1999; Marcus *et al*., 1999; Rogers *et al*., 2000). There have been several prospective studies on the relationship between bone turnover markers and fracture risks in postmenopausal women, including risks of hip, spine, and nonspine fracture. Bone resorption markers above the premenopausal reference range have been associated with a doubling in the risk of hip fracture, as shown by Garnero *et al*. (1996) and Epidos. this is Garnero 96which discussed Epidos This association is as strong as that of a one standard deviation decrease in proximal femoral neck BMD. Thus, a low BMD measurement and a high bone resorption marker put a woman at a particularly high risk of fracture. The increase in risk of fracture with high bone resorption markers is independent of bone mineral density.

Risk Factors Related to Increased Bone Loading

Although some osteoporotic fractures, such as vertebral compression fractures, may occur independent of a fall, most fractures occur in the setting of a fall. The likelihood of falling increases with aging (Winner *et al*., 1989). Most falls do not, however, result in fracture (fewer than 5%). The pathogenesis of falls is complex with causation related to extrinsic factors such as environmental barriers, i.e., loose carpets, electrical cords, and so on (Schwartz *et al*., 2001), as well as intrinsic factors such as reduced sensory input, balance problems, impaired strength, and drug side effects. Reduced sensory input includes changes in vision such as blindness, diabetic retinopathy, cataracts, etc. Balance and gait problems include peripheral neuropathy, neuromuscular diseases, residual hemiplegia, or hemiparesis following stroke, etc. Drugs such as sedatives or psychotropic medications increase the risk of falling and fractures (Schwartz *et al*., 2001). Postural hypotension from antihypertensive medications may also be associated with increased risk of falling and fractures.

Unfortunately, it is difficult to prevent falls. Gillespie *et al*. (2004), in a recent Cochrane review, showed that meta-analysis found only a reduction in risk of falling of 14 to 27% with multidisciplinary intervention and little influence on risk of fracture.

THE NEW WHO ALGORITHM

Assessing Fracture Risk Using the New WHO Risk Factors

In an effort to identify candidate clinical risk factors that could be integrated in a clinically useful way, a WHO scientific group first reviewed the literature by using meta-analysis to identify risk factors for fracture independent of BMD. Second, a meta-analysis was done by combining data from 12 cohorts around the world, including studies in Dubbo, Australia (Jones *et al.,* 2006); Hiroshima, Japan (Fujiwara *et al.*, 1997, 2003); multiple studies in Europe: such as EVOS/EPOS (Felsenberg *et al.*, 2002; Oneill *et al.*, 1996; Ismail *et al.*, 2002), EPIDOS (Rollins *et al.*, 2005), Gothenberg I (Svanborg, 1977) and II (Johannson *et al.*, 1998), Rotterdam (van der Klift *et al.*, 2004), Sheffield (Sheffield***)Kuopio in Finland (Honkanen *et al.*, 1995); and the OFELY study in France (Albrand *et al.*, 2003); as well as the CaMos in Canada (Sawka *et al.*, 2005) and Rochester in the United States (Melton *et al.*, 1998, 2003a). This global cohort analysis consists of approximately 250,000 person-years, approximately 60,000 patients, and more than 5000 fractures. Using this analysis, eight candidate risk factors have been proposed (Silverman, 2006). To be chosen, each of these risk factors needed to be validated in multiple populations; adjustable for age, sex, and type of fracture; intuitive; amenable to therapeutic manipulation; and readily accessible for primary practitioners. Thus, factors such as calcium deficiency and risk of falling, which would not be easy for primary care physicians to measure were not considered (WHO Meeting on Fracture Risk, Brussels, May 5 to 6, 2004).

As listed on Table III, the eight independent risk factors that have been suggested by the WHO include age (Dawson-Hughes *et al.*, 1993), prior fracture (Kanis *et al.*, 2004a), BMD of the femoral neck (Johnell *et al.*, 2005) or BMI (DeLaet *et al.*, 2005a), past or present corticosteroid use (Kanis *et al.*, 2004a), family history of fracture (Kanis *et al.*, 2004b), current smoking (Kanis *et al.*, 2005a), and ingestion of more than two units of alcohol per day (Kanis *et al.*, 2005c), as well as secondary osteoporosis associated

TABLE III Proposed WHO Risk Factors and Their Gradient of Risk

		Relative Risk (RR)		
Risk Factor	Reference	Any Fracture	Osteoporotic Fracture (hip, shoulder, vertebral, or forearm)	Hip Fracture
Age	Dawson-Hughes *et al.*, 1993		*T* score <–2.5; 13.9% 10-year probability at age 50 and 34.4% at age 80	*T* score <–2.5; 2.9% 10-year probability at age 50 and 23.8% at age 80
Prior fracture	Donnelly *et al.*, 1994	1.86 (95% CI = 1.75–1.98)	RR 1.86 (95% CI = 1.72–2.01)	RR 1.85 (95% CI = 1.58–2.17)
BMD femoral neck	Eastell, 2007		RR/SD decrease in BMD: 1.41 (95% CI = 1.33–1.51) in men 1.38 (95% CI = 1.28–1.48) in women	RR/SD decrease in BMD: 2.94 (95% CI = 2.02–4.27) in men 2.88 (95% CI = 2.31–3.59) in women
BMI	Espallargues *et al.*, 2001	RR per unit higher BMI 0.98 (95% CI = 0.97–0.99)	RR per unit higher BMI 0.97 (95% CI = 0.96–0.98)	RR per unit higher BMI 0.93 (95% CI = 0.91–0.94)
Ever corticosteroid use	Ettinger *et al.*, 1999	RR varies with age from 1.98 at age 50 to 1.66 at age 85	RR varies with age from 2.63 at age 50 to 1.71 at age 85	RR varies with age from 4.42 at age 50 to 2.48 at age 85
Family history of fracture	Ettinger *et al.*, 2005	RR 1.17 (95% CI = 1.07–1.28)	RR 1.18 (95% CI = 1.06–1.31)	RR 1.49 (95% CI = 1.17–1.89)
Current smoking	Felsenberg *et al.*, 2002	RR 1.13 (95% CI = 1.15–1.36)	RR 1.29 (95% CI = 1.13–1.28)	RR 1.60 (95% CI = 1.27–2.02)
Alcohol more than 2 units/day	Fujiwara *et al.*, 2003	RR 1.23 (95% CI = 1.06–1.43)	RR 1.38 (95% CI = 1.16–1.65)	RR 1.68 (95% CI = 1.19–2.36)
Secondary OP (rheumatoid arthritis)	Fujiwara *et al.*, 1997			RR 1.95 (95% CI = 1.11–3.42)

Reprinted with permission of Current Osteoporosis Reports (Silverman, 2006).

with disorders such as rheumatoid arthritis (Kanis *et a*l., 2001). BMI may be used where there is limited access to DXA scanning, as BMI correlates with BMD in terms of fracture prediction. These clinical risk factors can be used to generate probabilities of fracture with or without BMD. The clinical risk factors may be added (De Laet *et al.*, 2005b), but the sum would vary depending on the risk factors added because they have differing weights. These risk factors will improve the prediction of fracture risk by improving the gradient of risk. The addition of clinical risk factors to BMD may be most valuable for younger women and least valuable for those who are elderly. If information on a patient's BMD is not available, these eight risk factors can help clinicians decide which patients to treat.

In the proposed WHO model, 10-year probability of fracture is used rather than relative risk because relative risk decreases with age. For example, by age 80 the relative risk of fracture (compared with other 80-year-old women) for a woman with a *T* score of −2.5 is reduced because many women of this age are at high risk of fracture independent of BMD. Similarly, lifetime fracture risk is not used because it begins to decline at about age 70 because of the competing risk of death. Ten-year fracture probability has therefore been chosen as an intermediate point.

The risk of different clinical fractures is combined in this model. Rather than simply adding the risk of a single osteoporotic fracture, the morbidity and mortality associated with each fracture is weighed, using a utility measurement compared with the utility of a hip fracture, so-called hip fracture equivalents (Silverman, 2006). This technique will reduce fracture risks to a common currency. The model assumes that the direct cost of a fracture is proportional to the utilities measured. The proposed new WHO algorithm would predict the probability that a patient will experience a hip fracture or a clinical fracture (shoulder, hip, clinical vertebral, or forearm fracture) over the next 10 years.

Deciding on the Need for Treatment

How will this information be used to decide whom to treat? Currently, there are numerous guidelines. There is general agreement that older patients with osteoporosis need treatment (Lewiecki, 2005; Kanis *et al.*, 2000), but there is no consensus about other risk groups. Most guidelines do not distinguish between risk factors that are BMD-dependent and BMD-independent. No guidelines consider the interaction between risk factors in determining who is to be treated. No current guidelines use assessment of fracture probability. Most guidelines agree that patients with *T* scores below −2.5 should be treated and those with *T* scores above −1.5 should not be treated, but the guidelines differ for the group with *T* scores between those numbers (Lewiecki, 2005).

Worldwide, there are currently two basic case-finding strategies. The International Osteoporosis Foundation (IOF) strategy is based on clinical risk factors. If the BMD femoral neck T score is less than −2.5, the patient should be treated (go to www.iofbonehealth.org). In the United States, the strategy is more liberal. The National Osteoporosis Foundation (NOF) (go to www.nof.org) recommends treatment for a postmenopausal woman with a *T* score below −2 or a *T* score below −1.5 with risk factors.

The availability of the new WHO risk platform suggests the possibility of a new, global case-finding strategy. Based on fracture probability and clinical risk factors, it should be possible to identify patients who are at low risk and should not be treated, patients at high risk who can be treated without BMD measurement, and those with intermediate risk, who will need BMD measurement before they are reassessed for risk. Those at high risk would then be treated and those with low risk would not be treated. A slightly different model may be considered in North America and Australia, where patients are initially screened for BMD and clinical risk factors are added to then determine which patients will be treated. In the United States, DXA scanning is more easily available than in the rest of the world and regulatory agencies have decided that it is medically appropriate to screen any woman above age 65 or any woman above 60 with risk factors (U.S. Preventive Systems Task Force at: www.ahrq.gov/clinic/uspstfix.htm).

To target osteoporosis treatment at those patients who will receive the greatest benefit, clinicians need to decide on an intervention threshold. There is growing consensus that an intervention threshold should be based on absolute fracture risk rather than on a diagnostic threshold (Kanis *et al.*, 2002). The intervention threshold need not be consistent globally, however, as there are regional differences in the ability and willingness to pay. In the United Kingdom, treatment is cost-effective for a 50-year-old woman with a 1.1% 10-year probability of hip fracture and an 85-year-old woman with a 9% 10-year probability (Kanis *et al.*, 2005c). In Sweden, treatment of a 50-year-old woman with a 1.2% 10-year probability of hip fracture is cost-effective, as is treatment of an 80-year-old woman a 7.4% probability (Kanis *et al.*, 2005b). In these two societies, approximately 8% of the gross domestic product (GDP) is earmarked for health care. In the United States, which spends 13% to 14% or more of its GDP on health care, the threshold for cost-effectiveness may be lower.

How will the new osteoporosis model affect clinicians? For most patients, there will probably be little change from current clinical practice. Looking at the 10-year probability of hip fracture in patients with no prior fracture, treatment of those with *T* scores of approximately −2 is cost-effective, as is treatment of those with prior fracture and a *T* score of −1.5. This threshold closely resembles the current NOF guidelines. Clinicians will continue to treat older women with osteoporosis or older women with fracture. The younger woman who is at relatively low risk of osteoporotic fracture may not be targeted for therapy, however. In a study by Ettinger *et al*. (2005), it was shown that physicians seeing the same clinical data but given no absolute risk data may be less likely to prescribe a bisphosphonate for younger women at low risk. It is also likely that younger patients who are told that they have only a 2% to 3% risk of fracture would not be convinced to take a medicine that would reduce their risk by half, to 1%.

CONCLUSION

Individuals at high risk of osteoporotic fracture can be identified by clinical risk factors or a combination of clinical risk factors and BMD. With the availability of a diverse but expensive menu of osteoporosis therapies, it is clear that clinicians will be unable to treat everyone. Clinicians need to treat those individuals at high risk of fracture and not treat those who are at low risk. The use of fracture risk assessment using both BMD measurement and clinical risk factors in a case-finding approach will hopefully help us target our therapies to those who need it most.

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Part III

Pharmacological Mechanisms of Therapeutics

Chapter 78

Pharmacological Mechanisms of Therapeutics: Parathyroid Hormone

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INTRODUCTION

Parathyroid hormone (PTH) is an 84-amino-acid peptide hormone released in a pulsatile fashion by the parathyroid gland *in vivo*, and regulates serum-ionized calcium levels through its actions on kidney and bone. In this chapter, PTH will either refer generically to the whole class of parathyroid hormone peptides or, more specifically in some sections, to the intact 84-amino-acid hormone [PTH(1–84)].

Until fairly recently, PTH was considered to be primarily a bone-resorbing hormone. However, it is now appreciated that PTH exerts its effects on bone primarily through the osteoblast, and its resorptive effects are only part of the PTH stimulation of bone turnover (resorption and formation). Although bone resorption is a prominent histological event seen in bone sections obtained from patients suffering from pathological secretion of PTH, osteoclasts do not present PTH receptors, and the enhanced bone resorption accompanying pathological PTH secretion appears to require the presence of osteoblasts (McSheehy and Chambers, 1986; Parfitt, 1976). Patients with primary hyperparathyroidism may be at increased risk for ongoing bone loss and fractures, together with evidence for increased bone turnover (Christiansen *et al.*, 1999; Grey *et al.*, 1994; Khosla *et al.*, Parisien *et al.*, 1995; Silverberg *et al.*, 1999).

The bone anabolic properties of exogenously injected PTH (bovine parathyroid gland extracts) were first reported in a series of serendipitous findings dating back to 1931 (Pehue *et al.*, 1931; Selye, 1932), in both human and animal situations. Some 45 years later Reeve and colleagues, citing circumstantial evidence in both clinical hyperparathyroid states and animal data, used a synthetic human (h) PTH fragment comprising the amino-terminal 34 amino acids [referred to in this text as PTH(1–34), or teriparatide], to treat a small group of elderly individuals with osteoporosis (Reeve *et al.*, 1976, 1980). They reported a dramatic histological improvement in bone turnover and structural trabecular growth.

Initially, hPTH(1–34) was obtained by solid-phase peptide synthesis, a relatively expensive process that meant the supply of drug for clinical trials remained limited. However, the small number of clinical trials evaluating the potential of exogenous PTH therapy for osteoporosis consistently demonstrated a beneficial anabolic effect. (Bradbeer *et al.*, 1992; Finkelstein *et al.*, 1994; Hesch *et al.*, 1989; Hesp *et al.*, 1981; Hodsman and Fraher, 1990; Hodsman *et al.*, 1991, 1993b, 1997; Lindsay *et al.*, 1997; Neer *et al.*, 1987, 1991; Reeve *et al.*, 1981, 1987, 1990, 1991, 1993; Slovik *et al.*, 1981, 1986; Sone *et al.*, 1995). Commercial development of PTH as an osteoporosis therapy awaited the technological advances needed for inexpensive large-scale manufacture of peptides by fermentation/recombinant DNA synthesis, and the first commercial randomized clinical trial (RCT) of recombinant hPTH(1–84) was carried out in the mid-1990s, a dose-finding placebo-controlled 12-month evaluation of the effect on bone mineral density (BMD) (Hodsman *et al.*, 2003). Shortly thereafter, a large, fracture outcome clinical trial of recombinant hPTH(1–34) was begun by Eli Lilly (Neer *et al.*, 2001).

ADVANTAGES OF ANABOLIC AGENTS FOR REVERSAL OF OSTEOPOROSIS

There are strong epidemiological associations between BMD measurements at either central (lumbar spine) or

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appendicular sites (femoral neck, distal radius, os calcis) and the current or future risk for osteoporotic fractures (Cummings *et al.*, 1993; Marshall *et al.*, 1996; Ross *et al.*, 1991). There is approximately a twofold increase in the risk of fracture for every 1.0 standard deviation below average age- and sex-adjusted BMD, increasing dramatically if there is a preexisting fragility fracture in an individual with a low BMD (Marshall *et al.*, 1996; Ross *et al.*, 1991). Relatively small BMD increments reported in published placebo-controlled clinical trials translates into an incident fracture reduction of approximately 50% within 1 to 4 years of therapy. The evidence for this is best illustrated in the randomized controlled clinical trials (RCTs) of the bisphosphonates (Black *et al.*, 1996; Cummings *et al.*, 1993; Harris *et al.*, 1999; Liberman *et al.*, 1995; McClung *et al.*, 2001; Reginster *et al.*, 2000).

There are currently two therapeutic strategies to treat osteoporosis. These strategies are illustrated in Figure 1. For the purposes of this figure a fracture threshold has been indicated, but the gradient of risk is continuous (as it is for many biological variables such as blood pressure and cholesterol in ischemic vascular disease). The most important role for an anabolic agent in bone would be the rapid induction of new bone formation in the skeleton to reduce the risk of fragility fractures. It is against this background that the role of PTH therapy is discussed.

Antiresorptive Agents (Also Termed "Anticatabolic" or "Antiremodeling" agents)

This approach relies on the inhibition of osteoclastic activity during the bone mineralization unit (BMU) remodeling activity, allowing osteoblastic function to continue unimpeded. For example, the RCTs of the bisphosphonates, alendronate and risedronate, have defined the benefits of this strategy, resulting in an increased BMD and significant reductions in incident fracture risk (Black *et al.*, 1996; Cummings *et al.*, 1993; Harris *et al.*, 1999; Liberman *et al.*, 1995; McClung *et al.*, 2001; Reginster *et al.*, 2000). Evidence from observational cohort studies suggests that estrogen also reduces osteoporosis-related fractures by up to 50% (Eiken *et al.*, 1996; Kiel *et al.*, 1987; Maxim *et al.*, 1995; Naessen *et al.*, 1990); however, only one short-term RCT supported this observation (Lufkin *et al.*, 1992), until publication of the Women's Health Initiative study of postmenopausal hormone therapy (Cauley *et al.*, 2003).

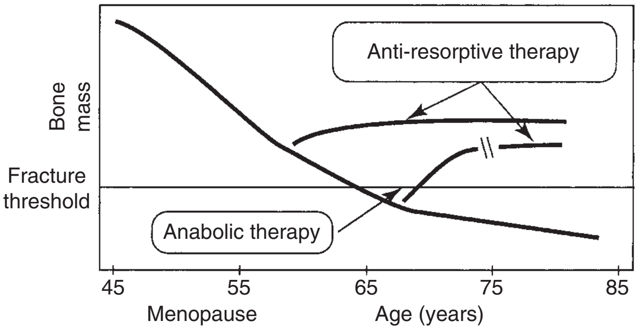

FIGURE 1 Depicts the relative efficacy of different strategies to maintain bone mass. (Upper) Antiresorptive strategy. Various agents (e.g., estrogens, bisphosphonates, and calcitonin) will arrest ongoing age-related and postmenopausal bone loss. Reductions in bone resorption and turnover are observed, and gains in skeletal bone mass may occur owing to the reduced bone resorption and perhaps to a reversal of the remodeling deficit., (Lower) Anabolic therapy. Agents such as PTH and sodium fluoride directly stimulate osteoblastic activity to increase bone mass, despite concurrent increases in bone turnover (i.e., there is an increase in both bone formation and resorption). The rapid increase in bone mass should accelerate the reduction in future fracture risk more effectively than the use of an antiresorptive agent alone.

The large-scale RCT with raloxifene (a selective estrogen receptor modulator) demonstrated comparable antifracture efficacy for vertebral (but not for peripheral) fractures, after a gain in vertebral BMD of less than 3% over 36 months (Ettinger *et al.*, 1999). Another antiresorption agent, calcitonin, was shown to reduce incident vertebral fractures without any significant increment in BMD (Chestnut *et al.*, 2000). These RCTs of antiresorptive therapy have called into question that increments in BMD automatically translate into a reduced fracture risk. Nonetheless, overall gains in BMD at an axial measurement site following bisphosphonate therapy (typically 4–8% at the lumbar spine over 3 years) may reduce incident fractures by approximately 40% to 50%. Detailed reports of antiresorptive therapies are reviewed in other chapters.

Anabolic Agents

Two principal agents have been shown to stimulate osteoblast function within bone: sodium fluoride and analogues of PTH. Sodium fluoride and its slow-release formulations are discussed elsewhere in this publication. The landmark publication of Riggs *et al.* (1982) suggested that sodium fluoride significantly reduced vertebral fracture rates. However, although a subsequent placebo-controlled trial demonstrated a rapid and linear gain in lumbar spine BMD of 9% per year over 4 years, this study provided no evidence for a reduction in incident fractures (Kleerekoper *et al.*, 1991). Fluoride now has very limited use in osteoporosis.

Recently, strontium ranelate has been postulated to be able to combine modest effects of increasing bone formation with reduced bone resorption, resulting in steady increments in bone density, and reduction in fractures (Meunier *et al.*, 2004). The anabolic effect of this agent is somewhat confounded by the effect of a higher atomic weight strontium replacing calcium in bone mineral, and thereby amplifying the increase in BMD and the postulated anabolic effects on bone.

By whatever outcome measure used, PTH analogues have been shown to be anabolic, resulting in comparable gains in bone mass by comparison with fluoride. These gains are considerably more rapid than those seen with antiresorptive agents. However, fluoride therapy has not supported the dictate that gains in BMD translate into lower fracture rates. Because there are as yet few controlled trials reporting incident fractures in patients treated with PTH analogues, it is necessary to evaluate the potential mechanisms by which PTH has seemingly paradoxical anabolic effects, focusing on the preclinical data in osteopenic animal models, as well as the available clinical evidence.

BONE HISTOLOGY EVIDENCE OF ANABOLIC ACTION OF PTH

Anabolic agents work by a fundamentally different cellular mechanism of action than antiresorptive agents. As noted earlier, a more appropriate term for the latter would be "antiremodeling agents" because, although their initial effect is to lower resorption, they secondarily inhibit formation. Indeed, inhibition of remodeling is the primary mechanism by which this class of drugs works. Lowering the remodeling rate has several effects that improve bone strength, including (1) a modest increase in bone density by reducing the remodeling space; (2) maintenance of cancellous bone microarchitecture; (3) a decrease in the number and size of resorption cavities, which act to concentrate stress and trigger mechanical failure; (4) an increase in the mineralization density (amount of mineral per unit volume); and (5) a decrease in cortical porosity (Chavassieux *et al.*, 1997; Dufresne *et al.*, 2003; Boivin *et al.*, 2000; Seeman and Delmas, 2006; Ettinger *et al.*, 1999; Chesnut *et al.*, 2000; Parfitt, 1991; Roschger *et al.*, 2003, Hernandez *et al.*, 2001).

By contrast, PTH treatment ultimately stimulates the bone remodeling rate and thereby stimulates net bone formation. With teriparatide treatment, the amount of bone laid down in each remodeling unit is increased, which is confirmed by an increase in osteon thickness (Bradbeer *et al.*, 1992; Hodsman *et al.*, 2000; Dempster *et al.*, 2001, Ma *et al.*, 2006). This distinguishes the effects of PTH treatment from other high-remodeling states, such as estrogen deficiency, which result in loss of bone structure and strength. The combination of an increase in the osteon thickness and an increase in the number of osteons being formed per unit time provides a mechanism for ongoing gains in the amount of bone tissue, including an increase in trabecular thickness (Bradbeer *et al.*, 1992). In addition to the stimulation of bone formation through this mechanism, referred to as "remodeling-based formation," changes in bone histology can be detected in animal experiments very early on in the course of PTH therapy. There is histomorphometric and biochemical evidence that, during the early stages of treatment, teriparatide stimulates formation directly, that is, without prior resorption (Ma *et al.*, 2006; Hodsman and Steer, 1993a; Lindsay *et al.*, 1997, 2006, 2007). Hodsman *et al.* (1993b) reported that after only 28 days of hPTH(1–34) injections, changes in both formation and resorption surfaces were increased two to five times those measured in a control panel of osteoporotic bone biopsies. Because tetracycline bone markers were given on days 14 and 28, there had not been sufficient time for a *de novo* resorption/reversal/formation cycle to be completed. This is referred to as modeling-based formation and may occur by activation of lining cells on previously quiescent bone surfaces (Jiang *et al.*, 2003), or by osteoblasts that were engaged in remodeling-based formation annexing resting bone surfaces surrounding the resorption cavity (Figs. 2 and 3) (Lindsay *et al.*, 2006, 2007).

Treatment with teriparatide or intact PTH not only leads to an increased trabecular thickness but also improves trabecular connectivity as demonstrated by microcomputed tomography of iliac crest bone biopsies (Dempster *et al.*, 2001; Jiang *et al.*, 2003; Fox *et al.*, 2005) (Fig. 4). The underlying mechanism for the improvement in trabecular

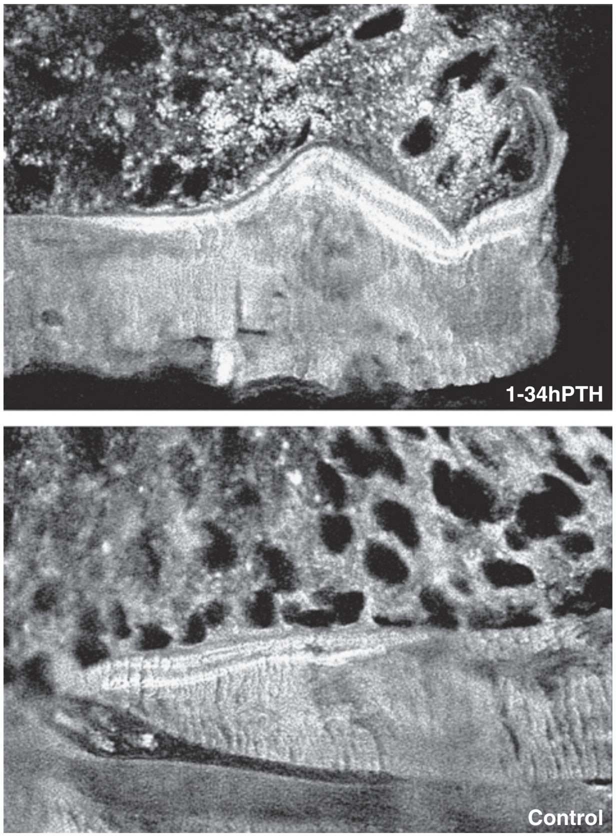

FIGURE 2 Quadruple tetracycline labels in a patient treated with teriparatide (Upper) and a control patient (Lower). Note that in the teriparatide-treated patient the second set of labels, which were incorporated during teriparatide treatment longer than the first set, which were incorporated before treatment began. This is not the case in the control subject. (Reproduced from Lindsay et al., 2006, with permission).

connectivity is still unclear but may involve thickening of trabeculae followed by intratrabecular tunneling (Jerome *et al.*, 2001; Fox *et al.*, 2007; Allen and Burr, 2006) (Fig. 5). The ability of anabolic agents to improve cancellous bone structure is in sharp distinction to antiresorptive agents, which maintain, but do not improve cancellous bone integrity (Seeman and Delmas, 2006).

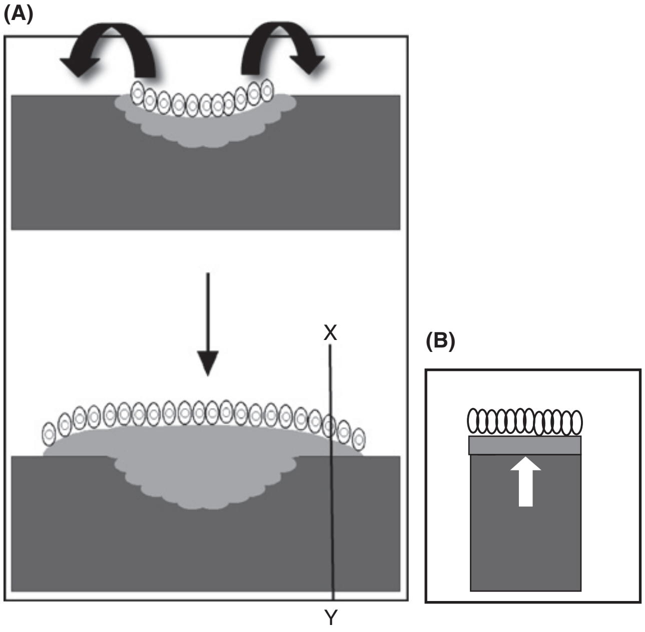

FIGURE 3 (**A**) Diagram illustrating a proposed mechanism whereby, under the influence of teriparatide treatment, bone formation is extended beyond the limits of the remodeling unit and annexes the adjacent previously unresorbed territory. (**B**) A section taken orthogonal to the plane of the paper along the line XY would display a smooth reversal line and may be erroneously interpreted as indicating that bone formation occurred on a smooth bone surface (arrow) that was temporally and spatially unrelated to a prior resorptive event. (Reproduced from Lindsay *et al.*, 2006, with permission).

There was an early concern that PTH therapy might have a negative effect on cortical bone, with the notion that gains in cancellous bone may be achieved at the expense of cortical bone (Reeve *et al.*, 1980b). This was not confirmed in animal studies, which showed that teriparatide improved cortical thickness and strength by stimulation of formation on both the endosteal and periosteal surfaces (Allen and Burr, 2006; Hirano *et al.*, 2000; Jerome *et al.*, 1999; Burr *et al.*, 2001; Mashiba *et al.*, 2001; Fox *et al.*, 2006) (see Fig. 5). Biopsy studies in humans also showed an increase in cortical thickness (see Fig. 4) and stimulation of bone formation with a decrease in eroded perimeter on the endosteal surface (Dempster *et al.*, 2001; Lindsay *et al.*, 2006, 2007; Jiang *et al.*, 2003). However, the concept that teriparatide is capable of stimulating periosteal bone formation and increasing bone diameter in humans remains controversial. Two studies, which employed different noninvasive imaging techniques on the same subjects, one cross-sectional and the other longitudinal in design, yielded conflicting results on the effects of teriparatide on bone diameter (Zanchetta *et al.*, 2003; Uusi-Rasi *et al.*, 2005). However, there is recent histomorphometric evidence for stimulation of bone formation at the periosteal surface of the ilium following teriparatide treatment (Ma *et al.*, 2006; Lindsay *et al.*, 2007) with an increase in tetracycline uptake and insulin-like growth factor expression on the periosteal surface.

The possibility that PTH may be able to increase bone size is noteworthy because the strength of a tube is dependent on the fourth power of its radius. Consequently, small increments in bone size may have disproportionately greater effects on bone strength. Bone size increases with age, which compensates for the age-related loss of bone mass (Garn, 1970; Duan *et al.*, 2001a, 2001b). PTH treatment may accelerate this natural process. Thus, the long-standing belief that intermittent PTH treatment might be harmful to cortical bone appears to be losing ground. With

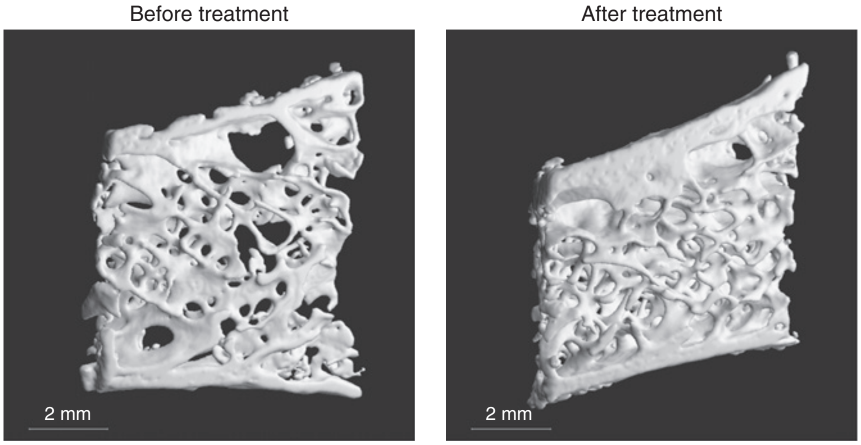

FIGURE 4 Microcomputed tomography images of paired biopsies from a 64-year-old woman before (Left) and after (Right) treatment with teriparatide. Note improvement in cancellous and cortical bone structure. (Reproduced from Dempster *et al.*, 2001, with permission).

that, however, comes the recognition that in order to assess the effects of PTH treatment in a clinical setting, we need to be careful when interpreting BMD changes, particularly areal measurements obtained by DXA. For example, a decrease in BMD owing to enhanced cortical porosity may not necessarily indicate loss of bone strength. Increased porosity under the influence of PTH treatment is limited to the inner aspect of the cortex where it has little effect on bone strength, and weakness that results may be offset by an increase in cortical thickness and bone size, as well as improvements in trabecular architecture. Another reason that areal DXA may underestimate improvement in bone mass and strength following PTH treatment is that there is more new bone matrix, which has a lower mineral content (Misof *et al.*, 2003). There is, therefore, a need to evaluate other noninvasive imaging modalities, such as those discussed in this textbook, to monitor the effects of anabolic treatment.

PHYSIOLOGICAL MECHANISMS OF ANABOLIC ACTION OF PTH

The physiological action of PTH is to maintain the ambient concentration of ionized calcium in blood (1) by influencing calcium reabsorption from the glomerular filtrate of kidney tubule cells and (2) indirectly by enhancing calcium absorption from the gut through increased activity of renal vitamin D-1-hydroxylase, which produces $1\alpha,25(OH)_2D_3$ (Bilezikian *et al.*, 1994; Brabant *et al.*, 1992). PTH also initiates a series of events that result in release of skeletal calcium by osteoclasts.

In 1931, Pehue *et al.* described the case history of an 8-year-old Parisian child who had succumbed to anemia owing to the obliteration of his marrow space by bone; he was subsequently discovered to have had hypertrophic parathyroids (Pehue *et al.*, 1931). Subsequently Selye suggested that, in contrast to the findings in primary hyperparathyroidism, PTH could have a potent anabolic effect (Selye, 1932). Selye was able to mimic this clinical situation in 30-day-old albino male rats with daily injections of 5 IU of Lilly parathyroid extract for 30 days. These low doses of parathyroid extract resulted in the production of osteopetrotic bone with no sign of osteoclastic bone resorption. In contrast to the catabolic effect of continuous infusion of PTH, as perhaps exemplified by primary hyperparathyroidism, numerous investigators have since confirmed the anabolic action of PTH (used as either the 1–84 intact hormone or various amino-terminal analogues) when administered as multiple intermittent doses. This effect has been termed “the PTH paradox” (Morley *et al.*, 1997).

The amino-terminal third of the PTH molecule appears to contain most of the biological activity in bone. Both intact PTH(1–84) and synthetic PTH(1–34) act through dual signaling pathways in target cells. In osteoblasts, the type 1 PTH/PTHRP receptor is coupled to both the adenylate cyclase activating G protein, Gs, and the phospholipase C activating Gq protein. PTH requires the first two amino acids and some part of the amino acid 25–34

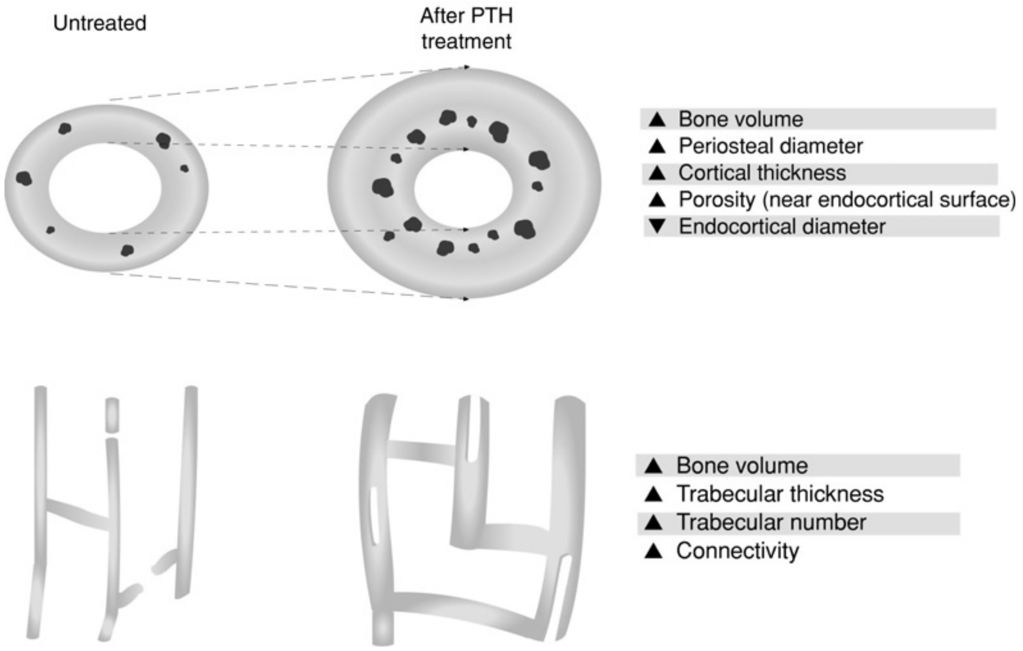

FIGURE 5 Diagram illustrating the effects of PTH treatment on cortical bone (Upper) and cancellous bone (Lower). (Reproduced from Allen and Burr, 2006, with permission).

region to activate Gs, but only the 28–32 portion to activate Gq (Whitfield *et al.*, 1998). The literature suggests that cAMP generation is positively linked to the anabolic effect of PTH. PTH fragments such as (1-desamino) hPTH(1–34), hPTH(8–84), or hPTH(28–48) that stimulate membrane-bound PKC, but not adenylate cyclase, in isolated osteoblasts do not stimulate bone formation in the oophorectomized rat model of osteoporosis (Jouishomme *et al.*, 1994; Takasu and Bringhurst, 1998). The carboxyl-terminal truncated analogue PTH (1–31) and its amide derivative (Ostabolin), are highly anabolic in the ovariectomized (OVX) rat and activate cAMP (Whitfield *et al.*, 1996, 1998; Neugebauer *et al.*, 1995; Whitfield *et al.*, 2000). Inhibition of GPCR kinase (GRK) prolongs cAMP activation and results in bone anabolism in the rat (Spurney *et al.*, 2002). In a mouse model of osteoporosis, only PTH peptides with an intact adenylyl cyclase-activating domain were anabolic to bone (Yang *et al.*, 2007), thus confirming the importance of this signaling pathway in the bone-building action of PTH.

The exact cellular mechanisms and principal mediators of the action of PTH on osteoblasts have not been fully elucidated, but it is likely to result from a combination of activating growth factors available in the immediate bone marrow environment, the recruitment of new populations of preosteoblasts from marrow stromal cells, interactions between cells in the bone microenvironment, and the recycling and trafficking of the type I PTH receptor (PTH1R). There is strong evidence that PTH stimulates both the insulin-like growth factor (IGF) and transforming growth factor beta (TGF-β) systems in bone (Watson *et al.*, 1995; McCarthy *et al.*, 1989; Canalis *et al.*, 1991; Hodsman *et al.*, 2005; Ishizuya *et al.*, 1997; Lee and Lorenzo, 1999; Wu and Kumar, 2000; Dempster *et al.*, 1993).The insulin-like growth factor regulatory system is composed of two growth factors (IGF-I and IGF-II), two receptors, six binding proteins that regulate IGF bioavailability, and IGF-binding protein-specific proteases. Following binding to the PTH receptor on osteoblasts and the generation of cAMP these cells produce more IGF-II , IGFBP-1, -4, and -5, and the IGFBP-3 and IGFBP-5 proteases (Johansson and Rosen, 1998). PTH also directly stimulates the synthesis of TGF-β by mature osteoblasts (Canalis, 1996). IGF-I, IGF-II, and TGF-β secreted by osteoblasts are bound to the collagen matrix of bone where they reside as a growth factor bank. During cycles of bone remodeling, when PTH induces osteoclastic bone resorption, these factors are released into the immediate environment where they can act on the recruitment and development of resident osteoblasts (Canalis, 1996). Intermittent (anabolic) treatment with PTH also results in the appearance of multistacked active osteoblasts (whose origin is uncertain) at sites of bone formation. They may be osteoprogenitor cells from marrow precursors, PTH-responsive postmitotic cells that have been recruited to the site by chemotaxis, or bone lining cells that have been induced to reenter proliferative cycles and pile up (Dobnig and Turner, 1995; Watson *et al.*, 1995).

Studies of the role of the osteocyte in sensing and transducing a bone anabolic response to mechanical loading have also opened the possibility of PTH modulating this response. The osteocyte-derived inhibitor of osteoblast-mediated bone formation, sclerostin, has been recently shown to be regulated by PTH (Leupin *et al.*, 2007). In UMR-106 cells, PTH downregulated expression of sclerostin via inhibiting MEF2 transcription factor activity or expression. This, in turn, would be expected to increase osteoblastic bone formation.

Mathematical modeling suggests that the ontogeny, or life span, of osteoclasts and osteoblasts is relevant to the anabolic effect of PTH. In this model, anabolic PTH treatment induces waves of remodeling where waves of short-lived osteoclasts initiate new BMUs followed by waves of much longer lived osteoblasts, resulting in a net shift of the remodeling balance toward formation (Komarova, 2005). In addition, although intermittent PTH treatment acts by enhancing formation in established remodeling units, some part of its effect owes to *de novo* bone synthesis on quiescent surfaces (Hodsman and Steer, 1993; Hodsman *et al.*, 2005; Lindsay *et al.*, 2007; Samadfam *et al.*, 2007). There are also mathematical models that suggest that intermittent PTH does not result in desensitization of the PTH1R and leaves the receptor relatively unoccupied and sensitized for new cycles of activation (Potter *et al.*, 2005).

Differences in bone cell gene expression evoked by intermittent versus continuous PTH have been documented. Onyia *et al.* (2005) utilized microarrays to explore the genes and pathways regulated by intermittent versus continuous PTH(1–34) for 1 week in 6-month-old female rats. The effect of each PTH regimen was confirmed by histomorphometry, and mRNA from metaphyseal bone was analyzed. Both PTH treatments coregulated 22 genes including collagens, osteocalcin, decorin, and osteonectin. Intermittent PTH regulated 19 additional genes, and continuous treatment regulated 173 additional genes.

Locklin *et al.* (2003), using cultured marrow cells from 6-week-old C57BL/6 mice, demonstrated that 4 days of intermittent PTH treatment (6 hours exposure every 48 hours) increased mRNA for osteoblast differentiation markers [Runx2, alkaline phosphatase (AP), and type I procollagen (COL1A1)]. IGF-I mRNA was consistently higher than after continuous treatment. In contrast, continuous treatment resulted in increased gene expression of RANK ligand (RANKL), whereas that of osteoprotegerin (OPG) was decreased, with a 25-fold increase in the RANKL/OPG ratio and production of large numbers of TRAP-positive multinucleated osteoclasts. Intermittent PTH treatment therefore appears to enhance osteoblast differentiation through an IGF-I-dependent mechanism, whereas continuous PTH treatment enhances osteoclastogenesis.

None of these studies has completely answered the question of the mechanism behind the anabolic effect of PTH, which likely results from a combination of all of these physiological effects. There are still unexplained phenomena that are likely regulated by other factors, as yet unknown, or just beginning to be unraveled. The PTH1R may be targeted to the nucleus of receptive cells (Watson *et al.*, 2000a,b). Immunoreactive PTH1R was found in both the cytoplasm and the nucleus of cultured osteoblast-like cells (Watson *et al.*, 2000b) and cells of rat kidney, gut, liver, and uterus (Watson *et al.*, 2000a), suggesting that the receptor plays a nuclear role. Nuclear translocation of the PTH1R appears to be physiologically relevant because its translocation is cell cycle dependent and is regulated by the cellular transportation machinery (Watson *et al.*, 2000b; Pickard *et al.*, 2006, 2007). The cell cycle dependence of PTH1R translocation may indicate a role in the effects of PTH on bone cell ontogeny described earlier. New aspects of cellular regulation by the nuclear PTH1R may shed further light on the anabolic action of PTH.

ANIMAL MODELS OF PTH EFFECTS ON BONE METABOLISM

Detailed investigation of the anabolic action of PTH requires a suitable animal model. The ovariectomized (OVX) mature rat rapidly develops osteopenia that is characterized by a significant decrease in both cancellous bone mineral and bone mass, and a marked increase in osteoblast and osteoclast activity typical of increased bone turnover (Frost and Jee, 1992; Kalu *et al.*, 1989; Liu *et al.*, 1991; Hori *et al.*, 1988; Hock *et al.*, 1988; Wronski *et al.*, 1989., 1991; Liu and Kalu, 1990; Kalu, 1991). The OVX rat has been widely accepted as a model for human postmenopausal osteoporosis because it closely parallels the early, rapid phase of bone loss characteristic of postmenopausal human bone loss (reviewed in Liu *et al.*, 1991; Kalu, 1991). The osteopenia of OVX rats shares many characteristics in common with the bone manifestations of perimenopausal bone loss, including increases in both bone resorption and activation frequency, with a relative deficiency in bone formation leading to a negative modeling balance (Frost and Jee, 1992; Kimmel *et al.*, 1993; Liu and Kalu, 1990; Liu *et al.*, 1991; Wronski *et al.*, 1989).

The OVX rat has been extensively used as a preclinical model for the *in vivo* anabolic action of intermittent treatment with PTH and is covered elsewhere in this book. Collectively, these studies have shown that intermittent treatment of the OVX rat with PTH or its anabolic fragments both prevents bone loss following OVX and reverses preexisting bone loss induced by OVX (Hori *et al.*, 1988; Lane *et al.*, 1996; Meng *et al.*, 1996; Lane *et al.*, 1996; Meng *et al.*, 1996; Akhter *et al.*, 2001; Kalu, 1984, 1991; Shen *et al.*, 1992; Kimmel *et al.*, 1993; Li *et al.*, 1999; Shen *et al.*, 2000; Alexander *et al.*, 2001; Fox *et al.*, 2006; Iwaniec *et al.*, 2007). Dempster and colleagues have shown that the increase in bone formation rate after intermittent therapy with PTH is primarily caused by an increase in both total mineralization surface and the mineral apposition rate that occurs in the first week after the initiation of treatment (Dempster *et al.*, 1993; Meng *et al.*, 1996). Measurement of bone mineral density (BMD) in PTH-treated rats has also shown positive increments in femur, vertebrae, and tibia confirming the histological data (Mosekilde *et al.*, 1994; Gunness-Hey and Hock, 1989; Wronski *et al.*, 1988). All of these studies show a PTH-induced increase in bone formation chiefly by the induction of osteoblast function. Thus, intermittent PTH therapy increases osteoblastic bone formation via increased activation frequency resulting in positive bone balance (Wronski *et al.*, 1993).

More recently, mouse models have been employed to study the anabolic effect of PTH and explore its mechanism of action. In brief, mouse strains are more heterogeneous than rats in both their “normal BMD” and responses to OVX or PTH treatment, but they offer a number of advantages over the rat model including economization (in terms of drugs, housing costs, etc.), the availability of strains with differing endogenous BMD (somewhat similar to various human groups), and the ability to readily analyze and manipulate their genetics (Bouxsein *et al.*, 2005a; Beamer *et al.*, 2002; Akhter *et al.*, 2004a, 2004b; Bouxsein *et al.*, 2004). In particular, the ability to produce both knockout and transgenic mice (constitutive and conditional) has already allowed for the analysis of many physiological parameters in the anabolic pathway of intermittent PTH treatment (Rosen *et al.*, 2000, 2004; Dumble *et al.*, 2004; Silva *et al.*, 2004; Pogoda *et al.*, 2005; Bodine *et al.*, 2007; Sawakami *et al.*, 2006; Hurley *et al.*, 2006; Yu *et al.*, 2006; Miao *et al.*, 2005; Bouxsein *et al.*, 2005b; Kitahara *et al.*, 2003; Demiralp *et al.*, 2002). As conditional knockout/knockin technology becomes more widely available, genetically manipulated mouse models are sure to yield new insight into PTH anabolism in bone. Finally, few studies have been published on our primate relatives. Those published do, however, demonstrate that anabolic therapy with PTH peptides is as effective in primates as in the rodent models. Several studies have shown that PTH(1–34) treatment results in significant increases in primarily trabecular BMD in cynomolgus monkeys (Jerome *et al.*, 1999; Brommage *et al.*, 1999; Sato *et al.*, 2004). A more recent study in rhesus macaques concurs and demonstrates that PTH(1–84) treatment for 18 months increases both bone formation rates and BMD (Fox *et al.*, 2007). Another important consideration is the quality of the bone produced by PTH therapy. By necessity, bone needs to have an appropriate trabecular structure to provide adequate mechanical strength (reviewed in Dalle and Giannini, 2004; Turner, 2002). Data from the rat have shown that

PTH works primarily by increasing trabecular thickness, not number (Kimmel *et al.*, 1993; Lane *et al.*, 1995; Li *et al.*, 1995; Shen *et al.*, 1993; Sibonga *et al.*, 2007; Fox *et al.*, 2006; Iida-Klein *et al.*, 2006; Akhter *et al.*, 2001; Lane *et al.*, 2003). In the mouse, PTH appears to enhance trabecular number and connectivity, as well as thickness (Xiang *et al.*, 2006; Pierroz *et al.*, 2006; Bouxsein *et al.*, 2005b; Zhou *et al.*, 2003; Iida-Klein *et al.*, 2007; Iida-Klein *et al.*, 2006). Data from primates indicates that PTH may act primarily by increasing trabecular number and connectivity (Fox *et al.*, 2007; Jerome *et al.*, 2001). Finally, human data indicate that PTH increases trabecular bone volume and connectivity with a shift to plate-like structures and a reduction in rod-like structures (Dobnig *et al.*, 2005; Jiang *et al.*, 2003). More detailed information can be obtained in the review of the effects of PTH on bone remodeling and structure by Compston (2006). One last point to bear in mind is the biomechanical strength of PTH-induced bone formation, because nothing is gained if the newly formed bone does not provide the strength and support to protect against fractures [bone qualities that affect strength are reviewed in Davison *et al.* (2006)]. Numerous studies of bone strength have looked at load-to-failure rates in vertebral bodies, femoral shafts and femoral necks, and tibia from various species and all have consistently shown that bone from PTH-treated OVX animals consistently performs better than that from sham-operated controls (Fox *et al.*, 2006; Iida-Klein *et al.*, 2006; Sato *et al.*, 2004; Akhter *et al.*, 2001; Shen *et al.*, 1995, 1998; Li *et al.*, 1995; Mosekilde *et al.*, 1994; Sogaard *et al.*, 1994; Ejersted *et al.*, 1993; Fox *et al.*, 2007; Jerome *et al.*, 2001).

CLINICAL STUDIES

This will not be an exhaustive review of all literature in this area. Systematic reviews of the literature pertaining to the clinical use of PTH will be continually updated, and a recent review is typical of these (Cranney *et al.*, 2006). The focus of this chapter will be to refer to the pertinent literature that illustrates the utility of PTH therapy in a variety of clinical situations.

Since the early studies by Reeve *et al.* (1976a,b, 1980), there have been numerous clinical studies describing the outcomes of therapy with PTH (e.g., Adami *et al.*, 2007; Black *et al.*, 2003; Body *et al.*, 2002; Boonen *et al.*, 2007; Bradbeer *et al.*, 1992; Cosman *et al.*, 2001, 2005, 2007; Finkelstein and Arnold, 1999; Finkelstein *et al.*, 1994, 1998a,b, 2003, 2006; Fujita *et al.*, 1999a,b; Gallagher, 1999; Greenspan *et al.*, 2007; Hesch *et al.*, 1989b; Hesp *et al.*, 1981; Hodsman and Fraher, 1990; Hodsman *et al.*, 1991, 1993b, 1997, 2000, 2003; Kurland *et al.*, 2000, 2004; Lane *et al.*, 1998a,b, 2000; Lindsay *et al.*, 1997; Neer *et al.*, 1987, 1993, 2001; Orwoll *et al.*, 2003; Plotkin *et al.*, 1998; Reeve *et al.*, 1976a,b, 1980, 1981, 1987, 1990, 1993, 1994; Rittmaster *et al.*, 2000; Rehman *et al.*, 2003; Roe *et al.*, 1999; Saag *et al.*, 2007; Slovik *et al.*, 1981, 1986; Sone *et al.*, 1995). Treatment protocols have been very heterogeneous, over periods of 6 to 36 months. Most subjects have been women, over 60 years of age with a diagnosis of osteoporosis, but more recently there have been studies involving younger women with acute estrogen deficiency (Finkelstein and Arnold, 1999; Finkelstein *et al.*, 1998b, 1994), men with osteoporosis (Kurland *et al.*, 2000, 2004; Orwoll *et al.*, 2003), and glucocorticoid-induced osteopenia (Finkelstein and Arnold, 1999; Finkelstein *et al.*, 1998b; Lane *et al.*, 1998b, 2000; Rehman *et al.*, 2003; Saag *et al.*, 2007).

It is only recently that studies have involved a randomized design in which control subjects did not receive PTH (Black *et al.*, 2003; Body *et al.*, 2002; Finkelstein and Arnold, 1999; Finkelstein *et al.*, 1998b, 1994, 2003; Fujita *et al.*, 1999b; Gallagher, 1999; Greenspan *et al.*, 2007; Hodsman *et al.*, 2003; Kurland *et al.*, 2000; Lane *et al.*, 1998b, 2000; Hodsman *et al.*, 2003; Neer *et al.*, 1999, 2001; Rittmaster *et al.*, 2000; Rehman *et al.*, 2003; Ross *et al.*, 1990; Saag *et al.*, 2007). Many studies have included concomitant therapy with the specific intention of minimizing the resorptive properties of PTH; "cotherapy" with estrogen has been widely used (Bradbeer *et al.*, 1992; Cosman *et al.*, 2001; Lane *et al.*, 1998b, 2000; Lindsay *et al.*, 1997; Reeve *et al.*, 1990, 1991, 1993), but other studies have included calcitonin (Hesch *et al.*, 1989b; Hodsman and Fraher, 1990; Hodsman *et al.*, 1991, 1993b, 1997, 2000), calcitriol (Neer *et al.*, 1987, 1991; Reeve *et al.*, 1987; Slovik *et al.*, 1986), and bisphosphonates (Black *et al.*, 2003; Cosman *et al.*, 2005; 2007; Finkelstein *et al.*, 2003). Other studies have examined whether use of antiresorptive therapies following PTH will preserve the anabolic gains achieved with PTH (Adami *et al.*, 2008; Black *et al.*, 2005; Cosman *et al.*, 2007; Kurland *et al.*, 2004; Rittmaster *et al.*, 2000).

There are many issues to be addressed in the assessment of the utility of PTH peptides as osteoporosis therapy. We will discuss these clinical issues under the headings by which regulatory agencies currently examine data for the treatment of osteoporosis. One major problem in evaluating PTH therapy has been the short duration (18 months or less) of the two largest RCTs and most of the other studies with teriparatide. The reason for the short duration of the major trials is the result of a carcinogenicity study being carried out by the sponsor of the teriparatide clinical trial program (Eli Lilly) at the same time as the clinical trials. In that study, rats treated for near-lifetime exposure to extremely high doses of teriparatide were found to have a dose-dependent increased incidence of osteogenic sarcoma (Vahle *et al.*, 2004; Tashjian *et al.*, 2002). The sponsor stopped all clinical trials and temporarily suspended the clinical research program. When further data analysis was available, the drug was successfully submitted for regulatory approval, based on the data from the truncated clinical trials, but the premature termination of the studies also limited the FDA-approved duration of future studies of PTH to 18 to 24 months.

FRACTURES

Fracture risk reduction, in a randomized clinical trial (RCT), with a placebo-control arm, is the gold standard of efficacy for all osteoporosis treatments. The clinical trials with PTH peptides do not match the size or subject selection of the other major therapeutic agents for osteoporosis, such as the bisphosphonates, estrogen or raloxifene, but it is clear that PTH has antifracture efficacy. Two early trials of PTH mentioned fracture rates as secondary outcomes (Hodsman *et al.*, 1997; Lindsay *et al.*, 1997), but neither study included a placebo-control group for comparison. Hodsman *et al.* (1997) reported a vertebral fracture incidence of 4.5/100 patient years in a group of elderly patients with preexisting vertebral fractures and receiving eight 28-day cycles of hPTH(1–34) over 2 years (Hodsman *et al.*, 1997). Lindsay *et al.* (1997) reported an incidence of 2.5/100 patient years in a comparable group of patients receiving daily hPTH(1–34) injections over 3 years. Because both studies enrolled high-risk female patients with an average of more than two preexisting vertebral fractures, an average age of over 60 years, and comparable BMD measurements, they document fracture incidences in a high-risk population. A reasonable historical comparison can be made with the placebo (calcium-supplemented) arm of the Fracture Intervention Trials, in which new vertebral fracture incidence rates were documented at 10/100 patient years in a cohort of women with only one prevalent osteoporosis-related vertebral fracture who were monitored for 3 years (Black *et al.*, 1997).

More recently, a placebo (calcium and vitamin D supplemented)-controlled RCT of teriparatide, which was specifically designed to evaluate the antifracture efficacy of this analogue in postmenopausal women with prevalent vertebral fractures, was reported (Neer *et al.*, 2001). The significant trial outcomes for reduced incident vertebral and nonvertebral fracture rates together with BMD measurements can be compared with placebo-treated patients and are shown in Figures 6, 7, and 8. Unfortunately, as noted earlier, the clinical trial was stopped at a mean duration of 18 to 19 months of therapy, because of a dose-dependent increased incidence of osteogenic sarcoma in rats receiving near-lifetime high-dose therapy. Despite the shortened duration of the study, with 20 μg and 40 μg of PTH(1–34) the relative risk (RR) of new vertebral fractures was 0.35 (95% confidence interval [CI] 0.22–0.55) and 0.31 (95% CI 0.19–0.50), respectively [absolute risk reduction (ARR) 9– 10%]. Treatment with 20 μg of PTH(1–34) resulted in a RR 0.47 (95% CI 0.25–0.88) for nonvertebral fractures

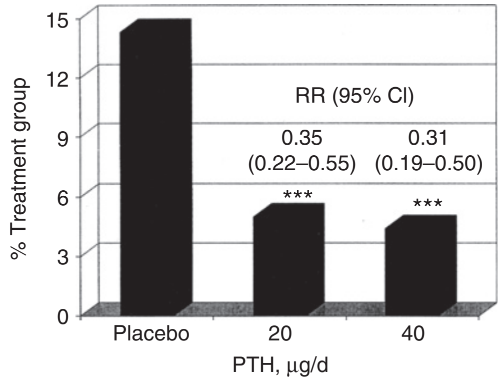

FIGURE 6 Reduction in vertebral fracture rates during a randomized controlled clinical trial of median duration 21 months. Compared with placebo ($n = 544$), both doses of PTH(1–34) (20 μg/day, $n = 541$; 40 μg/day, $n = 552$) significantly reduced the risk of one or more vertebral fractures (***$P < 0.001$). Adapted from Neer *et al.* (2001).

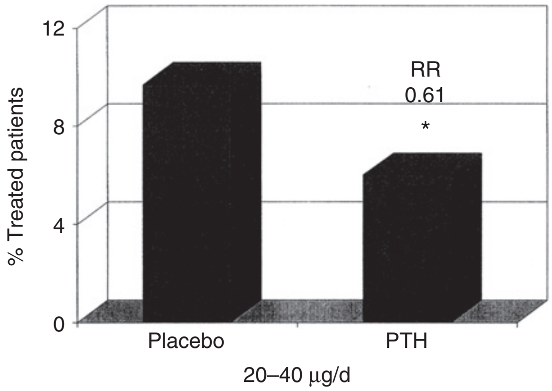

FIGURE 7 Reductions in all nonvertebral fractures during treatment with PTH(1–34) over 21 months. Adapted from Neer *et al.* (2001). Compared with placebo, new fractures were similarly reduced at both doses of PTH (*$P < 0.04$ to < 0.02).

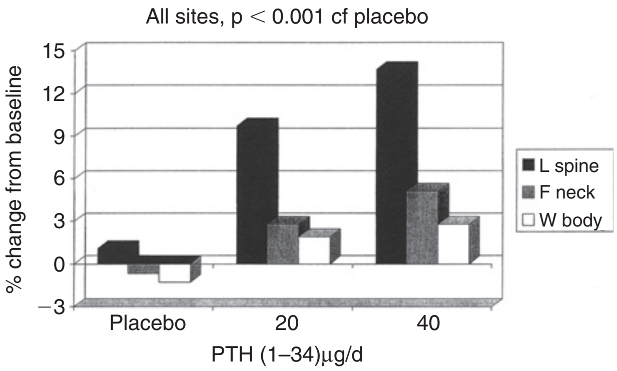

FIGURE 8 Changes in BMD measurements over 21 months during a randomized placebo-controlled clinical trial of PTH(1–34). Adapted from Neer *et al.* (2001).

and an absolute risk reduction of 3% (see Fig. 7). The study was not powered to look at subgroups of nonvertebral fractures, such as hip fractures, but it seems reasonable to conclude PTH therapy has the potential to reduce incident fractures not only in the spine, but also at other sites. An evaluation of the absolute fracture reductions with teriparatide in this study reveals a number needed-to-treat (NNT) estimate of only 10 and 27 women needing treatment to prevent one vertebral or one nonvertebral fracture, respectively. Recognizing differences in subject recruitment and duration of therapy, this compares favorably with the *post hoc* analysis of the Fracture Intervention Trials with alendronate reported by Ensrud *et al.* (1997); in women selected for alendronate therapy who are at comparable risk (prevalent vertebral fractures and femoral neck T score of less than 1.6), the NNT calculations to prevent one new vertebral fracture or any new clinical fracture were 16 and 26, respectively, albeit these estimates require up to 60 months of treatment.

The other large RCT of PTH utilized recombinant hPTH(1–84) (Greenspan *et al.*, 2007), which compared 100 μg of PTH to placebo in 2532 postmenopausal women with low bone density. The primary outcome variable was new or worsened vertebral fractures. The study was limited to 18 months of therapy because of the restrictions on duration of PTH therapy arising from the FDA conditions of approval of teriparatide as an osteoporosis therapy. The average age of patients participating in the study was 64 years, and less than 20% had a prevalent vertebral fracture on entry for the study. Because of difficulty recruiting older subjects at higher risk of fracture, the overall fracture rate was very low in this study. The incidence of new or worsened vertebral fracture in the placebo group was 3.4%, and 1.4% in the PTH-treated group. The absolute risk reduction was only 2%, but the relative risk of a new or worsened vertebral fracture in the treatment group was 0.42 (95% C.I. 0.24–0.72), with a NNT of 51. The number of nonvertebral fractures was similar for placebo and treatment groups. Nonvertebral fractures were only reported as adverse events and not fully adjudicated. Hypercalcemia was seen in 27.8% of the subjects receiving PTH compared with 4.5% of the subjects receiving placebo. This complication was usually mild, but contributed to the FDA decision not to grant full approval of the drug for the treatment of osteoporosis in the United States. The drug is, however, approved for this indication in Europe.

A small study by Body *et al.* (2002) compared teriparatide (40 μg/day) with therapy with alendronate (10 mg per day), and found a reduction in nonvertebral fractures compared with alendronate (4.1% versus 13.7%, $P = 0.042$). This was a randomized active treatment comparator study in 146 postmenopausal women, and it suffered from premature termination for the same reason as the major teriparatide study (Neer *et al.*, 2001). Although the reduction in fractures favoring teriparatide was significant, the investigators included fractures of the small bones of the hands and feet, which may or may not be representative of osteoporotic fractures.

An open-label follow-up study of the largest clinical trial of teriparatide (Neer *et al.*, 2001) in 1262 women provided evidence for continuing significant protection against vertebral fracture for at least 18 months after the cessation of teriparatide therapy (Lindsay *et al.*, 2004). The fractures were graded by radiologists blinded to subjects' treatment assignment. In a similar follow-up study of the original clinical trial of teriparatide in men with low bone density (Orwoll *et al.*, 2003), men who received teriparatide had a lower occurrence of all classes of vertebral fractures, but the numbers did not reach significance. However, when the analysis was restricted to moderate or severe vertebral fractures, there were significantly fewer ($P = 0.01$) in the TPTD-treated subjects (2 of 176 subjects) compared with those who received placebo (7 of 103). This effect was seen irrespective of whether the subjects received bisphosphonate or other osteoporosis therapy after teriparatide.

In a comparison of teriparatide and alendronate therapy for prevention of glucocorticoid-induced osteoporosis, 18 months of teriparatide therapy was associated with a significantly lower incidence of vertebral fractures in the teriparatide-treated subjects, but the overall fracture rate was similar between the two groups (Saag *et al.*, 2007).

In summary, there is strong evidence that parathyroid hormone therapy will prevent vertebral fractures in postmenopausal osteoporosis. Prevention of nonvertebral fractures was demonstrated for teriparatide, but not for intact PTH, which has not been tested in a population at high risk for nonvertebral fractures. There have been no adequately powered fracture outcome clinical trials testing PTH against an approved pharmacological agent for the treatment of osteoporosis, but the available trials suggest there might be a slight fracture prevention advantage to teriparatide over alendronate.

BONE MASS MEASUREMENTS

As an anabolic agent, PTH has shown dramatic effects on bone mass, predominantly in skeletal areas rich in trabecular bone, like the spine. Table I selectively summarizes the results of available studies of PTH therapy in which bone mass was measured. The data provided in the table are the percentage increase over baseline or placebo for the highest dose of PTH analogue used in the trial. The method of measurement of bone mass or density (BMD) has varied among these studies and includes both dual-energy x-ray absorptiometry (DXA) and quantitative computed tomography (qCT) for vertebral and femoral neck sites, as well as peripheral areal measurements of the forearm by DXA and qCT. Because these BMD measurements are not entirely comparable, only key points are summarized in this section.

TABLE I Selected Publications Outlining Changes in Bone Mineral Density Measurements during and/or Following PTH Therapy

Author[*] (year)	Age (years)	No. of subjects: treatment/ control	PTH dose[†]	Study duration (months)	Concomitant therapy	Therapy controls	BMD[‡] (% change from baseline)
Postmenopausal women							
Reeve *et al.*, 1990	64	12/12	500 units/day	12	Estrogen or androgen	12 patients on fluoride	QCT spine +50% Forearm ns
Lindsay *et al.*, 1997[§]	60	17:17	25 µg/day	36	Estrogen Calcium (Ca)	17 patients on estrogen	Spine +13% Total hip +2.7% Forearm ns, TBBM +8.0%
Hodsman *et al.*, 1997	67	16 (PTH + CT) 14 (PTH alone)	65 µg/day, 28-day cycles	24	Calcitonin	None	Spine +10.2% Femoral neck +2.4%
Hodsman *et al.*, 2003	64.5	162/55	PTH(1–84) 50,75,100 µg / day	12	Ca/vit D	None	Spine +6.9% Hip ns TBBM −0.9%
Fujita *et al.*, 1999a	70	220/0	15, 30, 60 µg/ week	11	None	None	Spine +8.1% Metacarpal cortical thickness ns
Roe *et al.*, 1999 [Abstract]	Postmenopause	37/37	40 µg/day	24	HRT Ca/vit D	HRT	QCT spine +79% Spine +29.2% Femoral neck +11%
Rittmaster *et al.*, 2000b	64	12,17,18,19	After 12 months PTH(1–84): 25, 50, or 75 µg, or placebo	12 on 12 off	None Alendronate (ALN) 10 mg/day Ca/vit D	66 women: all on ALN	PTH pretreated patients: Spine +14% Femoral neck +4.5% TBBM +3.3%
Neer *et al.*, 2001[§]	69	1093/544	20, 40 µg/day	21	None (Ca/vit D)	None	Spine +13.7% Femoral neck +5.1% Forearm −3.2% TBBM +1.0% (hologic) TBBM +4.5% (lunar)
Body *et al.*, 2002[§]	65.5	73/73 PTH vs alendronate	40 µg/day	14	Ca/vit D	ALN 10 mg/day	Spine +12.2% (PTH) vs +5.6% (ALN) PTH >ALN for femoral neck, total hip, and TBBM. Distal radius −4% (PTH) vs no change (ALN)
Black *et al.*, 2003	70	PTH 119 PTH/ALN 59 ALN 60	100 µg/day PTH(1–84) vs alendronate (ALN) or PTH plus ALN	12	Ca/vit D	ALN 10 mg/day	Spine +6.3% PTH; +6.1% PTH/ALN; +4.6% ALN Total hip +0.3% PTH; +1.9% PTH/ALN; +2.1% ALN QCT trab. Spine +25.5% PTH; + 12.9% PTH/ALN; +10.5% ALN

(*Continued*)

TABLE I (*Continued*)

Author[*] (year)	Age (years)	No. of subjects: treatment/ control	PTH dose[†]	Study duration (months)	Concomitant therapy	Therapy controls	BMD[‡] (% change from baseline)
Greenspan *et al.*, 2007[§]	64.5	1286/1246	100 µg/day PTH(1–84)	18	Ca/vit D	None	Change vs. placebo: Spine +6.9% Total hip +2.1% Distal radius –3.4% Whole body ns QCT trab spine +37%
Other causes of osteoporosis							
Finkelstein *et al.*, 1998a[1]	31	21:0	40 µg/day	12	Nafarelin	22 on nafarelin	Spine, hip, forearm TBBM[¶]; ns
Gonadotropin suppression							
Lane *et al.*, 1998a[2] Glucocorticoid	62	28:0	40 µg/day	12	Prednisone Estrogen	23 on prednisone + estrogen	QCT spine +35% Spine +11%, all other sites ns
Finkelstein *et al.*, 1999[1]	34	15:0	None Follow-up Observation Post-Nafarelin	12	None	Remained within original cohort	Both groups gained spine BMD after stopping nafarelin, but prior PTH-treated group gained more
Lane *et al.*, 2000[2] Glucocorticoid	62	28:0	40 µg/day	12 on + 12 off	Prednisone Estrogen	23 on prednisone + estrogen	QCT spine +45.9% Spine +11% Femoral neck +5.2%, Forearm ns
Kurland *et al.*, 2000 Males	50	0:10	40 µg/day	18	None	13 on Ca/ vit D	Spine +13.5% Femoral neck +2.9% Forearm −1.2% (ns)
Orwoll *et al.*, Males	59	290:147	20 µg/day; 40 µg/day	11 (median)	None	Ca/vit D	Spine +5.9 (20 µg), +9%(40 µg) Total hip +1.17 (20 µg [ns]); +2.33(40 µg) Distal radius ns Total body ns
Saag *et al.*, 2007[§] Glucocorticoid	57.6 (ALN); 56.1 (PTH)	214:214	20 µg/day	18	Prednisone	ALN 10 mg daily	Spine +7.2% PTH; +3.4% ALN ($P < 0.001$) Total hip +3.8% PTH +2.4% ALN ($P = 0.005$)

Note. TBBM, *total-body bone mineral (usually excludes skull); trab, trabecular. Data are significantly different from controls unless stated (ns).*

[*] *Superscript number by author indicates published data from the same study cohort.*

[†] *Analog is h*PTH(1–34) *unless otherwise stated.*

[‡] BMD *measurement made by* DXA *or similar areal method unless stated for* QCT *(quantitative computed tomography).*

[§] *Fracture prevention by* PTH *versus control or comparator.*

[¶] *Control group had a significant loss of* BMD *(i.e.,* PTH *prevented loss).*

In the Phase II RCT of hPTH(1–84) (Hodsman *et al.*, 2003), the total body bone mineral density (TBBM) and mineral content showed a decrease of 0.9% compared with placebo over the 1 year of treatment. This finding raises the concern that PTH acts to increase trabecular bone mass at the expense of the cortical bone envelope. Alternatively, it seems likely that PTH causes acceleration of cortical remodeling, resulting in an enlarged expansion of the remodeling space. Increments in cortical bone mass would be expected to occur later, as the anabolic action of PTH gradually leads to a net remodeling gain over time. Within this study, the subsequent treatment of approximately one-third of the study cohort with an antiresorptive agent, alendronate, for a second 12-month interval supports the latter alternative explanation (see later) (Rittmaster, *et al.*, 2000).

In general, it appears that BMD gains achieved with PTH therapy will slowly decline after the therapy is stopped. This BMD loss appears to be preventable by institution of another osteoporosis therapy: bisphosphonate (Rittmaster *et al.*, 2000, Black *et al.*, 2005), raloxifene (Adami *et al.*, 2008; Cosman *et al.*, 2007), or estrogen (Cosman *et al.*, 2001).

Lumbar Spine

Consistent with the anabolic effect on trabecular bone noted in histomorphometric analysis (see earlier), PTH causes a significant increase in BMD in the lumbar spine which exceeds that reported for all other available therapies except sodium fluoride (Riggs *et al.*, 1990) and strontium ranelate (Meunier *et al.*, 2004). The studies of PTH as a monotherapy are of relatively short duration (2 years or less) so it is impossible to determine whether this increase is dose- and duration-dependent or if it reaches a plateau in a manner similar to those of antiresorptive therapies (Riggs and Melton, 1992). There are three RCTs of hPTH(1–84) monotherapy: the Phase II and III studies of recombinant hPTH(1–84) (Hodsman *et al.*, 2003; Greenspan *et al.*, 2007) and the PaTH Study (Black *et al.*, 2003). The BMD response to teriparatide has been documented in a number of small studies over the past two decades, but the two larger Phase III studies of women (Neer *et al.*, 2001) and men (Orwoll *et al.*, 2003) are the most instructive.

In the Phase II study of hPTH(1–84), women with BMD criteria for osteoporosis (Kanis *et al.*, 1994) received daily subcutaneous injections of 50, 75, and 100 μg, in a randomized, placebo-controlled 1-year clinical trial sponsored by Allelix Biopharmaceuticals (Mississauga, ON, Canada). A dose-dependent increase in lumbar spine BMD measured by dual-energy x-ray absorptiometry (DXA) was seen over the baseline measurement, reaching 6.9% in the 100 μg/day group. Changes in bone mineral content were even greater, reflecting an apparent increase in the area of the vertebral bodies examined by DXA (Hodsman *et al.*, 2003).

In the 18-month Phase III RCT of hPTH(1–84) (Greenspan *et al.*, 2007), there was a relatively large number of subjects that discontinued or reduced treatment. A significant 6.9% increase in DXA BMD of lumbar spine was observed versus placebo, with 25% of subjects having more than a 10% increase. A subgroup had volumetric trabecular density of L3 measured by qCT, which showed a 38% increase over placebo (95% CI 24–53%), indicating a dramatic effect on spinal trabecular bone.

The PaTH Study, a three-arm RCT comparing one year of therapy with alendronate 10 mg daily to daily subcutaneous injections of 100 μg of hPTH(1–84) alone, or PTH in combination with alendronate, recruited 238 postmenopausal women with low bone mineral density at the hip or spine (a T score < -2.5, or < -2.0 with an additional risk factor for osteoporosis). BMD at the spine and hip was assessed by DXA and qCT. Spine BMD increased the most in the PTH-treated group, and the increase in qCT spine (volumetric density of trabecular bone) was double the amount seen with alendronate or alendronate in combination with PTH (Black *et al.*, 2003).

In the Eli Lilly Phase III RCT of hPTH(1–34) (Neer *et al.*, 2001), dramatic effects on BMD were observed. In the spine, the increase in BMD was 9.7% and 13.7% in the groups receiving 20 and 40 μg, as opposed to an insignificant increment of 1.1% in the placebo group (see Fig. 4). TBBM increased in the PTH-treated groups (Neer *et al.*, 2001). This is in contrast to the shorter 1-year study of hPTH(1–84) (Hodsman *et al.*, 2003), which reinforces the possibility that an initial decline of BMD reflects the opening of a remodeling space.

The general conclusion from all studies of lumbar spine BMD is that a progressive increase occurs for the duration of therapy (see Table 1). When PTH is stopped, two studies have shown a continuing gain in bone mass that may reflect the filling in of the increased remodeling space (Finkelstein and Arnold, 1999; Lane *et al.*, 2000). On the other hand, cessation of PTH therapy might be expected to result in reestablishment of the pretreatment pattern of bone losses. This is suggested in the spinal BMD measurements of patients participating in the study of Neer *et al.* (2001), if they did not take an osteoporosis drug during the 18 months of post-PTH follow-up (Lindsay *et al.*, 2004).

Hip

There are differences in the BMD responses to PTH among different regions of the skeleton. One of the major concerns about "anabolic agents" is that the increase in spinal (largely trabecular) bone mass may be at the expense of cortical bone, in particular, at important fracture regions such as hip and forearm. Some studies with sodium fluoride have shown dramatic increases in the spine but loss, or no improvement, at cortical bone sites. If PTH causes a marked increase in cortical bone remodeling, one might expect a decline in BMD in cortical sites during the early stage of PTH therapy, with a later increase if the net effect of PTH is increased bone formation. In reviewing the results of the clinical studies that included BMD measurements at the hip (see Table 1), a net positive effect of PTH is seen.

Several studies showed no change or a decline in femoral neck or total hip BMD within the first year of treatment (Finkelstein *et al.*, 1998a; Lane *et al.*, 1998a), and this is particularly clear in the 1-year study of hPTH(1–84) (Hodsman *et al.*, 2003), in which there was no change in hip BMD. However, in the latter study, there was an increase in the second 6 months of the study.

The few studies that have gone beyond 12 months of therapy have shown a gain in hip BMD (Hodsman *et al.*, 1997; Kurland *et al.*, 2000; Lindsay *et al.*, 1997; Neer *et al.*, 2001; Roe *et al.*, 1999). In the Phase III trial of hPTH (1–34) reported by Neer *et al.* (2001), there were significant BMD gains seen in the hip after just under 2 years of therapy. In the total hip BMD measurements, the placebo group lost 1.6% whereas gains of 2.6% (20 μg dose) and 3.6% (40 μg dose) were seen in the PTH-treated groups (see Fig. 4). Similar dose-dependent improvements in trochanter, femoral neck, and intertrochanteric regions were observed, and the 5.1% increase in femoral neck is greater than that reported for any antiresorptive therapy after 2 years. A modest but significant increase in hip density was seen after 18 months of treatment with PTH(1–84) (Greenspan *et al.*, 2007). In the study by Greenspan *et al.* (2007) DXA at the femoral neck and total hip increased over placebo by 2.5% and 2.1%, respectively, and volumetric BMD of the femoral neck increased by 4.7% over placebo. Clearly, the effects on BMD in the hip region are not as dramatic as those seen in the spine, but the earlier concern about loss of bone at the hip appears to simply be a function of opening a remodeling space, which is followed by a net increase in bone formation at the femoral neck.

Again, these observations can be explained by postulating the activation of an increased number of bone remodeling units, resulting in an increased remodeling space within the skeleton. During the early stages of PTH therapy, this may cause a decline in BMD in some areas, but as the enlarged remodeling space begins to be filled, increased hip BMD appears as the net result. The clinical trials were not powered to examine effect on hip fracture, but there is no evidence to suggest this initial opening of remodeling space is associated with any clinically important reduction in bone strength.

Forearm

The earlier studies of PTH have shown inconsistent results in the forearm, but the more recent and larger RCTs of both teriparatide and PTH have shown areal BMD by DXA declines. In reviewing studies in which PTH was not being used in combination with estrogen, three small studies showed no change (Hesch *et al.*, 1989b; Neer *et al.*, 1987; Reeve *et al.*, 1987), and three showed significant decline (Hodsman *et al.*, 1991; Neer *et al.*, 1997, 2001). One study, using a PTHrP analogue (semparatide acetate) reported by Gallagher (1999), also showed a decrease in forearm BMD. The best assessment of hPTH(1–34) effects on BMD of the forearm probably comes from the Phase III trial reported by Neer *et al.* (2001), which averaged 21 months of therapy. It showed a significant, though modest 2-3% decline in BMD of the forearm. This may be a concern, but the decline was not progressive in the second year of the study (Neer *et al.*, 2001), and was not associated with any trend to increased risk of wrist fracture. A more recent analysis of a subgroup of patients in the large teriparatide trial suggests that the apparent loss of BMD at the radius is really an artifact of the standard method of calculating areal bone density by DXA. Zanchetta *et al.* (2003), demonstrated that the area of the radius region of interest in the DXA measurements was significantly greater in the teriparatide-treated subjects than in the placebo group. Thus, although the DXA BMD decreased with teriparatide, the biomechanical result of increased bone area (cortical diameter and periosteal circumference) would actually be a stronger bone, which would be more resistant to fracture.

Total Body Bone Mineral

In the 1-year Phase II RCT of hPTH(1–84), there was a significant decline in TBBM content as assessed by DXA (Hodsman *et al.*, 2003). In the subjects receiving 75 and 100 μg of PTH(1–84), there was a slight but significant decline (0.3 and 0.9%, respectively) in TBBM, reflecting loss in the arms and legs. This loss was thought to represent a generalized increase in cortical bone turnover and would be expected to be transient. In the 18-month Phase III RCT of PTH(1–84), there was no change in whole-body DXA measurements in the treatment arm, and a non-significant trend to lower whole-body BMD in the placebo arm (Greenspan *et al.*, 2007). Longer-term studies with PTH(1–34) have demonstrated small increases in TBBM as shown in Table I and Fig. 4 (Lindsay *et al.*, 1997; Neer *et al.*, 2001).

Finite-Element Analysis

High-resolution QCT studies of bone have allowed use of sophisticated computer-generated modeling to estimate biomechanical properties of bone without having to destroy the bone in the process. Animal studies have indicated that PTH has improvements in mechanical strength out of proportion to that which would be expected simply through increased bone density (Kim *et al.*, 2003). Despite increasing cortical porosity in ovariectomized monkeys treated with teriparatide, the biomechanical strength of bone at the femoral neck was markedly increased (Sato *et al.*, 2004). Recently, in a clinical trial comparing alendronate and teriparatide, a subset of patients (28 teriparatide; 25 alendronate), who had QCT scans of the spine at baseline and under treatment, were assessed by using finite-element modeling to estimate vertebral body strength changes elicited by the two therapies. Both therapies increased estimated strength of the vertebrae at 6 months in at least 75% of patients compared with baseline, with increased average volumetric density and increased strength in the trabecular bone, but the median percentage increases for these parameters were 5- to 12-fold greater for teriparatide (Keaveny *et al.*, 2007).

PTH IN COMBINATION WITH OTHER TREATMENTS

Several methods of combining PTH with other osteoporosis therapies have been tested. On theoretical grounds, an attractive approach to osteoporosis therapy would be to combine an anabolic agent like PTH, which increases both resorption and formation, with an anticatabolic agent that would reduce the resorption stimulated by PTH while allowing the increased bone formation to continue. Sequential therapy with an anabolic agent (PTH), followed by an anticatabolic agent to preserve the gains achieved with PTH makes eminent sense, and recent evidence supports this approach (Rittmaster *et al.*, 2000; Cosman *et al.*, 2001; Black *et al.*, 2005). In practice, and dictated in part by the high purchase price of PTH therapy, PTH is usually started after the patient has undergone therapy with an anticatabolic agent, often for many years preceding the use of PTH. It is only in the past 8 years that these approaches to combination therapy have been tested systematically in RCTs.

Since the earliest years of study, PTH therapy has been used in a heterogeneous fashion: as a single agent, with or without nutritional calcium and vitamin D supplements (Fujita *et al.*, 1999a; Hesp *et al.*, 1981; Kurland *et al.*, 2000; Neer *et al.*, 2001; Reeve *et al.*, 1980a,b, 1981, 1976a,b), with estrogen or androgen as a concurrent antiresorptive agent (Bradbeer *et al.*, 1992; Lane *et al.*, 1998a; Lindsay *et al.*, 1997; Reeve *et al.*, 1990, 1991; Roe *et al.*, 1999; Cosman *et al.*, 2001), with raloxifene (Deal *et al.*, 2005; Cosman *et al.*, 2007), with calcitriol (Neer *et al.*, 1987, 1991, 1993; Reeve *et al.*, 1987; Slovik *et al.*, 1986), and in several cyclical protocols with or without calcitonin (Hesch *et al.*, 1989b; Hodsman and Fraher, 1990; Hodsman *et al.*, 1991, 1993b, 1997; Reeve *et al.*, 1987).

Despite the heterogeneity of these protocols, the overall effect of PTH has been remarkably consistent: an increase in trabecular bone (as measured by BMD or histomorphometry), with somewhat smaller changes in bone mass in the appendicular skeleton. Some of the studies of combination of PTH with bisphosphonate therapy have raised concerns about diminished response of the skeleton when the bisphosphonate precedes or is concurrent with PTH (Ettinger *et al.*, 2002; Finkelstein *et al.*, 2003, Black *et al.*, 2003; Boonen *et al.*, 2007).

Most reported studies of combination therapies have had small samples of treated subjects and thus a low power to dissect the interactions of concurrent therapies. None have been adequately powered to identify any additional antifracture benefits of the combination therapies being tested.

Estrogen

Estrogen appears to have the best evidence that it does not interfere with the anabolic effects of PTH, and may even augment them. The early literature is confusing. Cosman *et al.* (1993) infused PTH over 20 hours (approximately 800 units to each of 17 estrogen-treated and 15 estrogen-deficient postmenopausal women with osteoporosis). The estrogen-deficient women had a significantly higher excretion of bone resorption markers (urinary OH-proline and deoxypyridinoline) suggesting a protective effect of estrogen on resorption. However, in a similar experiment, Tsai *et al.* (1989) infused 400 units/day for 3 days to three groups of subjects, premenopausal and postmenopausal women with and without osteoporosis. They found no differences in serum calcium or urinary OH-Pro excretion and concluded that estrogen did not have such a protective effect. Using an alternative approach of calcium deprivation to induce endogenous 2° hyperparathyroidism, the same group reached similar conclusions (Ebeling *et al.*, 1992). Marcus *et al.* (1992) gave acute (20 min) infusions of graded doses of PTH to 15 postmenopausal women before and after starting estrogen replacement, and no differences were found in serum 1,25(OH)$_2$D increments or urinary cAMP excretion as a result of estrogen therapy. They concluded that the renal–endocrine axis was not affected by estrogen deficiency. The flaw in these arguments is the fact that clinical responses to PTH infusion favor bone catabolism, whereas intermittent injections favor anabolism (Hodsman *et al.*, 1993b).

In the study by Reeve *et al.* (1991), daily PTH injections and concurrent estrogen therapy were combined in nine women. By comparison with historical controls (women treated with PTH alone) (Reeve *et al.*, 1980b), calcium balance studies were significantly improved; cotreatment with estrogen actually led to a 12% decrease in urinary calcium (Reeve *et al.*, 1991), rather than the 14% increase seen historically (Reeve *et al.*, 1980b). To date, there are no controlled factorial studies to test the estrogen effect independently of PTH.

However, many clinical studies of PTH therapy have incorporated concurrent therapy with estrogen, or estrogen/progesterone therapy (HRT; see Table I). The intent of these combinations has been to limit the ongoing bone resorption induced by PTH. In this context, Lindsay *et al.* (1997) reported a progressive total 3-year net gain over baseline lumbar spine BMD of 13%. In a study of glucocorticoid-treated women, who were all on stable HRT, Lane *et al.* (1998a) reported similar increments of 11% in the spine (35% by QCT) over 1 year. In a more recent RCT, employing the same dose of PTH(1–34) (40 μg daily) in postmenopausal women receiving HRT, Roe *et al.* (1999, 2000) reported average gains of BMD in the lumbar spine of 29% over 2 years (79% increments at this site as measured by QCT). It is difficult to reconcile the dramatic differences in BMD increments, on the one hand, between the two studies reported by Lindsay *et al.* (1997) and Lane *et al.* (1998a) and, on the other hand, by Roe *et al.* (1999, 2000), despite the reported dose of PTH being similar for all three studies. Possibly the absolute BMD of patients reported by Roe *et al.* (1999, 2000) who were approximately 8 years older was much lower than in the other studies, leading to a much higher relative gain. Alternatively the specific activity of the preparation used by Roe *et al.* may have been higher, leading to a larger functional dose.

More recently, in an expanded follow-up study to that of Lindsay *et al.* (1997), Cosman *et al.* (2001) demonstrated that following PTH therapy with continued HRT preserved PTH-stimulated gains in BMD. Patients who had been taking HRT for 2 years were recruited to receive 3 years of HRT and teriparatide 25 μg/day, or continue HRT. Both groups were then monitored for an additional year receiving HRT alone. The PTH plus HRT group had an increase in BMD by 13.4 ± 1.4% in the spine, 4.4 ± 1.0% in the total hip, and 3.7 ± 1.4% in the total body. One year after stopping the teriparatide, BMD was stable, and vertebral fractures were still significantly fewer in the group that continued HRT than in the control group receiving HRT alone for the 4 years of study.

The weight of evidence would suggest that, at worst, postmenopausal estrogen or estrogen/progestogen therapy has no effect on the skeletal response to PTH, when the two therapies are given concurrently. Under the same conditions, when PTH is stopped but the estrogen therapy is continued, there is minimal change in bone density, indicating that estrogen prevents the expected bone loss when PTH is stopped. There have been no RCTs comparing PTH alone with PTH plus estrogen or a placebo. Consequently, although the gains in bone density appear to be at least the equivalent of what has been reported in the RCTs of PTH alone, it is not clear that estrogen enhances the response to PTH.

Selective Estrogen Receptor Modulators (SERMs)

There have been two clinical trials indicating that the SERM, raloxifene (60 mg daily, taken concurrently), does not inhibit the anabolic response to teriparatide (Deal *et al.*, 2005; Cosman *et al.*, 2007). In a six-month randomized-double blind trial comparing teriparatide plus raloxifene versus 20 μg daily of teriparatide plus placebo, biochemical markers of bone turnover indicated that raloxifene reduced the bone resorption response to teriparatide, whereas the increase in the biochemical marker of bone formation was similar in both treatment groups (Deal *et al.*, 2005). The combination therapy showed a trend to a greater increase in lumbar spine density, and the increase in total hip BMD was significantly greater in the combination group than in the teriparatide-alone group. Interestingly, the teriparatide-induced increase in serum calcium was blunted by the combination therapy with raloxifene. This small study supports the concept that use of an agent, like estrogen or a SERM, acting through the osteoblast to reduce bone resorption without causing major changes in bone formation, does not interfere with the actions of PTH, and might even augment them.

This observation was confirmed in a smaller but longer-duration study by Cosman *et al.* (2007), in which 42 osteoporotic postmenopausal women being treated with raloxifene were randomized to continue on raloxifene alone or take teriparatide 25 μg daily for 12 months while continuing raloxifene. They were then monitored for another 12 months on raloxifene alone. After one year of therapy with teriparatide, spine bone density increased by 9.6% and total hip 2.7%, whereas a 4.3% reduction in BMD at the radius was observed. These changes are typical of, and at least as great as what would be expected with PTH alone. In the year after PTH withdrawal, continued raloxifene prevented significant decline in bone density at the spine or hip, although the BMD at the radius remained significantly lower than in those who never received teriparatide. As noted earlier, change in radius DXA BMD may be partly explained by an increase in cross-sectional area.

In a RCT of one year of teriparatide (20 μg daily) followed by one year of raloxifene or placebo (Adami *et al.*, 2008), the placebo group showed a 4% loss of lumbar spine BMD in the first year after teriparatide, but the loss was 1% in the raloxifene-treated subjects. A second year of raloxifene therapy (open label) for both groups reversed the LS BMD decrease with a placebo, resulting in similar decreases 2 years after randomization (−2.6 ± 0.4% (raloxifene-raloxifene) and −2.7 ± 0.4% (placebo-placebo). Raloxifene therefore appears to prevent the usual loss of lumbar spine bone density after stopping teriparatide therapy, but does not result in further gains; whereas a modest increase in hip BMD occurs when teriparatide is followed by raloxifene.

Bisphosphonates

Concurrent or prior use of bisphosphonates with PTH is an area of major concern and controversy in considering PTH as a therapy for osteoporosis. The data for bisphosphonate interactions with PTH in animal models are unclear, with contradictory evidence that the concurrent use of bisphosphonates may blunt the anabolic agents of PTH (Cheng *et al.*, 1995; Delmas *et al.*, 1995; Mashiba *et al.*, 1995), although this is not an invariable finding (Mosekilde *et al.*, 1995). This is an important issue.

Concurrent Therapy: PTH and Bisphosphonates

At the time of the first writing of this chapter, there were no clinical trials of PTH in combination with a bisphosphonate. Now there have been several studies, but the results are not entirely consistent nor have they been uniform with respect to patient selection. Although, in theory, the use of an antiresorptive agent might allow the anabolic effects of PTH to predominate, and this may, in fact, be the case with estrogen and raloxifene, the results with the bisphosphonate alendronate have been very different. In 2003, two studies indicated that the bone density response to teriparatide appeared to be blunted by prior and continued therapy with alendronate. The PaTH study of PTH, given with or without alendronate to treatment-naïve individuals, showed a better bone density response by DXA and QCT when PTH was given as a monotherapy. The trabecular bone response to one year of PTH(1–84) was significantly greater at the lumbar spine than the response to alendronate alone or alendronate plus PTH in combination. Volumetric QCT analysis at the spine showed a significantly greater response to PTH alone than PTH in combination with alendronate or alendronate alone. At the hip, alendronate's effect on bone density was slightly superior to that seen with PTH alone.

In a study of men with low BMD, Finkelstein *et al.* (2003) gave teriparatide 40 μg s.c. daily to men with low bone density for 24 months. After prior therapy with alendronate 10 mg daily for 6 months, the subjects were randomly assigned to teriparatide, teriparatide plus continued alendronate, or alendronate alone. Teriparatide caused the greatest increase in BMD by DXA in all areas studied except the distal radius. It was apparent from both spine and hip DXA, as well as volumetric spinal QCT studies, that the combination of alendronate with teriparatide resulted in a diminished bone density response in comparison with teriparatide alone. The BMD response to combination therapy was intermediate between PTH alone and alendronate alone.

In contrast, Cosman *et al.* (2005) randomly assigned patients to alendronate 70 mg p.o. once weekly, weekly alendronate plus teriparatide 25 μg s.c. once daily, or weekly alendronate plus cyclic treatment with teriparatide (three months of daily teriparatide 25 μg s.c. followed by three months off therapy). The subjects were postmenopausal osteoporotic women, and the groups had been receiving alendronate for an average of 2.8 to 3.5 years prior to study entry. Cyclic or continuous teriparatide caused similar increments in BMD (DXA) of the lumbar spine of approximately 5% above the patients who continued alendronate, and there was no apparent blunting of the response to teriparatide by the alendronate, although the response to teriparatide was not as great as might be expected in treatment naïve patients. Biochemical markers of bone formation were increased throughout the 18-month trial, although for the cyclic teriparatide group, the marker of bone resorption was not significantly greater than the alendronate-alone group at 18 months. This suggests that the weekly alendronate may not inhibit bone remodeling as significantly as daily, and that cyclical PTH may be as effective as continuous therapy in the presence of continued bisphosphonate therapy. Although the alendronate may have some inhibiting effect on the anabolic response to PTH, the BMD response to teriparatide while continuing weekly alendronate therapy is still quite significant.

Most investigators believe PTH's effect on bone is somewhat blunted by prior or continued therapy with bisphosphonate, despite the apparent retained response documented by the study of Cosman *et al.* (2005). The recent publication of the EUROFORS study (Boonen *et al.*, 2007) indicates that prior therapy with a bisphosphonate, although perhaps diminishing the bone density effect of teriparatide, does not eliminate it. Further, there is a suggestion that in the final six months of two years of therapy following bisphosphonate treatment, there is a further increment in bone density (Boonen *et al.*, 2007).

There are many cautions in attempting to compare the studies of combination of prior and/or concurrent bisphosphonate therapy and PTH. The study designs, dose, and choice of PTH peptide differ among the available studies. Further, the studies are not powered or designed to look at fracture outcomes. Also, bone biopsies have not been reported in studies comparing PTH therapy with a combination PTH and bisphosphonate, and as noted earlier, DXA may not always be relied upon in assessing the response to PTH.

If prior bisphosphonate therapy does indeed cause blunting of PTH response, this is unfortunate, because most patients currently being treated with PTH have received bisphosphonate therapy first. This is, in part, because PTH therapy has only recently been approved as a therapy for osteoporosis. But because it is much more expensive than bisphosphonate therapy, PTH tends to be reserved for patients who have more severe osteoporosis, or patients who are considered to have failed to respond to other osteoporosis therapies (usually a bisphosphonate among them). Indeed, the regulatory agency approval of PTH in many countries includes an indication for patients who have failed to respond to other osteoporosis therapies. Further, because of the concern about osteogenic sarcoma in carcinogenicity studies performed in rats, most countries' approval of PTH peptides limits the duration of therapy to 18 or 24 months. If a bisphosphonate reduces or delays response to teriparatide, a longer duration of therapy might be required in those individuals, but is not currently allowed under the terms of most countries' regulatory agency approval.

Although clinical trial results have not been consistent, current data indicate concurrent therapy with a bisphosphonate does not offer additional benefit to the actions of PTH, although it is not certain that bisphosphonate therapy eliminates the response (Cosman *et al.*, 2005). Prior therapy with a bisphosphonate may reduce the BMD response to PTH, although some would argue that the apparent reduction simply is because of the patient already having experienced a positive effect of the bisphosphonate, thus leaving less room for improvement by PTH (Ettinger *et al.*, 2004).

Consecutive Therapy: PTH Followed by Bisphosphonate

Given the increase in skeletal bone remodeling induced by an activation drug such as PTH (at the BMU level), it is likely that the remodeling space within all skeletal bone envelopes will be enlarged as a transient state (Parfitt, 1980). The enlarged remodeling space could be exploited by antiresorptive drugs as maintenance therapy after discontinuing PTH. This hypothesis has been tested in only one published study.

Approximately one-third of the subjects in the Phase II RCT who received PTH(1–84) for 1 year (Hodsman *et al.*, 2003) were subsequently treated with open-label alendronate, 10 mg daily (Rittmaster *et al.*, 2000). Following PTH with alendronate caused a further rapid increment in BMD at all sites, such that the aggregate gain over 2 years in BMD at the highest dose of PTH(1–84) was 14.1% at the spine, 4.5% at the femoral neck, and 3.3% for the whole body. Because there was a slight negative change in whole-body BMD during the 12 months of PTH treatment, the subsequent rapid reduction of bone turnover induced by alendronate, accompanied by a rise in whole-body BMD, was consistent with closing down and filling in of the skeletal remodeling space.

This augmentation of BMD by bisphosphonates after PTH therapy has been stopped was confirmed in the second year of the PaTH study, in which three treatment groups (PTH, PTH plus alendronate, and alendronate alone) received alendronate 10 mg daily for one more year, and one of the PTH arms received placebo instead of alendronate in the second year (Black *et al.*, 2005). After PTH therapy alone, a dramatic further increase in spine and hip BMD was seen with alendronate therapy (4.9% and 3.6%, respectively). The PTH-induced increase in volumetric spinal trabecular density was maintained. In contrast, the group that received PTH and alendronate in combination in the first year saw minimal further gain in BMD at the spine and hip, and the group receiving 2 years of alendronate showed a similar modest improvement of BMD at spine and hip. The PTH-treated group that received placebo in the second year had no change in spinal BMD. All PTH-treated subjects lost BMD at the radius in the first year, and showed no further loss in the second year while taking alendronate.

In follow-up studies of the two large teriparatide RCTs in postmenopausal women (Neer *et al.*, 2001) and men (Orwoll *et al.*, 2003), subgroup analyses showed that those subjects who received bisphosphonate therapy after stopping the teriparatide had modest increments in spine and hip BMD (Lindsay *et al.*, 2005; Kaufman *et al.*, 2005).

All of the available evidence would suggest that, after a course of PTH therapy has been completed, the use of a bisphosphonate will retain or even enhance the gains in bone density that have been achieved (Rittmaster *et al.*, 2000; Black *et al.*, 2005; Lindsay *et al.*, 2005; Kaufman *et al.*, 2005).

Calcitriol

The early studies by Neer and coworkers combined concurrent calcitriol with daily PTH injection therapy (Neer *et al.*, 1987, 1991; Slovik *et al.*, 1981, 1986). This strategy was adopted because the earlier dietary calcium balance studies of Reeve and coworkers demonstrated minimal adaptations of dietary calcium absorption despite obvious histomorphometric restoration of trabecular bone and increased urinary Ca excretion. Because exogenous PTH increases serum $1,25(OH)_2D$ levels in the short term (Hodsman and Fraher, 1990; Lindsay *et al.*, 1993), and is associated with increased fractional ^{45}Ca absorption, the need for the addition of calcitriol therapy remains unproven. The changes in lumbar BMD reported by Neer and coworkers are comparable to other studies but theirs was the first group to report significant losses of BMD at the radius in a controlled clinical trial (15 patients on calcium, 15 patients on PTH + calcitriol 0.25 μg/day, over 2 years) (Neer *et al.*, 1991). The control group lost 1.7% and the experimental group lost 5.7% in radial BMD, all in the first year of treatment. The greater and abrupt loss of appendicular bone mass in the experimental group might indicate a permanent loss to cortical bone but an alternative explanation includes the possibility of an effect consistent with increased intracortical modeling, or an increase in bone size out of proportion to the degree of mineralization.

Cyclical Therapy with PTH and Calcitonin

Cyclical therapy with PTH and an antiresorptive agent, calcitonin, is of interest insofar as it might provide insights as to how the hormone might ultimately be given in the most economical fashion. The concept for the cyclical use of PTH should be distinguished from its direct anabolic effects on the skeleton. Because PTH also activates bone remodeling, cyclical protocols have attempted to exploit the ADFR hypothesis (A, activate remodeling; D, depress resorption in the activated bone modeling units; F, a treatment-free period of bone formation; R, repeat the treatment cycle). In fact, none of the cyclical protocols can be considered as true ADFR protocols and should be regarded as hybrid anabolic protocols.

Hesch and Hodsman have combined cyclical PTH (1–34) and PTH(1–38) with concomitant or sequential cycles of calcitonin (Hesch *et al.*, 1989b; Hodsman and Fraher, 1990; Hodsman *et al.*, 1991, 1993b, 1997, 2000). There is little evidence that the antiresorptive action of calcitonin provides any additional clinical benefit. Short cycles of PTH (400–500 units/day, approximately 40 μg) for less than 14 days provide little evidence of skeletal activation of bone turnover or anabolism (Hodsman and Fraher, 1990; Hodsman *et al.*, 1991; Reeve *et al.*, 1987), although biochemical responses are detectable within this time frame. However, 28-day cycles of high-dose PTH (800 units, or 65 μg daily) appear to provide a strong stimulus for activation of bone turnover (Hodsman *et al.*, 1993b) and anabolic effects on bone mass (Hodsman *et al.*, 1993b, 1997). The reported changes in lumbar spine BMD, with small increments in femoral neck BMD with 28-day cycles of high-dose PTH may provide an alternative approach to harnessing the anabolic effects of PTH.

USE OF PTH IN CONDITIONS OTHER THAN POSTMENOPAUSAL OSTEOPOROSIS

Glucocorticoid-Induced Osteoporosis

It is clear from the action of PTH on osteoblasts that it would be likely to be of benefit in glucocorticoid-induced osteoporosis. Small studies have indicated that this is the case. As noted previously, PTH has been used successfully to improve BMD in estrogen-treated women with glucocorticoid-induced osteoporosis (Lane *et al.*, 1998a). Of note in that study, BMD continued to increase at the spine and hip measurement site during the 12 months of observation following 12 months of PTH therapy, suggesting that treatment with continued HRT as an antiresorptive therapy effected similar reductions in bone turnover to those seen previously with alendronate (see Table 1) (Lane *et al.*, 2000).

More recently, a larger RCT comparing teriparatide to alendronate in glucocorticoid-induced osteoporosis has shown teriparatide to be at least as effective in preventing glucocorticoid-induced bone loss as a bisphosphonate (Saag *et al.*, 2007).

Hypogonadism

PTH(1–34) has also been shown to prevent the large and rapid loss of bone mass following "medical oophorectomy" induced by the gonadotropin antagonist, nafarelin (Finkelstein *et al.*, 1994). After cessation of nafarelin and PTH therapy, bone mass continued to increase during 1 year of follow-up, such that, in the subjects previously treated with PTH, BMD in the spine was actually higher than it had been at baseline 2 years earlier (see Table I).

Osteoporosis in Men

In early studies, PTH appears to have equivalent anabolic effects in men with osteoporosis (Kurland *et al.*, 2000; Slovik *et al.*, 1986), although the numbers of men studied were very small (see Table 1). The largest RCT of teriparatide in men, sponsored by Eli Lilly, was terminated before most of the subjects had completed a year of treatment, so conclusions are limited. However, comparing the results of the male osteoporosis trial to the first year of the fracture prevention study in postmenopausal women (Neer *et al.*, 2001), the increment in BMD of spine was similar (Orwoll *et al.*, 2003). The investigators enrolled 437 men with BMD more than 2 standard deviations below the young adult male mean, randomized to 20 or 40 μg of teriparatide daily or placebo, but averaged only 11 months of therapy. The study was too short and underpowered to examine fracture prevention, but in 355 men monitored for 30 months after stopping teriparatide or placebo, there were fewer vertebral fractures in the teriparatide treatment group than placebo arm, and this difference was significant if only moderate or severe vertebral compression fractures were counted (Kaufman *et al.*, 2005).

Fracture Healing

Recently, there has been growing interest in PTH as an aid to fracture healing. In animal models of fracture healing, the mechanical strength of callus and fracture union (both cortical and cancellous bone) after osteotomy, with increased osteoblastogenesis and decreased adipocytogenesis (Komatsubara *et al.*, 2005; Nozaka *et al.*, 2008). Local application of teriparatide to a fracture site in rat tibiae increased postfracture callus volume by 20% ($P < 0.01$) 14 days postfracture (Rozen *et al.*, 2007).

In a 28-day rat model of fracture healing, Nakazawa *et al.* (2005) showed daily s.c. teriparatide increased chondrogenesis in the early stages of fracture healing (day 14) but this was not evident at later stages (days 21 and 28). In the earlier stages of chondrogenesis, cell proliferation, expressed as the rate of proliferating cell nuclear antigen-positive cells, was increased in mesenchymal (chondroprogenitor) cells but not chondrocytes in the PTH-treated group compared with controls, suggesting that PTH induces a larger cartilaginous callus, but does not delay chondrocyte differentiation (Nakazawa *et al.*, 2005).

In a 16-week rat tibia fracture healing model, daily s.c. injection of PTH fragments hPTH(1–34), hPTH(1–31), and monocyclic [Leu27]cyclo (Glu22-Lys26)hPTH(1–31) for 8 weeks resulted in increased fracture strength and callus volume, and the three peptides were equally effective (Andreassen *et al.*, 2004).

In general terms, the animal studies have consistently suggested that systemic therapy with teriparatide enhances fracture healing (Holzer *et al.*, 1999; Alkhiary *et al.*, 2005). These studies raise the possibility of a role for PTH therapy in enhancing bone healing after fracture or orthopedic procedures, and support the design of clinical trials in humans.

BIOCHEMICAL MARKERS OF BONE METABOLISM

Biochemical markers of bone turnover provide noninvasive indicators of the balance between bone resorption and bone turnover (Delmas *et al.*, 1991; Garnero *et al.*, 1994; Riis *et al.*, 1995). Because PTH is regarded as a hormone with potent bone-resorbing properties, leading to concomitant increases in urinary calcium excretion, other markers of bone resorption are of great importance. In this section, discussion of biochemical markers of bone resorption discussed will be collagen breakdown products: urinary hydroxyproline (OH-Pro), deoxypyridinoline (DPD), and the type I collagen cross-linked amino (N)- or carboxyl (C)-telopeptides (NTx and CTx). Markers of bone formation will focus on bone-specific alkaline phosphatase (BSAP), osteocalcin, and the N- or C-terminal propeptide of type I collagen (P1NP, P1CP).

In the initial studies of the biochemical response to PTH, the stimulation of bone resorption was an obvious concern, because clinicians traditionally link hyperparathyroidism with bone loss. However, the early reports suggested fairly minimal increases in bone resorption accompanied the anabolic effect. Reeve *et al.* (1980b) reported a 26% rise in OH-Pro excretion in patients treated with daily PTH, but either no change or a 100% fall in urinary OH-Pro in a small group of 12 patients who were treated with estrogen or androgen as concurrent antiresorptive therapy (Reeve *et al.*, 1987, 1991), lending support to the concept of combining an anabolic with an anticatabolic agent. However, other investigators have variously reported increments in either urinary OH-Pro, DPD, or NTx excretion that are 50% to 350% over baseline (Finkelstein *et al.*, 1998a; Greenspan *et al.*, 2007; Hodsman *et al.*, 1993b; Kurland *et al.*, 2000; Lane *et al.*, 2000; Lindsay *et al.*, 1997; McClung *et al.*, 2005; Neer *et al.*, 2001; Sone *et al.*, 1995). Markers of bone resorption tend to reach maximal levels within the first 6 months of PTH treatment, falling back toward baseline values toward the end of the first year of therapy, and they do not rise any further over longer periods of continuing therapy.

Increments in markers of bone formation, P1NP, P1CP, BSAP, and osteocalcin, are consistently seen earlier than the rise in markers of resorption (Dobnig *et al.*, 2005; Finkelstein *et al.*, 1998a; Fujita *et al.*, 1999a; Greenspan *et al.*, 2007; Hodsman *et al.*, 1997; Kurland *et al.*, 2000; Lane *et al.*, 2000; Lindsay *et al.*, 1997 McClung *et al.*, 2005; Neer *et al.*, 2001). These increases in formation markers are better correlated with increments in bone mass and histological changes than the markers of resorption (Dobnig *et al.*, 2005). More recent studies have suggested that P1NP is a very sensitive indicator of PTH effect on bone, increasing by over 1000% after 6 months in one study (Finkelstein *et al.*, 2006). It has been suggested that if a patient's P1NP level does not increase by 10μg/L from baseline in 1 to 3 months, the patient should be questioned regarding storage or injection techniques, medical problems interfering with the response to therapy, or adherence (Eastell *et al.*, 2006).

In clinical trials, increments in biomarkers of bone formation precede those of bone resorption, usually by 1 to 3 months, but again are maximal within 6 to 12 months and fall toward baseline values in the longer studies. The earlier rise in markers of bone formation compared with markers of resorption has been characterized as an "anabolic window" by Bilezikian (Canalis *et al.*, 2007). This may reflect the time period in which the imbalance between formation and resorption is at its highest, but the apparent continued anabolic action of PTH on histology (Fox *et al.*, 2005), and BMD (Lindsay *et al.*, 1997), after more than a year of therapy would suggest that the decline of biochemical markers, and the "catching up" of the markers of bone resorption, does not signal a loss of net bone accretion.

As noted earlier, prior treatment with a bisphosphonate appears to reduce or delay the anabolic effects of PTH, and this is reflected in the biochemical markers. In a randomized three-arm (alendronate, teriparatide, or both) study of 63 men with BMD below a *T* score of −2.0, Finkelstein *et al.* (2003) documented that 6 months of prior therapy with alendronate reduced the BMD response to teriparatide 37μg s.c. daily, but also markedly blunted the response of the biochemical markers osteocalcin, P1NP, and the resorption marker NTx (Finkelstein *et al.*, 2006). Ettinger *et al.* (2004), in a small study of patients started on teriparatide after long-term prior therapy with alendronate or raloxifene, showed that the response of osteocalcin, P1NP, BSAP, and NTx to teriparatide therapy was reduced by prior therapy with alendronate, but raloxifene did not seem to reduce the response to teriparatide. Boonen *et al.* (2008) showed that prior therapy with bisphosphonates did not prevent a rise in P1NP after one month of teriparatide, although the increment following alendronate therapy was less than if the prior bisphosphonate therapy had been risedronate. This observation has been confirmed in an abstract report of the OPTAMISE clinical trial of teriparatide following 24 months of alendronate or risedronate (Delmas *et al.*, 2007).

In contrast to bisphosphonates, other anticatabolic agents, notably estrogen and raloxifene, do not appear to reduce the rise in formation markers stimulated by PTH, although there has only been one study comparing the combination of these agents with PTH alone. The reduction in the rise in PTH-stimulated bone resorption markers by cotreatment with raloxifene (Deal *et al.*, 2005) might suggest a mechanism for amplifying the anabolic effect of PTH, but in the absence of longer-term clinical trial data, this remains speculative.

The wide variation in BMD response to PTH therapy is somewhat unusual, and certainly greater than the response to alendronate. The investigators of the PaTH study (Sellmeyer *et al.*, 2007) found that levels of biochemical markers at baseline, or following therapy, could not explain the variability. The PTH-stimulated increase in serum 1,25-$(OH)_2$ vitamin D was the serum parameter best correlated with the BMD response, but only explained 16% of the variance.

In summary, the reported measurements of biochemical markers for bone resorption and formation support the rapid onset of skeletal activation and bone turnover in response to PTH as reported histologically by Hodsman *et al.* (1993b, 2000). Increments in bone formation markers are consistent and of considerable magnitude, in keeping with direct osteoblastic stimulation and bone anabolism (Hodsman *et al.*, 1997; Lindsay *et al.*, 1997). After the first year of therapy, a decline in biochemical markers of bone turnover appears to be a consistent observation, and there are few studies of PTH action on BMD beyond two years, so it is possible that skeletal resistance to the anabolic effects of PTH peptides might develop during the first three years of therapy.

CALCIUM BALANCE STUDIES

Before 1980, there were few methods to document changes in bone mass in response to any agent. Bone biopsies yield important information, but the measured parameters are not precise (typical CV±20%) (Chavassieux *et al.*, 1985). Therefore, early studies relied on classical dietary calcium balance studies and radioisotopic techniques of assessing skeletal calcium accretion to determine the extent to which new therapies for osteoporosis improved skeletal bone mass. The methodology of the reports discussed later is not easy to follow, but the cited references provide a source for such documentation. Moreover, the reader should appreciate that the cited clinical protocols vary widely with respect to dose of PTH (preparations also varied in units of biological activity), duration of therapy, and concurrent medication. Given these limitations, the results indicate enough consistency to describe the results in general terms.

In the initial report by Reeve *et al.* (1976a), four patients treated with PTH (500 units/day), demonstrated an improved dietary calcium balance averaging 7.3 mmol/day. Net diet absorption of ^{47}Ca averaged 3.4 mmol/day. Measured accretion of ^{47}Ca by the skeleton averaged 6.3 mmol/day. These data seemed to confirm that the observed improvements in bone histology were indeed caused by an anabolic effect of the injected hPTH(1–34). A subsequent report on the short-term changes ($<$1 month) measured in the same subjects defined dose-dependent effects, with calcium balance improving over doses of 500 to 1000 units of hPTH(1–34) per day, but deteriorating at doses of 1500 units/day (Reeve *et al.*, 1976b). In a small group of four patients treated with 450to 750 units of hPTH(1–34) over 1 month reported by Slovik *et al.* (1981), a similar dose dependency was observed, with positive dietary calcium balance (2.2 mmol/day) seen only at the lower doses of PTH.

In summary, kinetic calcium data demonstrate trends toward positive dietary calcium balances during a variety of therapeutic PTH protocols. These techniques have not been as sensitive to changes in bone mass as dual-energy absorptiometric measurements, but they comprise the only existing evidence that prolonged PTH treatment does not result in consistent total body calcium depletion. However, two reports suggest that higher doses of PTH ($>$1000 units/day) may be deleterious to total body calcium balance (Reeve *et al.*, 1976b; Slovik *et al.*, 1981).

EFFECTS ON SERUM AND URINARY CALCIUM

The biological effects of PTH when given by continuous intravenous infusion, intravenous bolus, or subcutaneous injection are not the same. For example, continuous infusion of hPTH(1–34) caused marked bone resorption in the rat (Tam *et al.*, 1981). When infused into osteoporotic subjects, Hodsman *et al.* (1993b) reported that a given dose of hPTH(1–34) caused significantly larger increments in total serum calcium over 24 hours than the same dose given by a single subcutaneous injection. The PTH infusion led to a significant fall in biochemical markers of bone formation, suggesting, if anything, that continuous intravenous infusion of PTH is antianabolic (Hodsman *et al.*, 1993b). Similar findings in osteoporotic subjects were reported by Cosman *et al.* (1993), albeit that concurrent estrogen therapy might have mitigated the catabolic effects of PTH infusion.

Increments in serum calcium following subcutaneous PTH injection are delayed and do not peak until 4 to 8 hours postdosing (Hodsman *et al.*, 1993b; Lindsay *et al.*, 1993), but they are generally within the normal physiological range. Because the clearance of injected PTH is so rapid, no obvious safety concern has arisen after more than 20 years of PTH therapy. However, persistent hypercalcemia may occur during daily PTH injections. The increment in serum calcium owing to PTH therapy appears to be relatively constant, so that individuals starting with a high basal level of serum calcium will be at higher risk of hypercalcemia (Antoniucci *et al.* 2007). Dose reduction because of sustained hypercalcemia within the dose ranges chosen in most published trials was seldom necessary, and cessation of nutritional calcium supplementation will often be sufficient to manage increments in serum calcium above the normal physiological range. It is certainly possible for a forgetful patient to accidentally give a second or even third dose in the same day, so there is always a risk of hypercalcemia owing to overdosage of PTH. This appears to be the case in at least one of the serious hypercalcemia episodes in the large clinical trial of PTH(1–84) (NPS Pharmaceuticals, personal communication).

In the largest RCT evaluating teriparatide against placebo, serum calcium measurements 24 hours after daily injections of PTH in a dose of 20 μg/day (approximately 250 units) were mildly elevated ($<$11.5 mg/dL) in about 10% of patients, and not sustained in a third of these women (Neer *et al.*, 2001). The FDA approval of this drug in the United States did not require monitoring of serum calcium, but many clinicians do so, testing serum calcium 20 to 24 hours postdose, within the first month of therapy.

In the clinical trials of PTH(1–84) 100 μg daily, the incidence of hypercalcemia has been variable, and therefore more of a concern. In the major Phase III trial, 28% of subjects receiving PTH had hypercalcemia, but almost all of these cases resolved with second testing or reduction in calcium intake (Greenspan *et al.*, 2007). A major contributing factor to the high incidence of hypercalcemia in this trial was likely the fact that the recruitment criteria allowed inclusion of patients with mild hypercalcemia (10.5–10.7 mg/dL). In other clinical trials with PTH(1–84), patients with mild hypercalcemia were excluded (Hodsman *et al.*, 2003; Black *et al.* 2003; Antoniucci *et al.*, 2007) and the frequency of hypercalcemia in those studies was approximately the same as described in the teriparatide study by Neer *et al.* (2001) (14% in PaTH vs. 11% in the 20-μg arm of the teriparatide study). It should be noted that the definition and time of testing for hypercalcemia were not the same in the teriparatide and PTH trials—hypercalcemia was defined as $=$10.7 mg/dL in the teriparatide study and $>$10.5 mg/dL in the PTH studies, whereas the serum calcium was tested 4 to 6 hours after teriparatide and 20 to 24 hours after PTH (Antoniucci *et al.*, 2007).

Increased fractional absorption of dietary calcium (Hodsman and Fraher, 1990) and hypercalciuria (Hodsman *et al.*, 1993b; Lindsay *et al.*, 1993) are probably or partially contributed to by increments in serum $1,25(OH)_2D_3$, which have been reported after subcutaneous hPTH(1–34) (Fujita *et al.*, 1999a; Hodsman and Fraher, 1990; Lindsay *et al.*, 1993). However, of the three studies reporting long-term biochemical changes in response to hPTH(1–34) injections, sustained increments in serum $1,25(OH)_2D_3$, and hypercalciuria were not of clinical importance (Hodsman *et al.*, 1997; Lindsay *et al.*, 1997; Neer *et al.*, 2001). Miller *et al.* (2007) recently reported the calciuric response in the two major Eli Lilly placebo-control studies of teriparatide 20 or 40 μg daily in 1637 women and 437 men. Urinary calcium excretion was increased for as long as 12 months of therapy, compared with placebo and baseline values, but the magnitude of these changes was felt to be unlikely to be clinically relevant or to warrant a recommendation for routine urinary calcium monitoring.

PHARMACOKINETICS OF PTH ADMINISTRATION

Because most therapeutic protocols have reported the response of the skeleton to daily subcutaneous injections, this section focuses on the pharmacodynamic responses to PTH when given by this route.

In a small study involving four healthy young subjects, Kent *et al.* (1995) reported that after subcutaneous administration of 1250 units hPTH(1–34) (100 μg, 20 nmol) the time to reach maximum plasma concentration $T_{\max}$ was approximately 15 min, with immunoreactive PTH concentrations decaying to baseline after 180 to 240 min. Two additional reports, in estrogen-treated postmenopausal osteoporotic subjects and in both young and untreated postmenopausal subjects (Fraher *et al.*, 1993; Lindsay *et al.*, 1993) provided very similar data, i.e., peak immunoreactive increments in serum hPTH(1–34) levels at 20 to 30 min postinjection, increasing 10- to 15-fold over baseline (depending on injected dose), and decaying within a $T_{1/2}$ of about 75 min (Fig. 9). Chu *et al.* (2007) conducted a single-dose study in healthy adult Chinese volunteers, and for the 20 μg subcutaneous dose, they confirmed a rapid peak and a slightly shorter $T_{1/2}$ plasma elimination of approximately 60 minutes.

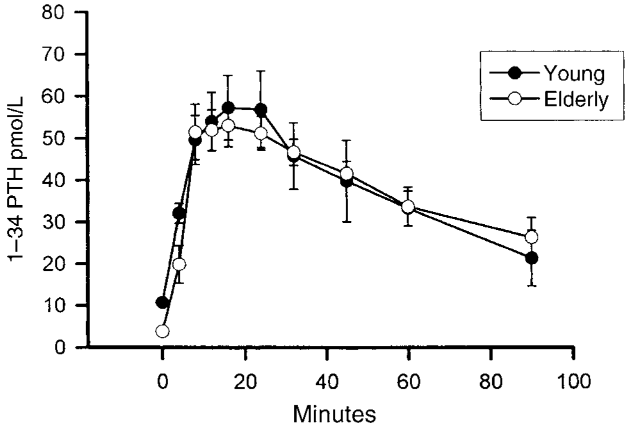

FIGURE 9 Pharmacokinetics of PTH(1–34) given by subcutaneous injection. Serum concentrations of hPTH(1–34) after subcutaneous injection of 800 IU PTH peptide in 10 young adults aged 25±9 (SD) years and 9 elderly women with osteoporosis, aged 67±11 years. Adapted from Fraher *et al.* (1993).

The only detailed pharmacodynamic study published for other PTH peptides is a dose-finding single subcutaneous injection report on hPTH(1–84). Schwietert *et al.* (1997) reported minimal changes in total serum calcium in response to single injections of up to 5 μg of hPTH(1–84)/kg (approximately 0.5 nmol/kg). However, with this peptide, absorption appeared to be associated with a double peak in $C_{\max}$ with an early peak occurring less than 20 min postinjection and a second peak appearing 1 1/2 to 2 hours later. This pharmacokinetic profile occurred at all doses of hPTH(1–84) and appears to be different from that observed after hPTH(1–34) dosing. The pharmacokinetics of hPTH(1–84) were recently reviewed by Moen and Scott (2006), and more recent studies have only been published in abstract form or have been made available by the manufacturer. Essentially, the observation of a double peak was again observed in patients receiving daily injections, with a mean serum elimination half-life of 2.5 hours. In addition to the double peak, this half-life appears to be longer than that described for teriparatide. This raises the possibility that teriparatide and PTH(1–84) may have slightly different biological effects. If the anabolic action is dependent upon bone cells receiving a short duration, intermittent exposure to PTH, a longer duration of exposure might enhance the resorptive action of the peptide, and treatment with the intact hormone might therefore have a less potent effect on bone formation.

IMMUNOLOGICAL RESPONSES TO EXOGENOUS PTH

Of the available reports in which antibody formation was deliberately sought, 67 of 1168 patients apparently developed anti-hPTH(1–34) antibodies in low titers (Hesch *et al.*, 1989b; Hodsman *et al.*, 1997; Neer *et al.*, 2001; Reeve *et al.*, 1976a, 1987, 1991). Four patients discontinued hPTH(1–34) therapy because of generalized urticarial reactions or local irritation at the injection site (Reeve *et al.*, 1976a, 1980b). Although the 6% incidence of anti-PTH(1–34) antibodies may seem high, and presence of other impurities in the early preparations is a possibility, it should be pointed out that hPTH(1–34) is not a naturally occurring peptide. In the more recent clinical trial with teriparatide (Neer *et al.*, 2001), antibodies to PTH (1–34) were detected in one woman in the placebo group (<1), 15 (3%) women in the 20-μg and 44 (8%) of the 40-μg treatment group, suggesting a dose-dependent immune response, but there was no evidence of biological effect of these antibodies.

In the large clinical trial of PTH(1–84), 2 (0.2%) placebo-treated and 36 (3.5%) of the PTH-treated subjects had developed positive PTH antibody titers after 12 months, but one-third of these had negative titers at 18 months (Greenspan *et al.*, 2007). Whether these antibodies bind in a manner to reduce biological activity is not known.

OTHER ANALOGUES AND DELIVERY SYSTEMS

The principal analogue used in the reported clinical literature was the presumed bioactive amino-terminal fragment of PTH, namely hPTH(1–34). Several have used hPTH (1–38) (Hesch *et al.*, 1984, 1989a,b; Hodsman and Fraher, 1990; Hodsman *et al.*, 1991), but there is no compelling reason to believe it might be more potent than hPTH (1–34). Other analogues include hPTH(1–31) amide and its lactam (Ostabolin and Ostabolin-C). These analogues stimulate adenyl cyclase with equivalent potency to the holohormone PTH(1–84), but seem to have no effect on the phospholipase C messenger system; in animal models of anabolic bone formation they appear to be equipotent to PTH(1–34) (Whitfield *et al.*, 1997). There is a single report of continuous intravenous infusion of PTH(1–31) amide in young human subjects (Fraher *et al.*, 1999). When compared with an equimolar dose of infused PTH(1–34), comparable urinary and plasma cyclic AMP responses were seen, but the PTH(1–31) amide resulted in no increase of serum and urinary calcium or in urinary NTx. This suggests that analogues can be designed with more selective potency for bone formation over bone resorption.

The parathyroid hormone-related peptide analogue PTHrP(1–36) was evaluated in a small and short-term double-blind randomized placebo-control clinical trial (Horwitz *et al.*, 2003). A significant 4.7% increment in BMD of the lumbar spine was seen, with an increase in osteoblast activity as reflected by serum osteocalcin, but no increase in markers of bone resorption over placebo in the 3 months of therapy. This certainly supports a primarily anabolic effect of this peptide when given by daily s.c. injection.

Although all PTH peptides are currently under clinical development using the subcutaneous injection route, alternative delivery systems include:

1. Automated injection or transcutaneous delivery
2. Intranasal delivery
3. Oral, or buccal delivery
4. Transpulmonary delivery

Alternative delivery systems for PTH peptides are in the early stages of clinical evaluation (Morley, 2005). Intranasal delivery devices have been demonstrated for both PTH and calcitonin peptides, using lyophilized, microparticulate delivery systems (Deftos *et al.*, 1996; Nomura *et al.*, 1996). Transpulmonary systems have been developed in which aerosol delivery to the bronchoalveolar tree leads to 30% to 48% bioavailability of PTH peptide compared with that seen through intravenous routes of administration, with profiles of peptide absorption that are very similar to those delivered by subcutaneous injection (Patton *et al.*, 1994; Pfutzner *et al.*, 2003). Although noninjectable routes of PTH peptide delivery are still in the realm of experimental medicine, there is no reason to believe that this compliance barrier cannot be crossed in the near future. In the short term, research using injectable PTH peptides has not been a significant barrier to patient compliance.

ADVERSE EFFECTS OF PTH THERAPY

More than 5000 patients have received PTH in published clinical trials. No serious adverse reactions have surfaced beyond the obvious effects on serum and urine calcium concentrations, the majority of which can be handled by appropriate dose reductions during the initial weeks of treatment (Neer *et al.*, 2001; Greenspan *et al.*, 2007). Nausea, headache, dizziness, arthralgia, and leg cramps following injection occur in 5% to 15% of women, in a dose-dependent fashion, but are unrelated to changes in serum calcium and are usually transient (Neer *et al.*, 2001; Greenspan *et al.*, 2007). Hodsman *et al.* (1997) raised a concern about the long-term safety of PTH on renal function. In this report, 39 patients were treated with cyclical high-dose teriparatide (800 units/day for four 28-day cycles per year over 2 years). This patient group experienced a significant 10% increase in serum creatinine (albeit within the normal range of age-related serum creatinine). Thus, PTH-induced hypercalciuria might conceivably affect renal function, but this observation has not been confirmed in the large Phase III clinical trials of teriparatide and PTH.

Osteogenic Sarcoma

A major issue for most patients considering PTH therapy is whether there is a risk of osteogenic sarcoma. The approval of PTH peptides for osteoporosis in most countries requires a warning on the package label, indicating the drug has been shown to cause osteogenic sarcoma in rats, and it is recommended that patients at higher risk for osteogenic sarcoma not be given the drug (i.e., Paget's disease, prior radiotherapy, previous bone tumors, and pediatric patients who are still experiencing skeletal growth). This warning is given because the major clinical trials of teriparatide were prematurely terminated when a concurrent carcinogenicity study documented a consistent dose-dependent appearance of osteogenic sarcomas in Fischer 344 rats. These animals were treated with near-lifetime daily injections of PTH (1–34), and developed an increased incidence of osteosarcoma after 18 months of dosing, by which time teriparatide has caused severe osteosclerosis and compensatory extramedullary hematopoiesis to occur (Vahle *et al.*, 2004). PTH did not increase the incidence of nonskeletal tumors.

Osteosarcoma has also been reported in a similar 2-year carcinogenicity study with hPTH(1–84) (Jolette *et al.*, 2006). There was a dose-related incidence of osteosarcoma in the mid (50 μg), and high (100, 150 μg) dose groups, similar to the incidence seen in the teriparatide studies. However, there was no difference in the low dose (10 μg/kg/d) compared with controls, suggesting that the intact hormone may not be as potent a stimulus to bone neoplasia in the rat skeleton.

Osteogenic sarcoma has not been reported in other species after similar dosing protocols (Neer *et al.*, 2001), and there is reason to believe the tumorogenic potential of PTH is much greater in the rat than in humans. The rat skeleton has an unusually exuberant response to PTH, perhaps related to its continual growth throughout life; the rat skeleton lacks Haversian systems and the cortical remodeling that is characteristic of the human skeleton (Vahle *et al.*, 2004; Tashjian and Chabner, 2002).

Since teriparatide's approval as a treatment of osteoporosis, one case of osteosarcoma has now been reported (Harper *et al.*, 2007). However, because more than 300,000 patients have been treated with teriparatide, this is not outside the expected 1:250,000 incidence in the older adult population (Harper *et al.*, 2007). In the two naturally occurring clinical conditions in which long-term increments in circulating PTH are well documented (primary and secondary hyperparathyroidism associated with chronic renal failure) there is no reported incidence of osteosarcoma (Kaplan *et al.*, 1971; Koppler and Massry, 1988; Maissonneure *et al.*, 1999; Vamvakas *et al.*, 1998). However, these conditions are analogous to continuous rather than intermittent PTH therapy, and the anabolic effect of PTH is much more evident with the intermittent dose regime.

Although the mitogenic potential for PTH has not been confirmed outside of the near lifetime duration of high-dose PTH in rat studies, PTH does appear to act as a growth factor within skeletal tissue and its receptor is widely distributed in other tissues. Therefore the oncogenic potential of these peptides should not be dismissed.

SELECTION OF PATIENTS FOR PTH THERAPY

Given the availability of other very effective anticatabolic therapies, the high cost of 18 to 24 months of PTH therapy governs the definition of the appropriate patient selection guidelines. In countries where PTH therapy of osteoporosis is approved by regulatory agencies, the criteria for patient selection are some variation of "very high risk of fracture" or "patients who have failed other osteoporosis therapies." The ideal patient for PTH therapy would appear to be one with very low bone mass, or multiple previous osteoporotic fractures, and therefore at high risk of fracture, but the definition of what degree of risk constitutes "high risk" is left up to the prescribing physician in most countries. Age is a major contributor to fracture risk, and most experts would avoid prescribing the drug to younger adults (e.g., premenopausal women or men under age 60) whose only fracture risk is a low bone density.

Patients who have continued to fracture despite appropriate therapy with a bisphosphonate or other anticatabolic agent would also appear to be appropriate candidates for PTH. However, although this is a group of patients for whom many regulatory agencies have approved PTH therapy, no clinical trials with a fracture outcome have been conducted with PTH in patients who appear to have failed other osteoporosis therapies.

The following are typical guideline recommendations regarding the use of PTH (adapted from Hodsman *et al*., 2006):

- PTH should be considered as a first-line therapy for women 65 years or older who have prevalent vertebral fractures, or other fragility fractures, and low BMD (*T* score = −2.5). Ideally, these patients should be bisphosphonate naïve, given the possibility that bisphosphonates might blunt the effectiveness of PTH. If already taking bisphosphonate therapy, these patients should stop the bisphosphonate prior to starting PTH. However, on the basis of the results of cost-effectiveness studies (Liu *et al*., 2006; Stevenson, 2005), regulatory agencies will probably recommend that PTH should be reserved for the most severely affected patients (those with more than one fragility fracture and very low BMD).
- Other potential candidates for PTH include postmenopausal women with very low BMD (*T* score = −3.5) and those who continue to have fragility fractures despite an adequate trial of bisphosphonates (2-year period).
- PTH should be considered for men 65 years or older who have severe osteoporosis and prevalent fragility fractures.
- PTH should be considered for patients who are taking long-term corticosteroid therapy and have corticosteroid-induced osteoporosis and prevalent fractures.
- Therapy with an antiresorptive agent after completing teriparatide therapy is recommended to maintain or increase BMD.
- Baseline serum levels of calcium, PTH, uric acid, creatinine and 25-hydroxyvitamin D [25(OH)D] should be measured—and confirmed to be within acceptable normal limits—before PTH therapy is initiated. Serum calcium levels should be measured again, before injection, after the first month of therapy, because mild hypercalcemia will develop in a minority (about 10%) of patients during treatment. In patients with a history of renal stones, calcium levels in 24-hour urine collections should be measured.
- PTH should be avoided in patients who have primary hyperparathyroidism or who have significant renal impairment or vitamin D deficiency, and it should be used with caution in patients with a history of gout.

CONCLUSION

To date, the 25-year clinical experience of PTH therapy indicates that this is an important anabolic agent with the potential to reverse osteoporosis. Very few safety concerns have been raised over treatment periods of 2 to 3 years. The introduction of PTH peptides/analogues holds promise for a new class of anabolic agents that correct the bone mass deficit in addition to reducing fracture risk in osteoporotic individuals. A number of key points can be made:

1. PTH offers the promise of rapid increments in skeletal bone mass in osteoporotic subjects, and antifracture efficacy has been clearly demonstrated in Phase III clinical trials.
2. Data suggest that, although the effect of PTH treatment is most potent in increasing bone formation at trabecular sites, it is not detrimental to cortical bone and there is no evidence of increased risk of fracture in regions where cortical bone predominates.
3. Combining PTH with other osteoporosis therapies is not generally recommended, although it is not necessary to stop postmenopausal estrogen/progestogen therapy or SERMs when administering PTH. To date, clinical studies suggest that concurrent estrogen therapy and daily PTH injections result in significant increments in total body BMD as well as increments at axial sites, and if estrogen is continued, post-PTH bone loss is prevented. Similarly, it is likely that concurrent use of the SERM raloxifene would not interfere with PTH. In contrast, it is likely that concurrent (or prior) use of bisphosphonates (particularly alendronate) may be detrimental to the PTH effect, and certainly does not appear to add any benefit. Consequently, most guidelines would suggest stopping bisphosphonate therapy when giving PTH.
4. After PTH therapy is completed, there is gradual loss of the newly acquired bone mass. The addition of an anticatabolic therapy is therefore usually recommended after completion of a course of PTH. The bisphosphonate alendronate has been tested most often in this setting, and sequential therapy with alendronate results in further gains in both axial and appendicular bone mass. Other anticatabolic therapies (estrogen, SERMs) have also been shown to prevent BMD loss after PTH is stopped.
5. Cycles of PTH injections may be as effective as continued daily dosing, which may be an attractive approach, considering the cost of the available PTH preparations. This is an area for further research question has a significant impact on cost and compliance.
6. The long-term safety profile of PTH peptides remains to be established. Although hypercalcemia during the therapy is usually mild and transient, a small number

of patients have developed sustained hypercalcemia and hypercalciuria, and there is at least one report of declining renal function over time (Hodsman *et al.*, 1997).

7. Other unanswered questions:
   - a. Does the skeleton develop resistance to continued PTH therapy?
   - b. What is the optimal duration of therapy? The premature cessation of the first Phase III clinical trial, which showed fracture prevention, caused national regulatory agencies to limit duration of PTH therapy to the length of that study (1.5–2 years). Few (if any) investigators believe the occurrence of osteogenic sarcoma in rats receiving high-dose, near-lifetime PTH therapy has relevance to the use of PTH in osteoporotic humans.
   - c. Do we have the right dose? The current approved dose of teriparatide is 20 μg daily, but many of the smaller studies (some of longer duration) used 25 μg or higher doses, with significant bone benefit and no appreciable increased risk of side effects. Similarly, for hPTH(1–84), perhaps 75 μg instead of 100 μg might have been as effective in preventing fractures and less likely to cause hypercalcemia.
   - d. Would interval rechallenge be effective? If prior bisphosphonate therapy diminishes the therapeutic response to PTH, is there value in delaying PTH therapy until the skeleton recovers from bisphosphonate suppression of turnover?
   - e. Although estrogen and SERMs do not seem to inhibit skeletal response to PTH and may even enhance it, what about other anti-catabolic agents currently in clinical trials, such as cathepsin K inhibitors (Kumar *et al.*, 2007) or RANKL inhibition with denosumab (McClung *et al.*, 2006)?
   - f. Will analogues of PTH offer advantages as anabolic agents over hPTH(1–34) or hPTH(1–84)?

DEDICATION

Dedicated to the memories of an esteemed mentor, Dr. Louis M. Sherwood, and a treasured friend and laboratory colleague, Howard Cheng, who both passed away in 2007.

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Chapter 79

Calcium

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BACKGROUND

Calcium in the Environment in Which Life Arose

Calcium is the fifth most abundant element in the biosphere, after iron, silicon, oxygen, and aluminum. Unlike silicon and aluminum, whose compounds are quite insoluble, calcium salts exhibit an intermediate solubility that both permits them to be present in solution in the waters in which life evolved and to support development of various hard, solid organs for use by evolving life forms. Calcium is dissolved in seawater, for example, at concentrations up to 10 m*M*, about 10 times that which is found in the extracellular fluid of mammals. Even fresh water, if it is to support an abundant biota, must have substantial dissolved calcium (~1 to 2 m*M*, roughly on the order of mammalian extracellular fluid concentrations). Dissolved calcium is needed to keep carbon dioxide in solution for the photosynthetic activity of algae and aquatic plants, thereby establishing the base of the aquatic food pyramid.

The radius of the Ca^{2+} ion is just right to fit naturally into the folds of many proteins, coordinating with up to eight oxygen atoms in the peptide chain and its side groups. Calcium thus stabilizes critical tertiary structures of both catalytic and structural proteins (Carafoli and Penniston, 1985). This binding to key peptides is true, of course, for many metal cations, but most tend to be highly specific for one or two configurations (e.g., Fe, Mn, Cu, Zn, etc.). Magnesium, calcium's closest relative in the cell, and like calcium a divalent alkaline earth element, has a smaller ionic radius and does not bind as strongly as does calcium. Calcium, by contrast, binds avidly to a profusion of proteins, ranging from actin to calmodulin to the various *gla* proteins. Calcium's functionality is so broad, in fact, that essentially all cells have found it necessary to restrict calcium concentration in the cell sap to something like four to five orders of magnitude below that in the extracellularfluid (ECF) surrounding the cell. These low cytosolic concentrations are the context that permits cells to use calcium as a nearly universal second messenger. They do so by admitting controlled quantities into critical cellular compartments when specific functions are to be activated, and promptly pumping it out, either into the extracellular space or into intracellular vesicles, when the cell action is to be shut off. Intracellular storage of calcium is the rule, rather than the exception. Examples include the sarcoplasmic reticulum of muscle and calcium phosphate crystals in mitochondria. Calcium is so critical to intracellular function that most cells have developed means to maintain their own supply. In general, their dependence on extracellular calcium is limited to initiating the cascade by opening calcium channels in the cell membrane. Movement of ECF calcium into the cytosol through these channels then activates a much larger release of calcium from intracellular stores, as in muscle contraction.

However, life at a higher, multicellular level requires integrative functions and, therefore, the maintenance of critical concentrations of many factors in the ECF of complex organisms. Here, a higher calcium concentration (in the range of 1.25 m*M*) is essential for a variety of functions, ranging from neuronal synaptic transmission to blood clotting. Although ECF $[Ca^{2+}]$ is essentially constant across the higher vertebrates, the means whereby it is stabilized vary. Fish and amphibia have access to the calcium in the surrounding water and buffer the concentration of calcium in their extracellular fluids by controlling fluxes across the gill membranes. But terrestrial vertebrates, dependent upon periodic food ingestion for their calcium, need an internal source and sink of calcium for homeostasis. It is partly in this context that bone enters the scene.

Calcium and Bone in Evolutionary Perspective

Although the most obvious feature of bone in terrestrial vertebrates, and particularly in humans, is its structural property, it is likely that bone served somewhat different purposes in the early marine vertebrates (Urist, 1964). The fossil record indicates that bone evolved independently several

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times over the millennia of evolution—as dermal armor, as teeth, and as internal stiffening, often dropping out again in more modern descendants of ancestral bony members of the same classes. The internal stiffening and mechanical strength provided by bone, so obviously important on dry land, is of less significance in a buoyant medium, and there is a trend for fish to become less bony over the course of evolution. The modern sturgeon, a true bony fish by evolutionary origin, is essentially boneless today (except for the gill covers), but the fossil record shows that early sturgeons were quite as bony as most other modern fish. The *chondrichthyes* (sharks, skates, and rays), to cite another example, have only a cartilaginous skeleton, and yet do not lack for mechanical engineering efficiency.

Evolutionary biologists consider that the skeleton probably served an important homeostatic function, helping the organism maintain the constancy of critical elements in its internal milieu (Urist, 1962). That function is clearly evident in modern mammals, where the ion most prominently buffered by bone is calcium. There is reason to believe, however, that calcium may not have been the critical ion in marine vertebrates, because, as already noted, the surrounding sea provided a relatively large surplus of calcium relative to the ECF in most higher vertebrates. But phosphorus is a trace element in seawater, and the phosphorus essential for intermediary metabolism, for DNA and RNA structure, and for so many catalytic functions, is acquired by marine vertebrates by ingesting the tissues of other organisms lower in the food chain. Hence, phosphorus needs to be rigidly conserved. Bone meets that need.

Although the mechanisms by which bone serves a homeostatic function in terrestrial vertebrates are well worked out, it is less clear precisely how bone functions in maintaining internal homeostasis in fish. Fish produce many of the hormones involved in mammalian calcium and phosphorus homeostasis (calcitonin is an obvious example), but these agents do not seem to have the same effect on ECF [Ca^{2+}] in fish as in mammals. Furthermore, it is not certain in fish how minerals are either added to the bony reserve or withdrawn from it.

Bone as the Body's Calcium Sink and Reserve

Without a constant supply of calcium in the fluid bathing the gill membranes, land-living vertebrates were confronted with the problem of maintaining constancy of extracellular fluid calcium levels in the intervals between feedings. This required a place to put extra calcium acquired from food in the absorptive phase and a place from which to draw calcium during fasting. Bone is that place. However, bone is not simply a passive reservoir. As noted elsewhere in this volume, calcium is stored in bone mainly in the process of mineralizing newly deposited bone matrix, that is, by adding new volumes of bone *tissue*; and it is withdrawn from bone mainly by resorption of old bone *tissue*. Thus, the reservoir functions of bone are mediated by modulating, on a moment-to-moment basis, the balance between new bone formation and old bone resorption.

A good example of this modulation is afforded by what happens during calcium absorption from milk in infant mammals. The quantity of calcium ingested in a short period of time, coupled with the efficiency of absorption in infants and the small volume of the extracellular water into which that calcium is dumped, are such that near-fatal hypercalcemia would ensue if there were not some way to damp the absorptive rise in ECF [Ca^{2+}]. This is accomplished by calcitonin-mediated suppression of osteoclastic bone resorption. Because bone formation is continuing, the mineralization of recently deposited bone matrix effectively soaks up the absorbed calcium. Then, as absorptive input decreases, bony resorption resumes. The result is a nearly steady input of calcium into the ECF, with the relative contributions of bone and gut varying inversely.

It is virtually certain that most of the skeletal control of extracellular calcium levels throughout life is exerted on the resorptive side of the remodeling apparatus, for the simple reason that osteoclastic activity is susceptible to very rapid response to humoral mediators, whereas mineralization is not. Once suitable crystal nuclei are formed (see later), mineralization is mainly passive. The mineralization of newly formed bony units actually constitutes the principal systemic drain to which the control systems must respond (rather than functioning as a rapid response mechanism itself).

An example of the reservoir function of bone is seen during annual spring antler formation in deer (Banks *et al.*, 1968). Rapid bone growth in the antler buds creates a greater calcium demand than the nutrient-poor late-winter and early-spring foliage can satisfy. Because intestinal input cannot meet the demand, parathyroid hormone secretion increases and a burst of bone remodeling occurs throughout the entire skeleton. The first phase of bone remodeling is resorptive; hence, a great deal of calcium is thereby released into the ECF, countering the drain of antler bone mineralization. Several weeks later, when the skeletal remodeling loci reach their own formative stage, more nutrient-rich summer foliage has become available and antler growth has slowed. The deer are then able to pay back the skeletal calcium loan from food calcium sources. Something similar is seen in the deposition of medullary "egg bone" early in the egg-laying cycle in birds. Such bone is then resorbed during later phases of egg formation, when its calcium both supports shell formation and helps solubilize protein being transported to the oviduct. The same type of phenomenon occurs in preparation for lactation in most mammals, with pregnancy commonly being a time of more positive calcium balance than can be accounted for by fetal mineralization, and then with substantial loss of bone during the hypoestrin state of lactation (Heaney, 1996). For example, the nursing rat, across lactation of a full litter of pups, loses on the order of one-third of the skeletal mass she had at delivery (Brommage and DeLuca, 1985).

Thus, understanding of the comparative biology of the skeleton helps us to see that bidirectional remodeling imbalances (decreasing and then augmenting local bone mass) are capable of both removing and replacing calcium from bone by tiny minute-to-minute alterations of bone mass. Except for the accumulation of remodeling errors, this in-and-out process would not be expected to exert a deleterious effect on bone; in fact, it probably has a net beneficial effect on bone quality, inasmuch as evoked remodeling seems to focus preferentially on old, fatigue-damaged tissue, replacing it with fresh, new bone. However, when withdrawals are protracted or payback is incomplete or absent, structural elements may be lost (Fig. 1). When this occurs, the loss becomes irreversible, inasmuch as the scaffolding for osteoblastic replacement vanishes. Nutrient repletion can be expected only to fatten up existing structures, not replace lost ones. In this sense, calcium differs from a nutrient such as iron for which full repletion of functional iron mass is possible even after loss of a major fraction of total body iron stores.

There is an important asymmetry about this bidirectional flux of calcium in and out of bone through the remodeling cycle. Although bone can be resorbed essentially without limit, there is a practical ceiling that governs how much calcium can be stored. Given an environmental abundance of calcium, bone mass is normally regulated not by nutrient availability but by a mechanical feedback loop that works to maintain bony strain under load at a constant level in all skeletal parts and regions. The bone setpoint (or "mechanostat") is poorly understood (as is true for most biological setpoints), but appears to involve the lipoprotein receptor-5 (LRP-5) that, in turn, controls osteoblast formation through the canonical Wnt signaling pathway (Robinson *et al.*, 2006). Animals with functional mutation of the LRP-5 gene produce more bone in response to mechanical loading than do animals with the wild-type gene (Robinson *et al.*, 2006).

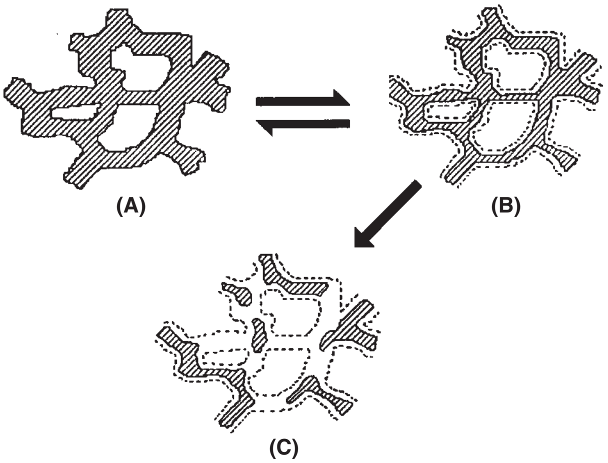

FIGURE 1 Schematic representation of the bidirectional flux of calcium in and out of the skeleton in a small volume of cancellous bone (**A** to **B**). Calcium is mobilized by resorbing bone tissue. So long as the basic structure is preserved (**B**), lost bone can be fully replaced. But when whole structures are resorbed (i.e., trabecular plates penetrated and trabecular beams severed i.e., **C**), replacement of lost bone becomes difficult or impossible. [Copyright Robert P. Heaney 1995. Reproduced with permission].

The regulation of this setpoint is complex and poorly understood, but in addition to mechanical loading, it is influenced, at least in part, by estrogen [which explains why bone tissue is added to the skeleton at puberty (Gilsanz *et al.*, 1988) and during pregnancy (Heaney and Skillman, 1971) when estrogen levels rise, and withdrawn from it at menopause and during lactation (Brommage and DeLuca, 1985) when estrogen levels fall]. But under a hormonal steady state, bone balance will become zero when the optimal level of strain is reached. Additional dietary calcium cannot be stored. (See "Defining the Calcium Requirement".) The upshot is that, although the organism can withdraw calcium virtually without limit, storage is limited by the extent to which the skeleton continues to be subject to normal loading stresses, contingent upon preservation of structural elements and consistent with the setpoint of the bone mechanostat.

Calcium in Bone

The adult human body contains, on average, slightly more than 1 kg of calcium, better than 99% of which is in the form of bone and teeth. Calcium is the principal cation of bone, comprising slightly less than 40% of the mass of the bone mineral and slightly less than 20% of the dry weight of bone. Calcium exists in bone in a mineral form that is usually characterized as hydroxyapatite, i.e., $Ca_{10}(PO_4)_6(OH)_2$. This is only approximately correct, and it is more accurate to say, after Posner (1987), that bone mineral is a "structurally imperfect analogue of hydroxyapatite." Bone mineral contains, for example, carbonate, citrate, potassium, and magnesium, among other ionic species. Carbonate content is especially high. It varies fairly substantially from species to species, in humans comprising in the neighborhood of 6% to 9% of the mass of the mineral. Because the carbonate ion does not fit well into the hydroxyapatite crystal lattice, it is generally presumed to be located in superficial positions on the mineral crystals. The lability of bone carbonate, as reflected in changes in carbonate content with metabolic acidosis, suggests also that its location may be mostly on anatomic surfaces.

The crystals of bone exist in intimate association with the collagen fibers of the bone matrix. They are long and needle-shaped, are ~70 Å in diameter and from 200 to 3000 Å long, and are constrained in size and orientation by the dense, orderly packing of the matrix collagen fibrils, which are laid down before mineralization begins. Matrix, as deposited, consists of about half protein and half water. As mineralization proceeds, mineral crystals displace the water, aligning themselves with the collagen fibrils. Ultimately, at full mineralization, the extracellular bony material contains virtually no free water. One consequence is the fact that mineral ions deep to anatomical surfaces are frozen in place, neither exchanging with nor supporting the level of the corresponding ionic species in solution in the ECF.

The calcification of matrix presents an interesting illustration of the importance of specificity of crystal habitus and takes advantage of the marginal solubility characteristics of calcium phosphate. Both calcium and phosphate, the latter in the form of H_2PO_4 and HPO_4^{2-}, circulate in the body fluids in solution and do not precipitate either in the blood stream or in healthy body tissues. However, the same minerals, in exactly the same concentrations, support mineralization when the blood flows past a bone-forming site. The reason, very simply, is that the concentrations of calcium and phosphate in ECF are such that body fluids are approximately half saturated with respect to $CaHPO_4$ (the most likely crystal form at physiological pH and pCO_2). However, bone mineralization is not simple precipitation. Rather, it involves the creation of a template for formation of a crystal more like hydroxyapatite or tricalcium phosphate [$Ca_9(PO_4)_6$]. Such minerals would not form spontaneously below pH 8; they are much less soluble, and body fluid concentrations of calcium and phosphate are approximately twice their K_{sp}. Local creation of such crystal nuclei is the means by which vertebrates have been able to control deposition of mineral, placing it only in loci specifically prepared to receive it, e.g., where it is needed to provide the necessary rigidity for the structural role of bone.

Noncollagenous matrix proteins are believed to play the critical role in configuring Ca^{2+} and PO_4^{3-} ions in space so as to create the hydroxyapatite template. Alkaline phosphatase, produced by the osteoblast late in the matrix deposition process, is believed to function by hydrolyzing pyrophosphate and organic phosphate esters present in the medium, which both makes extra phosphate available and removes components that otherwise function as crystal poisons, inhibiting crystal growth (Whyte, 1989).

The chemistry of formation of the apatite mineral can be summarized as

$$10\ Ca^{2+} + 3.5\ H_2PO_4^{-} + 2.5\ HPO_4^{2-} + 2H_2O \rightleftarrows Ca_{10}(PO_4)_6(OH)_2 + 13.5\ H^{+}$$

The proportion of $H_2PO_4^{-}$ and HPO_4^{2-} in extracellular fluid at body pH is such that this reaction produces ~13 protons for each unit cell[1] of hydroxyapatite formed. These must, of course, be removed from the mineralizing environment, or the reaction would cease or tend to run in reverse. By the same token, ~13 protons must be produced by osteoclasts to solubilize one unit cell of hydroxyapatite during bone resorption.

THE CALCIUM REQUIREMENT

What the Requirement Ensures

The strength of bone, as with all material structures, resides in four features: the instrinsic strength of the mineral–matrix composite, the massiveness of the structure, the geometric arrangement of the bony elements in space, and the loading history of a structural element, expressed in accumulated fatigue damage. A major mechanism by which calcium is recognized to influence bone strength is through its effect on bone mass. Because bone functions as the calcium nutrient reserve, it follows inexorably that any depletion of that reserve (or failure to produce the genetically programmed skeletal mass during growth) will carry with it a corresponding decrease in bone strength.

Additionally, it has recently been shown that, at prevailing calcium intakes, bone remodeling occurs at a rate considerably in excess of the need to repair fatigue damage (Heaney, 2003; Parfitt, 2004), and that this excess remodeling is a more important fracture risk factor than had been previously recognized. It is likely that contemporary calcium intakes, which are low by Paleolithic standards, are a part of the cause. In any event, supplementation reduces bone remodeling and reduces fracture risk as well (Heaney and Weaver, 2005).

As already noted, the calcium needed for critical cell-metabolic functions is, in most cells, derived from intracellular stores of the mineral. Bone constitutes such a large reserve of calcium that cellular functions could virtually never deplete it, no matter how low the oral intake of the nutrient. The same is true for the maintenance of ECF [Ca^{2+}]. A mere 5 g of bone contains as much calcium as the entire extracellular fluid space of an adult human. The requirement for calcium relates thus, not to the metabolic role of the nutrient, but to building and maintaining the size of the calcium reserve, i.e., to its secondary function, bone strength.

Calcium in the Environment in Which *Homo sapiens* Evolved

The abundance of calcium in the biosphere, previously noted, is expressed in the relatively high calcium content of the foliage, tubers, nuts, and other plant foods available to herbivorous and omnivorous mammals. The massive skeletons of extinct herbivores, the exuberant racks of antlers of deer and elk, both prehistoric and modern, and the large volumes of calcium-rich milk produced by dairy cattle today are all testimony to the abundance of calcium in a plant-based diet. Omnivorous animals, in addition to the calcium from plants, had access to the calcium in bones of animal prey, calcium in the chitinous exoskeletons of insects, and calcium in insect larvae and grubs.

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[1] A unit cell, in this case containing 10 calcium ions, is the smallest, symmetrical repeating unit of the crystal lattice.

Analysis of the foods consumed on a year-round basis by chimpanzees, our closest primate relatives, indicates a calcium nutrient density in their diets of 2 to 2.5 mmol (80 to 100 mg) Ca/100 kCal (Eaton and Nelson, 1991). The Kung people of South Africa have been found to have diets very nearly as high [1.75 to 2.0 mmol (70 to 80 mg) Ca/100 kCal]. Studies of other hunter–gatherer peoples indicate that their diets, too, have calcium nutrient densities in the range of those of the high primates. The energy expenditure of a hunter–gatherer or chimpanzee, scaled up to the body size of contemporary Western peoples, translates these nutrient density figures to a total calcium intake in the range of 50 to 75 mmol (2000 to 3000 mg)/day from plant sources. Riverine peoples, with bony fish in their diets, had still higher calcium intakes. By contrast with these pretechnological kinds of intakes, the diet calcium density of urbanized or industrialized nations is commonly less than 0.6 mmol (24 mg)/100 kCal (Carroll *et al.*, 1983).

The reason for the difference between primitive and contemporary diets is expressed graphically in (Fig. 2), which depicts schematically the effect on calcium nutrient density produced by the agricultural and pastoral revolutions (which can be dated to roughly 10,000 years ago, at least in the fertile crescent, and much more recently in the Western hemisphere and northern Europe). Domestication of animals and the development of flocks led to a nomadic lifestyle heavily dependent upon the milk produced by the pastoralists' herds. Farming, on the other hand, exploited natural hybrid forms of cereal grasses, which required humans for their propagation (mainly because the excess starch of the seed kernels made them too heavy for efficient dispersal by the wind). At the same time, the extra starch of the hybrids made it possible to feed a great many more people. The hunter–gatherer lifestyle makes very inefficient use of the land, and population pressures were one reason for the shift to farming or herding.

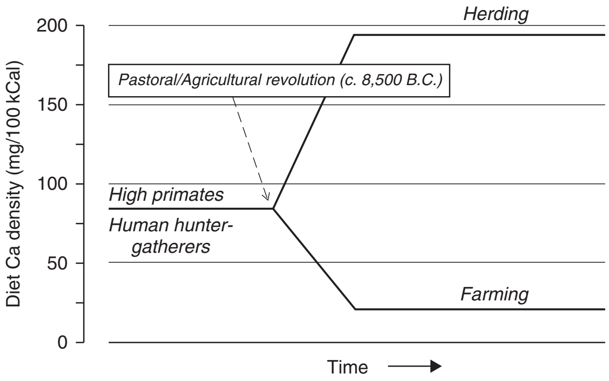

FIGURE 2 Schematic representation of the effect of the pastoral and agricultural revolutions on calcium nutrient densities of the diets of Paleolithic humans. [Copyright Robert P. Heaney 1995. Reproduced with permission.]

With this divergence, the pastoralists thus had a substantial increase in the calcium density of their food supply, whereas the agriculturists had a substantial decrease. By contrast with milks [at calcium densities in the range of 27.5 to 50 mmol (110 to 200 mg)/100 kCal], cereals typically have calcium densities less than 0.25 mmol (10 mg)/100 kCal. A contemporary example of a dominantly pastoral economy is provided by the Masai of East Africa, whose energy needs are largely met by fluid bovine milk, and whose calcium intakes, accordingly, are in the range of 150 to 175 mmol (6000 to 7000 mg)/day. It is likely that the actual calcium intake of the agriculturists was substantially higher than provided by the staple cereal grains, both because of other vegetables in the diet and because of adventitious calcium entering the food supply in the process of flour milling (Molleson, 1994). Grain was typically dehulled in limestone mortars, and the dehulled kernels then ground in limestone querns. Limestone is, of course, a calcium carbonate mineral; it is relatively soft and workable; and it undoubtedly added a great deal of calcium to the flour produced for bread making. Hence, it is likely that only with the development of a more advanced technology, which allowed large-scale milling and the construction of mill stones from harder minerals (silicon based rather than calcium based), that the calcium intake of technologically advanced, urban peoples fell to present levels.

Physiological Adaptations to a High Calcium Intake

The abundance of calcium in the environment in which hominids evolved undoubtedly influenced the physiological mechanisms for extraction of calcium from the food supply and for conservation of absorbed calcium. Mammals typically have low intestinal calcium absorption efficiency, unregulated cutaneous losses, and poor renal conservation. The significance of these features is perhaps seen most clearly by way of contrast with the physiological adaptations to another nutrient, one that was scarce in the early hominid environment, i.e., sodium, for which absorptive efficiency is complete and both cutaneous and urinary losses can be shut down to near zero. There is a cost to such conservation, of course, and it is likely that analogous mechanisms did not evolve for calcium because, given an environmental surfeit, they would have produced no selective advantage for their bearer. Further, efficient extraction of calcium from food sources would not only have been unnecessary; it would have been dangerous, for the excess would have had to be excreted, lest it lead to hypercalcemia and extraosseous calcification.

Exposure to chronic dietary calcium scarcity is a relatively modern phenomenon, as has been already noted, and fewer than 100 generations have elapsed for most of our lineages between the time of calcium surfeit and the present. This is far too few for the evolution of calcium-conserving mechanisms, particularly when the selective advantage of health at the end of life is much less strong than health during the reproductive years.

Studies Manipulating Calcium Intake

The importance of calcium intake on bone mass has been extensively documented in a number of species. As far back as 1929, Bauer, Aub, and Albright showed that cats developed severe bone loss when placed on low calcium diets. This work has been replicated in rats and dogs (e.g., Bodansky and Duff, 1939) and has been repeated several times in cats (e.g., Jowsey and Gershon-Cohen, 1964). Fig. 3, from one such study, depicts a classical nutritional experiment, in which a nutrient is first removed, the morbid result observed, and then the nutrient restored and recovery noted. The bone loss occurring under these circumstances is dependent upon intact parathyroid function (Jowsey and Raisz, 1968); in the absence of parathyroid hormone, the bone loss is prevented, although at a cost of severe hypocalcemia. These experiments, incidentally, highlight both the central role of bone as the calcium reserve for ECF calcium levels and the importance of parathyroid hormone in releasing calcium from bone to sustain ECF [Ca^{2+}].

Over 20 randomized controlled trials in humans have been published in recent years, establishing that elevating calcium intake enhances bone acquisition during growth, slows bone loss in postmenopausal women, and reduces fracture rates at spine, hip, and other extremity sites. These studies are reviewed in detail elsewhere (Heaney, 2000). One clear example of these effects is seen in the report by Chapuy *et al.* (1992) involving nearly 1800 French women, average age 84 (Fig. 4). Elevating calcium intake from ~13 mmol (520 mg)/day to ~42.5 mmol (1700 mg)/day reduced fracture rate at both hip and other extremity sites by 20% to 40% within 18 months of starting supplementation. The effect on bone mineral density (BMD) was fully as striking as the effect on fracture rate. Control subjects lost ~4.6% of the bone from the hip site over the 18 months of observation (an annual rate of ~3%/year), whereas the supplemented individuals gained 2.7%. The result was a greater than 7% difference in hip bone mass by 18 months. Clearly, 13 mmol Ca/day was not sufficient to prevent erosion of bone mass, whereas 42.5 mmol Ca/day was.

It may be that some of the benefit obtained in this trial was owing to vitamin D repletion, and some also to the added phosphorus contained in the supplement (e.g., on muscle function and coordination); however, the bone mass effect observed in this study was large enough to account for much or all of the fracture difference in its own right. Furthermore, in two further trials in which the control and the calcium-supplemented individuals were both given vitamin D supplements, a similar bone-sparing and fracture-reducing effect was observed in the

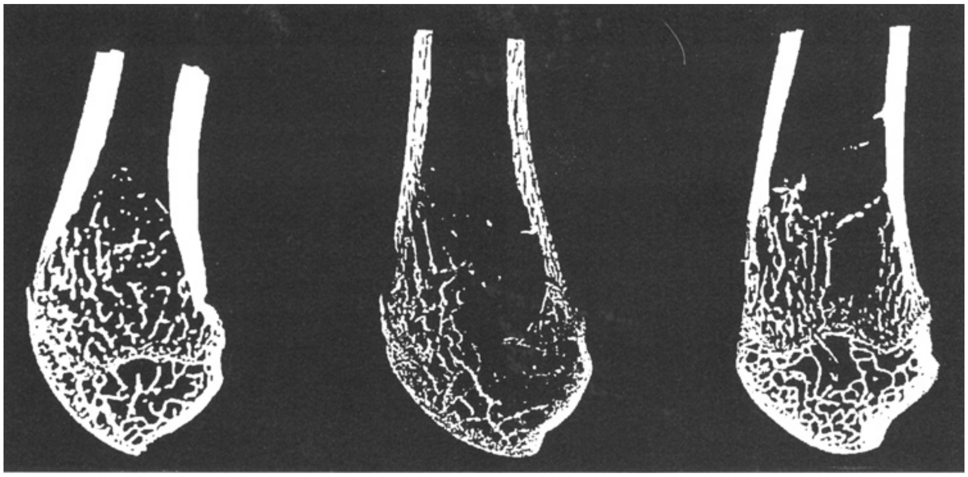

FIGURE 3 Effect of calcium deprivation and repletion on bone mass in cats, as revealed by micro x-rays of thin slices of undecalcified cat femora. The bone structure is shown (*left*) of a cat fed a normal, high calcium diet, a cat fed a low calcium diet for just 20 weeks (*center*), and a cat depleted for 10 weeks and repleted for 10 weeks (*right*). Bone loss in the central panel is evident. The right-hand panel shows partial healing. [Redrawn from Jowsey and Gershon-Cohen (1964).]

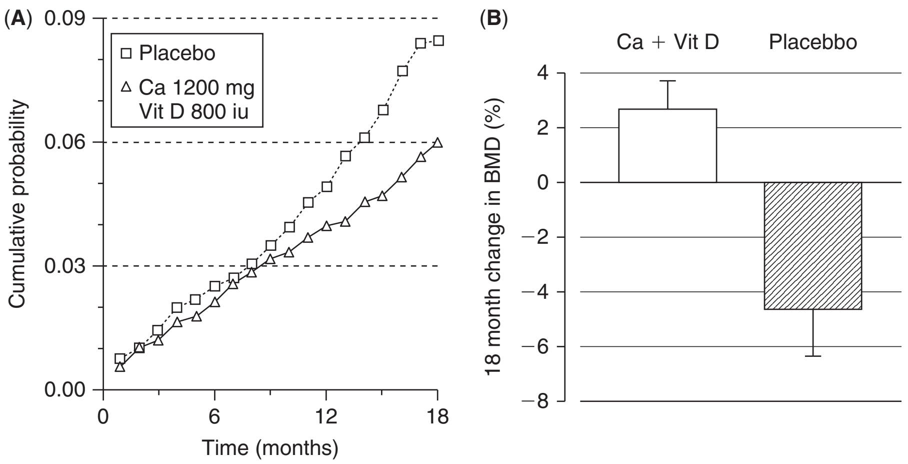

FIGURE 4 Effects of supplementation with calcium phosphate and vitamin D on hip fracture rate and hip BMD. The cumulative probability of fracture for the supplemented and unsupplemented groups is shown (**A**), as is the change from baseline in hip BMD for the two groups (**B**). [Redrawn from the data of Chapuy *et al.* (1992).]

calcium-supplemented individuals (Chevalley *et al.*, 1994; Recker *et al.*, 1996). In a more recent study, contrasting calcium and vitamin D, Peacock *et al.* (2000) showed that, although calcium alone was able to prevent bone loss entirely, vitamin D alone was not. [It should be noted that serum 25(OH)D levels in Peacock's study were substantially higher than in the study by Chapuy *et al.*, and thus some beneficial effect of vitamin D in the French women cannot be excluded.]

Although there is, obviously, a limit to how much fracture reduction can be achieved when one waits to ingest an adequate intake until old age, it is noteworthy that the elderly seem to be both more sensitive to calcium deprivation and more responsive to calcium supplementation than are younger individuals. This is illustrated, for example, in risk gradients for various ages reported by Hui *et al.* (1988) (Fig. 5). Although the risk of fracture rises with declining bone mass at all ages, risk rises also with age itself, even holding bone mass constant. In fact, as Figure 5 makes plain, the age effect is larger than the bone density effect. The gradient of risk on bone density also becomes steeper with age. Thus, whereas reducing bone loss in a 60-year-old woman would be expected to produce a modest fracture benefit, the same reduction in bone loss in an 85-year-old produces a much greater reduction in fracture risk. It is not certain why this should be so, but it is likely that the age effect itself is because of increased falling, poor reflex adjustment during the fall, decreased soft tissue padding over bony prominences, and increased intrinsic skeletal fragility from nonmass causes [trabecular disconnection, excessive remodeling, and accumulated fatigue damage (Heaney, 1993a)]. Interaction among these factors may explain the disproportionately large benefit of altering just one of them (i.e., bone mass or bone remodeling).

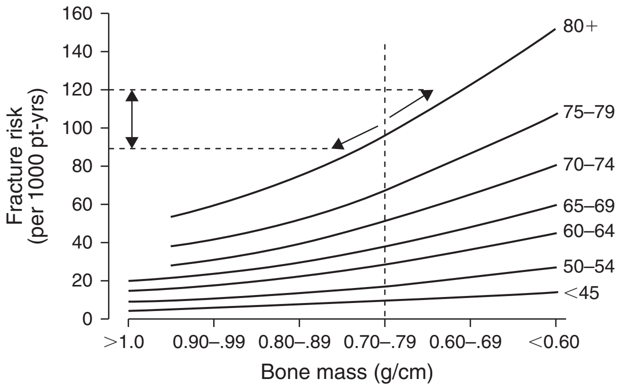

FIGURE 5 Age-specific fracture risk gradients for forearm BMD. Note the rise in risk with age, even holding BMD constant. Note also the increasing steepness of the gradients with age. A relatively small difference in BMD thus produces a greater fracture effect in the elderly than in younger individuals. [Redrawn from the data of Hui *et al.* (1988).]

Defining the Calcium Requirement

The calcium requirement can be defined as the intake needed to support genetically programmed acquisition of bone during growth and to sustain acquired bone during maturity and the declining years of life. Because of differing absorption and retention efficiencies, individuals will inevitably have differing requirements for a skeletal endpoint. A recommended dietary allowance (RDA) is a figure designed to be at about the 95th percentile of individual requirements. Hence, if everyone in a population were ingesting at least as much as the RDA, that intake would ensure that 95% would be getting as much as they need.

Calcium intake recommendations have undergone substantial revision over the past 15 years and the formats for

TABLE I Various Estimates of the Calcium Requirement in Women[*]

Age	1989 RDA[†]	NIH[‡]	1997 DRI[§]
1–5	800	800	600/1000
6–10	800	800–1200	1000/1600
11–24	1200	1200–1500	1600/1200
Pregnancy/ lactation	1200	1200–1500	1200
24–50/65	800	1000	1200/1450
65–	800	1500	1450

[*] *All values are given in milligrams, because this is how the respective bodies reported their recommendations. To convert to SI units, divide the values in the table by 40.*

[†] "*Recommended Dietary Allowances,*" 10*th Ed.*(1989). National Academy Press, Washington, DC.

[‡] *National Institutes of Health* (NIH) *Consensus Conference: Optimal Calcium Intake.* (1994). J. *Am. Med. Assoc.* **272,** 1942–1948.

[§] *Dietary Reference Intakes* (DRIs) *for Calcium, Magnesium, Phosphorus, Vitamin D, and Fluoride. Food and Nutrition Board, Institute of Medicine. National Academy Press, Washington,* DC, 1997. *The figures given are calculated as RDAs to facilitate comparison with earlier estimates. The published values were termed "Ais," which in this instance represent estimated average requirements* (EARs). *The RDA figures shown here are 20% higher than the corresponding EAR. The presence of two values in this column reflects the fact that the age categories for the DRIs overlapped those of the NIH.*

presenting them have been altered as well. Table I sets forth both the earlier and the current recommendations, using a standardized format to facilitate their comparison. Perhaps the most important of the recent changes is the recognition that the requirement rises substantially in the elderly. This elevated need reflects the age-related decline in ability to adapt to low intakes, and thus in a sense uncovers the optimal intake for all ages, i.e., the intake for which no adaptation would be needed. It is worth recalling, in this connection, that even though the recommended intakes may seem high by contemporary practice, they are still only a fraction of what the primitive human intake would have been for individuals of modern body size.

As previously noted, calcium is a threshold nutrient. This means that, at subthreshold intakes, calcium retention is less than optimal. Bone gain during growth is then a direct function of intake (i.e., it is less than is genetically programmed), whereas in the mature organisms, subthreshold intakes lead to actual bone loss. On the other hand, the threshold character of the relationship means that intakes above the threshold produce no additional bone effect. (See also "Bone as the Body's Calcium Sink and Reserve.") These relationships are depicted schematically in (Fig. 6). Because the approach to the threshold is asymptotic, it is difficult to define the precise point at which retention plateaus. For this reason, current recommendations are actually somewhat conservative. Some hint that the optimal intake may be substantially higher than even the newer recommendations is provided by several lines of evidence. One comes from the balance studies of Heaney *et al.* (1978), in which the *mean* requirement to maintain zero balance in estrogen-deprived women was ~37.5 mmol (1500 mg). An RDA would, inevitably, be higher still. Another pointer in that direction is found in a randomized controlled trial in elderly women supplemented with calcium for 3 years (McKane *et al.*, 1996). Unsupplemented individuals, with intakes averaging close to 20 mmol (800 mg)/day (the then applicable RDA for the age), exhibited the high 24-hour mean parathyroid hormone (PTH) levels, the enhanced PTH secretory reserve, and the elevated bone resorption that have all been taken to characterize the calcium and skeletal economies of the aged (Epstein *et al.*, 1986). By contrast, women supplemented to 60 mmol (2400 mg)/day decreased both their bone remodeling and parathyroid functional indices, restoring them to young adult normal values. The inference is that the increases in parathyroid cell mass and function represent not so much characteristics of old age as adaptive responses to longstanding calcium deprivation.

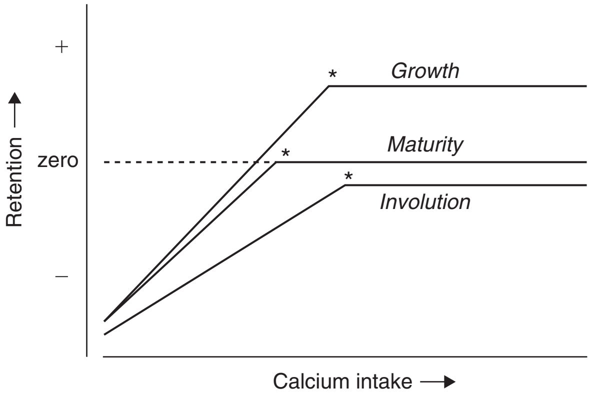

FIGURE 6 Schematic depiction of the relationship between calcium intake and skeletal retention at three life stages. Given sufficient intake, retention is positive during growth, zero at maturity, and variably negative during involution. Intakes above the threshold do not affect retention, but subthreshold intakes limit bone acquisition or lead to bone loss. The location of the minimum requirement is indicated by the asterisk above each line. (Copyright Robert P. Heaney 1998. Used with permission.)

Contributing causes for this increased parathyroid activity in the elderly, aggravating the effect of low calcium intakes, include a decline in vitamin D status with age (reflecting both decreased solar exposure and decreased efficiency of skin conversion of 7-dehydrocholesterol to previtamin D) and declining absorption and conservation efficiency for dietary calcium. Holding calcium intake constant at about 20 mmol (800 mg)/day, gross absorption efficiency declines from a mean at age 40 of ~0.24, at a rate of 0.002/year through at least age 65, with a one-time additional drop of 0.02 at menopause (Heaney *et al.*, 1989). Over that period, the decline thus amounts to 0.07, or a fall of ~30% in absorptive efficiency. Nordin and others have shown also that renal conservation of calcium deteriorates across menopause (e.g., Nordin *et al.*, 1991). The net result is that extraction of ingested calcium from food falls with age and conservation of that which is absorbed falls as well. These and similar findings from a number of investigators are the basis for the jump to 36 to 38 mmol (1450 to 1500 mg)/day in the 1994 and 1997 recommendations for the elderly in Table I.

It must be noted that the absorption values cited earlier are *gross* absorption, such as would be measured by calcium tracer flux from the intestinal lumen into the blood stream. They do not account for the contrary movement of calcium into the lumen in the digestive secretions and sloughed off mucosa (which turns over at a rate of ~20%/day). In adult women, this calcium from endogenous sources entering the intestinal stream amounts to ~3.5 to 3.75 mmol (140 to 150 mg)/day (Heaney and Recker, 1994). Most of this calcium is subject to the same absorptive probability as is ingested calcium, but given the low efficiency of calcium absorption in general, inevitably most endogenous calcium will be lost into the feces. Thus *net* absorption from intakes in the range of 20 mmol (800 mg)/day is on the order of only 10% to 15% of ingested calcium. Not all of even this small amount of net calcium gain from the intestine can be retained, because the tiny elevation of blood calcium produced when calcium is absorbed elevates the filtered load at the kidney and leads to an increase in urinary calcium loss.[2] The net result of the low net absorption and poor renal conservation is that, even during times of calcium need, only a small fraction of an ingested load is retained. Further, at the higher intakes achieved by calcium supplements, *fractional* retention is smaller still, because absorption fraction declines with the logarithm of the intake load size (Heaney *et al.*, 1990, 2000).

Nutritional Factors Influencing the Calcium Requirement

It is a truism of nutritional science that nutrients interact with one another and thereby alter their mutual requirements. Thus, requirements of certain of the B vitamins and of ascorbic acid vary with total energy intake (and expenditure). The lack of evolutionary development of efficient conservation of calcium has rendered this nutrient unusually sensitive to such nutrient–nutrient interactions. Most of this interaction is expressed in urinary calcium excretion, which, for example, is strongly influenced by the ingested load of, for example, sodium and by the acid/alkaline residue of the diet (Heaney, 1993b; Sebastian *et al.,* 1994). The sodium effect is owing to the fact that sodium and calcium share a common transport mechanism in the proximal tubule, and thus an increased filtered load of either ion will lead to increased excretion of the other. The magnitude of the sodium effect on the calcium economy is an increase of 0.5 to 1.5 m*M* of urine calcium for every 100 m*M* sodium ingested (Devine *et al.*, 1995; Nordin *et al.*, 1993, Itoh *et al.*, 1996).

These effects are linear across the full range of prevailing intakes from low to high, and are not thus consequences only of excessive intakes. With diets characteristic of typical North American and European women, nutrients such as sodium account for ~100 mg/day of obligatory calcium loss through the kidneys. Given the extremely poor net absorption efficiency for calcium, these nutrients can thus easily account for up to 1000 mg/day of the calcium requirement. It has been noted, for example, that individuals with low, but nutritionally adequate, intakes of sodium may have calcium requirements as low as 500 mg/day, whereas those with intakes more typical of contemporary patterns, may have intake requirements closer to 2000 mg/day. This quite extraordinary sensitivity of calcium to the intakes of other nutrients is but one more reflection of the lack of evolutionary acquisition of efficient mechanisms for calcium absorption and conservation.

The well-documented effect of the acid/alkaline residue of the diet (Berkelhammer *et al.*, 1988; Sebastian *et al.*, 1994) is probably a function of the “hard” anions (sulfate, chloride), either ingested with food or produced in metabolism. In any event, substitution of acetate or bicarbonate for chloride produces substantial reductions in urinary calcium loss and therefore in the ability to maintain calcium equilibrium on low calcium intakes.

It is important to stress this latter point, that these nutrient interactions negatively affect calcium balance mainly at low calcium intakes. This is because they limit an individual’s ability to adapt to a low intake. At the higher intakes typical of those that prevailed during hominid evolution, adaptation is easily possible, and high protein intakes, for example, do not then have a negative effect on the skeleton. [Indeed, such evidence as is available indicates that the net effect of protein on the skeleton is distinctly positive, overall (Delmi, 1990; Schürch *et al.*, 1998; Hannan *et al.*, 2000).]

Toxicity

It is possible to consume too much of any nutrient, and calcium is no exception. However, in healthy individuals, available evidence indicates that the toxic threshold is quite high. Many young adult males in the Unites States regularly consume more than 75 mmol (3000 mg)/day (Carroll *et al.*, 1983), and in pastoralist societies, such as the Masai of East Africa, calcium intakes regularly average more than

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[2] This sensitivity of urine calcium to variations in the filtered load is a reflection of the previously mentioned lack of evolutionary development of efficient calcium conservation mechanisms.

150 mmol (6000 mg)/day. In both instances, there are no known ill effects associated with such intakes.

However, there are special circumstances in which high calcium intakes can definitely be harmful. Even at the low-absorption fractions typical of high intakes, substantial quantities of calcium will enter the body and will need to be excreted in the urine (unless active bone building is occurring). Under conditions of dehydration or hypovolemia, perfusion of both bone and kidney can be sufficiently compromised so that absorbed calcium produces significant hypercalcemia. Likewise, under conditions of systemic alkalosis, large calcium intakes can produce renal calcinosis and severe impairment of kidney function. Furthermore, under these somewhat unusual and extreme circumstances, elements of positive feedback can cause the situation to deteriorate rapidly. Hypercalcemia impairs the ability of the kidney to retain water and thus aggravates a preexisting dehydration, with consequently worsening hypercalcemia. A fatal downward spiral can easily ensue. Published reports of such toxicity have generally involved intakes of more than 150 mmol (6000 mg)/day in individuals with preexisting impairments of water and electrolyte metabolism. Whatever the underlying cause, such problems can be easily handled by rehydration and by correction of the underlying abnormality, so long as these situations are recognized in time.

Kidney stones are perhaps the complication most often thought to be associated with high calcium intakes. However, this association has been largely speculative and is not based on clinical evidence. Most multiple regression analyses of risk factors for renal calculi have found either a very weak or no association at all for calcium intake. Two very large observational studies, in fact, found the converse (Curhan *et al.*, 1993, 1997). In the first, a prospective study of more than 45,000 men, there was an inverse correlation between calcium intake and stone formation; those with the highest calcium intakes had the lowest risk of kidney stones. This seeming paradox is explained by the fact that oxalate excretion in the urine is a more significant risk factor for calculi than is urine calcium. High calcium intakes bind oxalate both of dietary origin and that produced by bacterial fermentation in the gut before it can be absorbed; dietary calcium thereby lowers the renal oxalate burden. In fact, very large calcium supplements have long been standard therapy for the kidney stones occurring with intestinal hyperoxalosis, sometimes found in patients with short-bowel syndromes. A protective effect of high calcium intake on both oxalate loads and stone recurrence was seen in a randomized controlled trial in which those on a reduced calcium intake had twice the rate of stone occurrence as those on a high intake (Borghi *et al.*, 2002).

In summary, the levels of calcium intake discussed in the foregoing sections of this chapter, as well as those needed to support osteoporosis pharmacotherapy (see "Calcium and Osteoporosis Treatment"), are well within the safety limits for the ingestion of this nutrient.

Interpretation of Published Reports

The importance of a high calcium intake for bone health has often been questioned (e.g., Kanis and Passmore, 1989) and, even more often, has been termed "controversial." This is because some studies showed strong effects of calcium intake on bone status, but others showed no benefit at all. "Confusing" would have been a more accurate term. There are several reasons for these apparent discrepancies, now sufficiently understood to remove any confusion that may remain.

First, as already noted, osteoporotic fracture is a distinctly multifactorial affair. Nutrition, and particularly calcium nutrition, is only one of many contributing factors. Although calcium intake is, as the discussion above indicates, of critical importance in many individuals, it plays little or no role in others, just as iron intake is limiting in only certain forms of anemia. One example of this distinction is seen in menopausal bone loss, which is dominantly caused by estrogen deficiency and cannot be substantially influenced by calcium intake (although estrogen-related loss may be exaggerated if calcium intakes are very low) (Heaney, 1990). This is seen, for example, in the fact that even intakes of more than 75 mmol (3000 mg)/day, in the study by Elders *et al.* (1991), were able only to slow menopausal loss, not to prevent it.

However, menopausal loss is self-limited and is mostly confined to the 5-year period surrounding cessation of ovarian function. Every controlled trial of calcium supplementation in women more than 5 years postmenopausal has, without exception, shown a clear benefit on either bone loss or fracture rate or both. By contrast, roughly one of five observational studies performed on women in this same age group have failed to show a calcium effect (Heaney, 1993b, 2000); this is now understood to be because of the weak ability to estimate calcium intake from food records, or food frequency questionnaires, no matter how carefully these methods are applied, resulting in substantial classification bias (Heaney, 1991, 1997).[3] Usually in clinical science, one encounters positive results from observational studies, which are then sometimes not borne out in studies employing the more rigorous design of randomized controlled trials. With calcium, we have the paradoxical situation that the randomized controlled trials are essentially all positive, whereas some of the observational studies are not, and even those that are positive often show what appear to be weak effects. As the controlled trials show, it is not the effects that are weak, but the methods that must be employed in observational studies.

A final consideration relates to the uncritical use of transnational comparisons (e.g., Hegsted, 1986). Differences in fracture rate between ethnic and national populations have many bases, only some of them nutritional. An example is the lower hip fracture rates of contemporary Chinese and blacks, despite mean calcium intakes for both groups that are lower than those of North American or European Caucasians. Behind this difference there is, first, the large effect of the other nutrients in the diet, previously described. Thus, the predominantly vegetable-based and low protein intakes of many Third World populations would be expected to lower their calcium requirements. Similarly, there are major racial differences in ability to conserve calcium. American blacks, for example, both absorb at higher efficiency for any given ingested intake and conserve better at the kidney than do Caucasians (Abrams *et al.*, 1995; Bell *et al.*, 1985, Aloia *et al.*, 1998, Cosman *et al.*, 1997). Thus they have lower calcium requirements. Finally, there are important racial differences in bone geometry that have their own effect on fracture rate. Hip axis length, for example, explains most or all of the difference between the hip fracture rates of Caucasians on the one hand, and Chinese and Japanese, on the other, after adjusting for bone mass (Faulkner *et al.*, 1993; Nakamura *et al.*, 1994).

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[3] There are many sources of this estimation error. One of them, often ignored, is the fact that actual calcium contents of various foods vary over a roughly threefold range (from low to high) around the nominal values in the standard food tables.

Instead of making cross-cultural comparisons, the correct approach is to assess the effect of nutrient intakes *within* a cultural and ethnic group. When this has been done, for example, in both American blacks and in Chinese, bone mass and hip fracture rate are found to be inversely correlated with calcium intake, just as has been found for Caucasian populations (Anderson *et al.*, 1995; Hu *et al.*, 1993; Lau *et al.*,1988).

CALCIUM AND OSTEOPOROSIS TREATMENT

Effective osteoporosis treatments involve a combination of pharmacotherapy designed to reverse the negative remodeling balance prevailing in the skeleton and physical therapy designed both to increase function and to increase mechanical loading on the skeleton. Currently recommended calcium intakes are presumably adequate to sustain bone mass, but they may not be sufficient to support bone gain in most individuals for reasons just noted (i.e., net absorption from such intakes is just sufficient to offset obligatory losses). Furthermore, there is essentially no evidence that calcium alone, in any quantity, will lead to substantial gain in bone in individuals who already have osteoporosis. The small increase noted in the study by Chapuy *et al.* (1992) described earlier (see Fig. 4) probably reflects a remodeling transient (Heaney, 1994) and not a new steady-state rate of change. However, effective *pharmaco*therapy, including fluoride (Pak *et al.*, 1995; Riggs *et al.*, 1990), the bisphosphonates (Liberman *et al.*, 1995; Black *et al.*, 1996; Cummings *et al.*, 1998; Harris *et al.*, 1999), the selective estrogen receptor modulators (SERMS) (Ettinger *et al.*, 1999), and PTH (Kurland *et al.*, 2000; Neer *et al.*, 2001), does increase bone mass, and for some of these agents at a rate of up to 8% per year. To realize this potential gain, and particularly to optimize it, calcium intakes probably need to be above the maintenance level, i.e., for most osteoporotic individuals that means above 37.5 mmol (1500 mg)/day. Few controlled trials at such intakes have been done, so this conclusion is to some extent an inference from the data summarized in the foregoing. Also, it is worth noting that the antiresorptive agents (principally bisphosphonates and the SERMs, as well the RANK ligand and cathepsin K inhibitors), by blocking osteoclastic resorption, force higher levels of PTH secretion and hence better utilization of ingested calcium. Hence, the calcium intake requirement for optimal BMD response to these agents is unclear.

There is, however, some evidence. With respect to fluoride, Dure-Smith and associates (1996) showed, during effective fluoride therapy, an extraordinary degree of bone hunger, suggesting the need for calcium intakes during fluoride therapy of perhaps as much as 62.5 mmol (2500 mg)/day. Other clear examples of the importance of ensuring adequate calcium intake when using other bone active agents are provided by a series of recent papers. First is a meta-analysis of 31 controlled trials of estrogen replacement therapy (Nieves *et al.*, 1998), which showed that estrogen in trials using supplemental calcium (at an average intake of 1187 mg/day) produced bone responses more than twice as great as those that did not (at an average intake of 583 mg/day). Additionally, Recker *et al.* (1999) showed that a reduced dose of estrogen (0.3 mg of conjugated equine estrogens) produced a 5% increase in bone mass in women given supplemental calcium and vitamin D. By contrast, in at least three earlier trials without supplemental calcium, this estrogen dose was essentially ineffective.

SUMMARY

Calcium is the principal cation of bone, making up ~20% of its dry weight. Bone constitutes a very large nutrient reserve for calcium in terrestrial vertebrates, a reserve that has acquired a major mechanical function. The requirement for calcium is related to the protection of this mechanical function, not to the metabolic actions of calcium, which could be adequately protected by a reserve several orders of magnitude smaller. Calcium was abundant in the environment in which hominids evolved and in the foods they ate. Probably as a consequence, efficient calcium conservation mechanisms did not develop. Contemporary diets are low in calcium by comparison, and our Paleolithic physiologies are poorly adapted to them. Yet other features of the modern diet (e.g., salt) increase obligatory calcium loss and thereby reduce the ability to adapt to lowered intakes.

The evidence is compelling that inadequate calcium intake weakens bone and contributes to the growing osteoporosis problem. A fully adequate calcium diet for persons age 65 and older, consuming otherwise typical Western diets, would contain more than 37.5 mmol (1500 mg)Ca/day and perhaps as much as 60 mmol (2400 mg)/day.

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Chapter 80

Drugs Acting on the Calcium Receptor

Calcimimetics and Calcilytics

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INTRODUCTION

Bone and mineral-related diseases are a heterogeneous group of disorders affecting nearly every major organ system in the body. By far the most common disorder is osteoporosis, a disease characterized by reduced bone density and an increased susceptibility to fractures. Less common, but also associated with altered bone metabolism, are primary and secondary hyperparathyroidism. All these disorders are associated with aging, especially in women and, as the world population ages, these diseases are becoming more prevalent. Drugs that alter the circulating levels of parathyroid hormone (PTH) provide one means of treating hyperparathyroidism and possibly osteoporosis.

The secretion of PTH from parathyroid glands can be influenced by a number of factors acting in endocrine or paracrine fashion but the most important is serum calcium. Changes in the level of extracellular calcium are detected by a cell surface calcium receptor, a G protein-coupled receptor whose primary physiological ligand is ionized extracellular calcium (Ca^{2+}). Like many other G protein-coupled receptors, the calcium receptor has recently emerged as a viable drug target.

This chapter reviews the progress made in the discovery and development of calcimimetics and calcilytics, ligands that act as activators and inhibitors, respectively, of the calcium receptor. The most significant advance since the second edition of *Principles* is the regulatory approval of the first calcimimetic for treating some forms of hyperparathyroidism: cinacalcet•HCl. The development of calcilytics for treating osteoporosis has been more challenging but some progress has been made.

THE CALCIUM RECEPTOR

The calcium receptor is a G protein-coupled receptor that shares structural homologies with other members of subfamily C receptors. This subfamily includes the metabotropic glutamate receptors (mGluRs), γ-aminobutyric acid type B receptors ($GABA_BRs$), and some pheromone and taste receptors. All of these receptors are large by G protein-coupled receptor standards, particularly their extracellular domain, which binds the cognate physiological ligand glutamate, γ-aminobutyric acid, or extracellular Ca^{2+} (Brauner-Osborn *et al.*,1999; Hammerland *et al.*, 1999). The human parathyroid calcium receptor encodes a protein of 1078 amino acids and it is 92% and 93% identical with the rat kidney and the bovine parathyroid cell calcium receptors, respectively. At present, only one gene coding for the calcium receptor has been identified; no receptor subtypes have been found and splice variants of the calcium receptor are few and of low abundance (Garrett *et al.*, 1995a). Within family C, the most structurally homologous receptor is GPRC6A, which might use basic amino acids as physiological ligands (Conigrave and Hampson, 2006).

Calcium receptors are expressed on many of the tissues involved in systemic calcium homeostasis. In addition to parathyroid cells, parafollicular cells of the thyroid (C cells) and epithelial cells along the nephron express high levels of calcium receptor messenger mRNA and protein (Brown and MacLeod, 2001). Epithelial cells throughout the gastrointestinal tract and parietal cells and G cells in the stomach likewise express the calcium receptor (Hebert *et al.*, 2004). At all these sites the calcium receptor messenger RNA transcripts appear to be identical and, as assessed by Western blotting, the final protein products expressed at the cell surface are similar. The major differences between calcium receptors expressed in different tissues are post-translational and seemingly involve different patterns of glycosylation (Ray *et al.*, 1998; Bai, 1999). In C cells the calcium receptor

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regulates the secretion of calcitonin (Raue and Scherübl, 1996); in the kidney it is mostly involved in the transport of Ca^{2+} and Mg^{2+} (Ba and Friedman, 2004).

The calcium receptor mediates the initial events of stimulus-secretion coupling in parathyroid cells and is the key element in the primary physiological mechanism regulating PTH secretion. There are three separate lines of evidence supporting this conclusion. The first derives from molecular genetic studies of patients with the inherited disorders familial benign hypercalcemic hypercalciuria (FBHH) or autosomal dominant hypocalcemia (ADH; see Brown, 2007). These disorders result from point mutations in the calcium receptor gene that result in receptor proteins having altered sensitivity to extracellular Ca^{2+}. The mutated calcium receptors in FBHH patients are less sensitive (or completely insensitive) to activation by extracellular Ca^{2+} and this molecular defect is manifest in the phenotype: these patients are hypercalcemic, yet their plasma PTH levels are generally within normal limits. Conversely, the mutant calcium receptors in ADH are more sensitive to activation by extracellular Ca^{2+} and these patients are hypocalcemic, but again, their plasma PTH levels are normal. The second line of evidence is based on *in vitro* studies showing that the loss of responsiveness to extracellular Ca^{2+} of cultured bovine parathyroid cells correlates with loss of mRNA for the calcium receptor (Mithal *et al.*, 1995). Finally, and the focus of the present review, selective pharmacological manipulation of the calcium receptor promptly alters PTH secretion *in vitro* and plasma levels *in vivo*.

PHARMACOLOGY OF THE CALCIUM RECEPTOR

As originally defined, calcimimetic ligands are those that mimic or potentiate the action of extracellular Ca^{2+} at the calcium receptor, whereas calcilytic ligands are calcium receptor antagonists. Calcimimetics can be subdivided into type I and type II ligands. The former activate the calcium receptor in the absence of extracellular Ca^{2+} and behave as true agonists, whereas type II calcimimetics act as positive allosteric modulators to increase the sensitivity of the calcium receptor to activation by extracellular Ca^{2+}. These are operational definitions based on the ability of the ligand to activate the calcium receptor in the presence or absence of extracellular Ca^{2+}. The definition is based on functional cellular responses, typically changes in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$). Importantly, these terms do not imply any knowledge about the site on the receptor to which the ligand binds (Nemeth, 2002b).

Calcimimetics

From the outset, it was appreciated that the calcium receptor is rather promiscuous and responds to a variety of inorganic cations and structurally diverse molecules possessing a net positive charge. A number of inorganic di- and trivalent cations have been studied and include, in rank order of potency: $La^{3+} \cong Gd^{3+} > Be^{2+} > Ca^{2+} \cong Ba^{2+} > Sr^{2+} > Mg^{2+}$ (Nemeth, 1990). Similarly, many organic polycations activate the calcium receptor and include simple polyamines (spermine), aminoglycoside antibiotics (neomycin), polyamino acids (polylysine) or proteins (protamine), and arylamine spider toxins (araxins). All of these charged ligands behave as type I calcimimetics—they are agonists and fully activate the calcium receptor in the absence of extracellular Ca^{2+} (Nemeth and Fox, 1999).

In contrast to these type I calcimimetics are L-amino acids that act as allosteric activators of the calcium receptor (Conigrave and Hampson, 2006). Among the naturally occurring L-amino acids, those possessing an aromatic ring (phenylalanine, tryptophan) are the most potent and, at low millimolar concentrations, can potentiate secretion of PTH *in vitro* (Conigrave *et al.*, 2004). Regulation of calcium receptor activity by L-amino acids might well be physiologically relevant, particularly in the gastrointestinal tract after a meal (Conigrave and Brown, 2006).

Homology modeling of family C G protein-coupled receptors that detect L-amino acids as ligands and *in silico* analysis of the putative amino acid-binding pocket predicted the calcimimetic activity of the tripeptide glutathione (Wang *et al.*, 2006). Both the reduced and oxidized forms of glutathione are active at submicromolar concentrations. These peptides potentiate the effects of extracellular Ca^{2+} when the calcium receptor is studied in heterologous expression systems, but it is not known whether they activate the receptor in the absence of extracellular Ca^{2+}. Binding of radiolabeled glutathione to the secreted extracellular domain of the calcium receptor was dependent on the presence of extracellular Ca^{2+}, so this ligand could be acting as an allosteric modulator. Like the L-amino acids, there might be a physiological and/or pathophysiological role for glutathione that is mediated by the calcium receptor (Conigrave and Hampson, 2006).

Whereas all of these calcimimetic ligands have been useful in defining the pharmacology of the calcium receptor, they are far from therapeutic because they lack specificity and suitable pharmacokinetic properties. The first class of compounds that includes selective, drug-like ligands of the calcium receptor is the phenylalkylamines, typified by NPS R-568 and cinacalcet (Fig. 1). These compounds are selective and do not affect the activity of mGluRs or various other G protein-coupled receptors at concentrations that maximally activate the calcium receptor. Phenylalkylamine compounds, like the amino acids, are stereoselective and, depending on the response measured, the *R*-enantiomers are 10- to 100-fold more potent than the corresponding *S*-enantiomers. For example, NPS R-568 increases $[Ca^{2+}]_i$ and inhibits PTH secretion from bovine parathyroid cells *in vitro* at concentrations between 3 and 100 nM, whereas

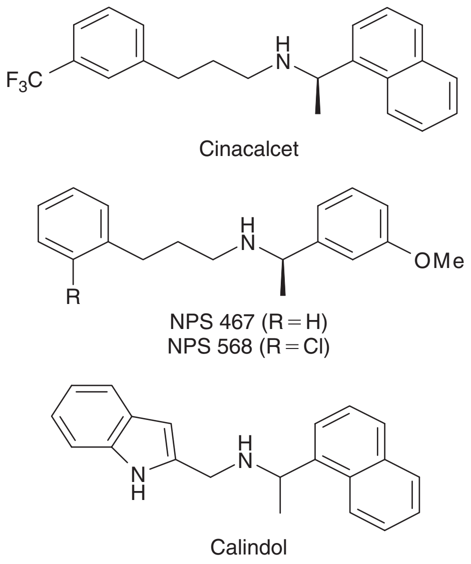

FIGURE 1 Structures of representative calcimimetic compounds. Shown are the *R*-enantiomers.

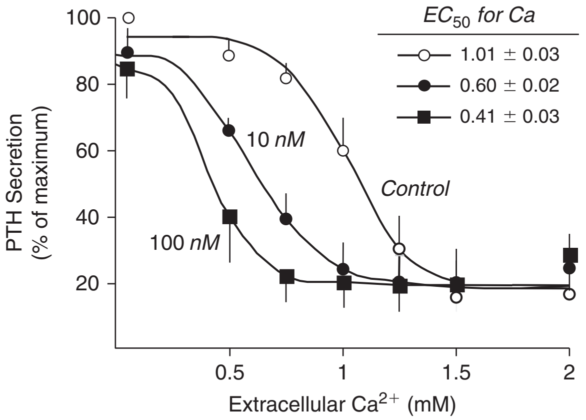

FIGURE 2 Cinacalcet potentiates the inhibitory effects of extracellular Ca^{2+} on PTH secretion. Primary cultures of bovine parathyroid cells were incubated for 20 minutes in the presence or absence of the indicated concentration of cinacalcet. The concentration-response curve for extracellular Ca^{2+} was shifted to the left without changes in the maximal or minimal response. This resulted in a dose-dependent decrease in the EC_{50} for extracellular Ca^{2+} as shown in the inset. (Fig. 7 in Nemeth *et al.,* 2004).

this compound is without effect on either response at concentrations less than 3 μ*M* (Nemeth *et al.*, 1998). These effects are dependent on the concentration of extracellular Ca^{2+} and NPS R-568 fails to alter parathyroid cell function when extracellular Ca^{2+} levels are reduced to levels below 0.1 m*M*. Type II calcimimetic compounds like NPS R-568 and cinacalcet are positive allosteric modulators and they shift the concentration-response curve for extracellular Ca^{2+} to the left without affecting the maximal or minimal responses (Fig. 2; Hammerland *et al.*, 1998; Nemeth *et al.*, 1998, 2004). This effect can be observed in heterologous expression systems, in authentic bovine parathyroid cells, and in rodents *in vivo* (Nagano, 2005).

Other calcimimetics have been synthesized of which the most studied is calindol (see Fig. 1; Dauban *et al.*, 2000; Kessler *et al.*, 2004a). This compound potentiates extracellular Ca^{2+}-induced increases in inositol phosphates and $[Ca^{2+}]_i$ in heterologous expression systems and is inactive in the absence of extracellular Ca^{2+} (Petrel *et al.*, 2004; Ray *et al.*, 2005). As such, it acts as a positive allosteric modulator. Calindol has not been studied *in vivo* and, although it probably does, it has not been shown to affect secretion of PTH *in vitro*.

The use of chimeric or domain-deleted receptor constructs and receptors with single or multiple point mutations has revealed sites within the calcium receptor where different calcimimetics act. Although it is generally true that these approaches provide information about the binding sites in the receptor, this is not always the case, particularly with point mutations. Very few studies have actually measured ligand binding; it is typically a functional response, such as formation of inositol phosphates that is measured. Of course, some calcimimetics, like the L-amino acids, simply lack the requisite potency to be studied in binding assays. Glutathione is one of the few calcimimetics that has been studied in a radiolabeled binding assay (Wang *et al.*, 2006). It does bind to the secreted extracellular domain of the receptor but it is unknown if it binds elsewhere in the receptor. Although the phenylalkylamines, calindol, and L-amino acids share a common mechanism of action, they do not all act at the same site within the calcium receptor. The L-amino acids, like extracellular calcium (Hammerland *et al.*, 1999; Brauner-Osborne, 1999; Silve *et al.*, 2005), bind to sites in the extracellular domain, whereas the phenylalkylamines act in the transmembrane domain (Hauache *et al.*, 2000; Ray and Northup, 2002; Zhang *et al.*, 2002; Miedlich *et al.*, 2004; Mun *et al.*, 2004). Calindol also binds to sites within the transmembrane domain of the calcium receptor (Ray *et al.*, 2005).

In normal rats, the oral administration of NPS R-568 or cinacalcet causes a rapid fall in plasma levels of PTH, which is accompanied by hypocalcemia (Fig. 3). The fall in plasma levels of PTH precedes that of serum calcium and the longer PTH levels are depressed, the greater is the magnitude and duration of the hypocalcemia that is achieved by a given dose of compound (Fox *et al.*, 1999a). It is remarkable that the effect of NPS R-568 on levels of plasma PTH in normal rats parallels that seen *in vitro*: the concentration-response curve for plasma Ca^{2+} and serum PTH is shifted to the left, thereby reducing the setpoint (Nagano, 2005).

Calcimimetics acting on renal calcium receptors might be expected to lower calcium reabsorption, as occurs in genetic disorders resulting from activating mutations of the calcium receptor (Brown, 2007). However, the hypocalcemic response elicited by NPS R-568 persists following an acute, total nephrectomy in rats (Fox *et al.*, 1999a). This finding does not necessarily mean that the compound

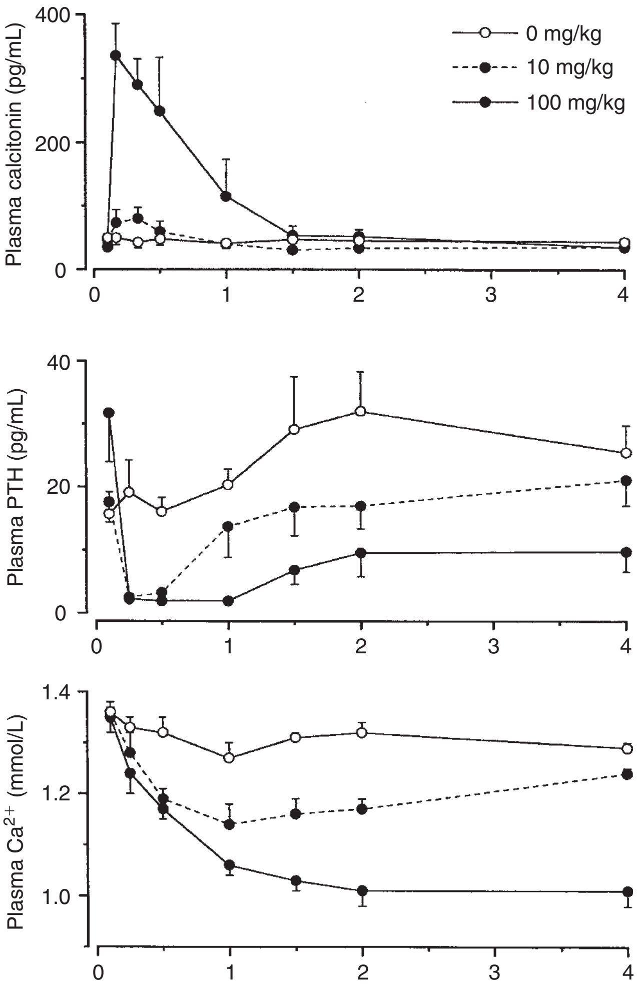

FIGURE 3 A calcimimetic compound decreases plasma levels of PTH and Ca^{2+} and increases those of calcitonin. NPS R-568 (or vehicle) was administered orally at the indicated dose to normal male rats. Note that the 10 mg/kg dose, which is close to the ED_{50} for depressing plasma PTH levels, is without effect on plasma levels of calcitonin. (Fig. 2 in Nemeth *et al.*, 1996).

is without effect on renal calcium receptors; only that any actions on these receptors are not required to explain the hypocalcemic effect observed *in vivo*. Selective surgical ablation of the thyroid, to eliminate C cells but leave the parathyroid glands intact, slowed the rate of onset but did not affect the magnitude of the hypocalcemia following administration of NPS R-568. In contrast, NPS R-568 failed to lower plasma levels of Ca^{2+} in thyroparathyroidectomized animals that were maintained at normocalcemia by continual infusion of PTH (Fox *et al.*, 1999b). Thus, hypocalcemia caused by administering phenylalkylamine calcimimetics results mostly from the ability of these compounds to inhibit secretion of PTH. In the rat, increased levels of calcitonin increase the rate of onset, but not the magnitude of hypocalcemia. With respect to the changes in circulating levels of PTH, calcium and calcitonin, the pharmacodynamics of cinacalcet and NPS R-568 are essentially the same. The differences between these two compounds reside in their pharmacokinetics, as described next.

Pharmacokinetics of Cinacalcet

Cinacalcet has more favorable pharmacokinetic properties than NPS R-568 and has, thus, become the calcimimetic of major clinical interest. NPS R-568 has poor oral bioavailability (5%) and it is metabolized mostly by the hepatic cytochrome P450 enzyme, CYP2D6. Approximately 5% to 7% of the population expresses an isozyme of CYP2D6 that metabolizes NPS R-568 poorly. Together with poor bioavailability, these properties can result in highly variable blood levels of NPS R-568.

The pharmacokinetics of cinacalcet has been assessed in rodents, monkeys, and normal humans (Kumar *et al.*, 2004) and in patients receiving hemodialysis or continuous ambulatory peritoneal dialysis (Harris *et al.*, 2004; Padhi *et al.*, 2005). The pharmacokinetic profile of cinacalcet is essentially the same in normal humans and in those receiving either form of dialysis. Cinacalcet has an oral bioavailability of 74% and maximal plasma concentrations (C_{max})

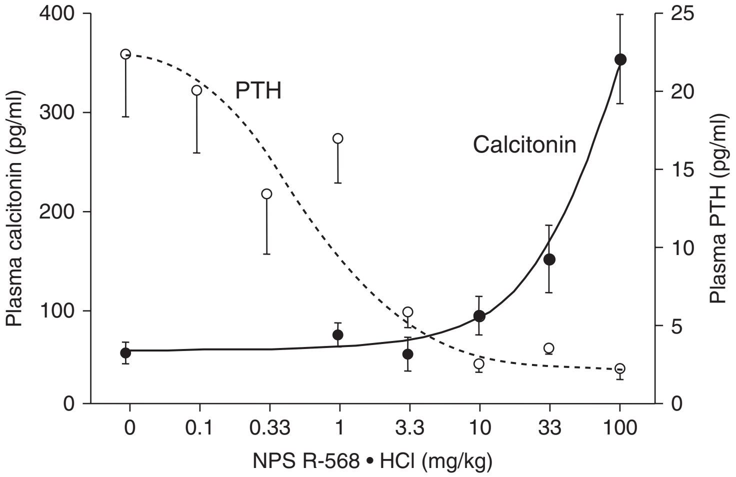

FIGURE 4 NPS R-568 preferentially acts on the parathyroid Ca^{2+} receptor. NPS R-568 was administered orally to normal rats and the changes in plasma levels of PTH and calcitonin determined 15 to 20 minutes later. (Redrawn from data in Fox *et al.*, 1999a,b).

occur within 2 to 3 hours after a single oral dose. There is a dose-proportional increase in C_{max} and area under the plasma concentration-time curve (AUC) with cinacalcet at doses up to 200mg once daily. These pharmacokinetic parameters and the plasma half-life ($t_{1/2}$) of cinacalcet were similar when estimated on days with and without dialysis. Moreover, the degree of renal impairment did not affect the pharmacokinetic profile of cinacalcet (Padhi *et al.*, 2005). The dose-proportional increase in exposure to cinacalcet correlates inversely with plasma levels of PTH. The plasma concentration of cinacalcet that results in a 50% decrease in serum levels of PTH is 6.2 ng/mL (about 15 n*M*), a concentration remarkably similar to that causing a 50% decrease in PTH secretion *in vitro* (27 n*M*; Nemeth *et al.*, 2004).

Cinacalcet is rapidly metabolized by several hepatic cytochrome P450 enzymes, primarily CYP3A4, CYP2D6, and CYP1A2 (Kumar *et al.*, 2004). The major routes of metabolism are by oxidative *N*-dealkylation and by oxidation of the naphthalene ring; none of the metabolites are biologically active on the calcium receptor and most (80%) are eliminated by renal excretion (Kumar *et al.*, 2004). Moderate to severe degrees of hepatic impairment will impede metabolism of cinacalcet and increase plasma exposure. Cinacalcet inhibits CYP2D6 *in vitro* and can therefore retard the metabolism of drugs that are metabolized by this enzyme, such as tricyclic antidepressants (Harris *et al.*, 2006).

Pharmacological Selectivity of Calcimimetics

Despite apparently identical calcium receptors on parathyroid cells and on C cells, their cellular pharmacology differs. An early example of this was noted with Mg^{2+}. Extracellular Mg^{2+} increases $[Ca^{2+}]_i$ bovine parathyroid cells and in HEK 293 cells expressing the human, bovine, or rat calcium receptor. Similar increases in the concentration of extracellular Mg^{2+}, however, fail to increase $[Ca^{2+}]_i$ in the medullary thyroid carcinoma cell lines 44-2 or 6-23 (Nemeth, 1990; Fajtova *et al.*, 1991). Nonetheless, increased concentrations of extracellular Mg^{2+} stimulate secretion of calcitonin from authentic C cells *in vitro* and *in vivo* (see references in Nemeth, 1996). Perhaps this discrepancy for a type I calcimimetic results from comparing medullary thyroid carcinoma cells with authentic C cells because these cells are likely to express different phenotypes. This explanation cannot, however, account for the preferential activity of NPS R-568 on secretion of calcitonin and PTH that is noted in normal rats *in vivo* (Fox *et al.*, 1999b). Similar results were obtained with cinacalcet. The calcium receptor mRNAs expressed in rat parathyroid cells and C cells are identical and the final protein products are likewise similar (Garrett *et al.*, 1995b). Nonetheless, NPS R-568 and cinacalcet depress plasma levels of PTH at doses 30- to 40-fold lower than those that increase plasma levels of calcitonin (Figs. 3 and 4).

The mechanism(s) explaining the preferential inhibitory effects of phenylalkylamine calcimimetics on serum levels of PTH is uncertain. It is now widely recognized that the same receptor, when expressed in different cellular contexts, can display quite distinct pharmacological properties. This so-called phenotypic pharmacology is especially prevalent among G protein-coupled receptors, and a number of different molecular mechanisms have been put forward to explain it (Kenakin, 2005). One relatively simple explanation is that different degrees of receptor occupancy may be required to alter secretion from parathyroid cells and from C cells. In this view, a lower fractional occupancy of the calcium receptor is required to depress PTH secretion than is required to stimulate calcitonin secretion. Cinacalcet is correspondingly more potent in depressing plasma levels of PTH. Alternatively, or additionally, the different signaling pathways linking the calcium receptor to PTH and to calcitonin secretion might also contribute to the preferential activity of phenylalkylamine calcimimetic compounds on parathyroid calcium receptors.

Calcilytics

The first substance, either atomic or molecular, shown to act as a calcium receptor antagonist is the amino alcohol NPS 2143 (Fig. 5). The inhibitory effects of NPS 2143 are not dependent on the properties of the ligand used to activate the receptor in either heterologous expression systems or authentic cells. The potency and efficacy of NPS 2143 are similar whether blocking cytoplasmic Ca^{2+} responses evoked by extracellular Ca^{2+} or NPS R-467. NPS 2143 is without on the cytoplasmic Ca^{2+} responses elicited by activation of various native or chimeric mGluRs nor does it affect those in cells coexpressing the GABABR1 and R2. Calcilytic compounds like NPS 2143, when used under appropriate conditions, are potent and selective pharmacological tools to study the function of calcium receptors expressed in normal and pathological tissues (Nemeth, 2002c).

The effects of NPS 2143 on PTH secretion from bovine parathyroid cells mirror those of the calcimimetics that act as positive allosteric modulators. In the case of the calcilytic, there is a shift to the right in the concentration-response curve that is unaccompanied by changes in either the maximal or minimal response (Fig. 6). NPS 2143 stimulates secretion of PTH from bovine parathyroid cells *in vitro* at concentrations similar to those that inhibit evoked increases in $[Ca^{2+}]_i$. These changes in the agonist concentration-response curves show that NPS 2143 decreases whereas phenylalkylamine calcimimetics increase the sensitivity of the calcium receptor to activation by extracellular Ca^{2+}.

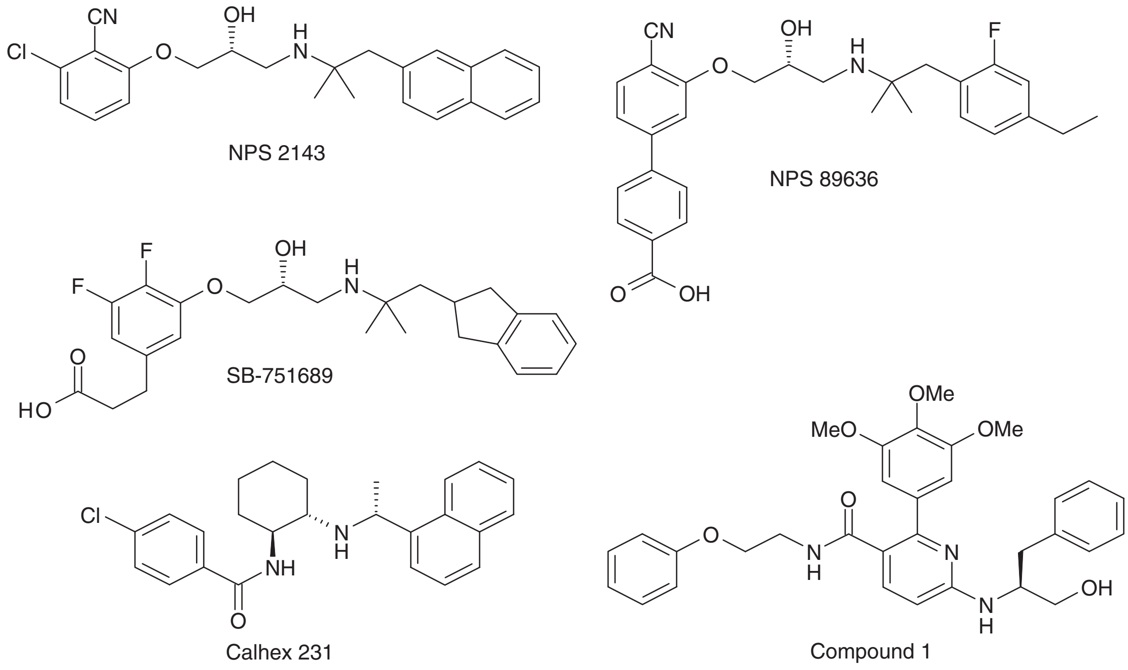

FIGURE 5 Structures of representative calcilytic compounds.

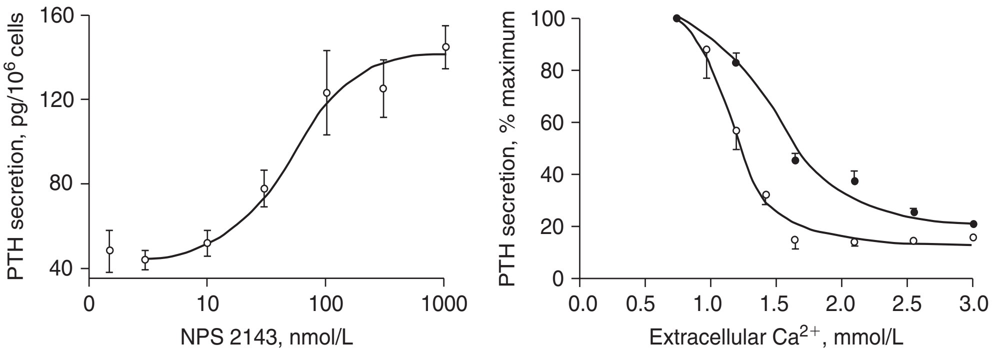

FIGURE 6 NPS 2143 stimulates PTH secretion from bovine parathyroid cells *in vitro*. In the right panel, parathyroid cells were incubated in the presence (closed symbols) or absence of 300 nM NPS 2143. (Fig. 7 in Nemeth *et al.*, 2001).

Although the rightward shift of the concentration-response curve could indicate competitive inhibition by NPS 2143, the shifts in the concentration-response curve shown in Fig. 6 would also be produced by a noncompetitive antagonist acting on a tissue with a large receptor reserve (Zhu, 1993). And, as discussed later, there might be a large reserve of calcium receptors on parathyroid cells.

When administered intravenously or by oral gavage to normal rats, NPS 2143 causes a rapid, 3- to 5-fold increase in plasma levels of PTH (Nemeth *et al.*, 2001). Other compounds of the amino alcohol chemotype (NPS 89636 and SB-751689) also increase circulating levels of PTH. These findings were comforting because it was never a certainty that a calcilytic compound would in fact stimulate PTH secretion. Although intuitively appealing, this assumption rests solely on the finding that lowering the level of extracellular Ca^{2+} stimulates PTH secretion. This is not, however, equivalent to blocking the activity of the receptor in a normocalcemic setting.

Other calcilytic chemotypes are shown in Fig. 5. These compounds were discovered by high-throughput screening of compound libraries or by progressive modifications to the amino alcohol structure of NPS 2143 (Kessler *et al.*, 2004b, 2006; Shcherbakova *et al.*, 2005a, b; Arey *et al.*, 2005). All these compounds are antagonists in *in vitro* assays using heterologous expression of the calcium receptor and all act within the transmembrane domain of the calcium receptor (Petrel *et al.*, 2003; Hu *et al.*, 2006). Compound 1 has been shown to act as negative allosteric modulator (Hu *et al.*, 2006) but the binding of radiolabeled compound 1 was not displaced by an amino alcohol calcilytic, so the binding sites of these two chemotypes differ (Arey *et al.*, 2005). Compound 1 is the only other calcilytic reported to affect plasma levels of PTH *in vivo*. It caused a dose-dependent, 5- to 6-fold maximal increase in circulating levels of PTH when administered intravenously to rats (Arey *et al.*, 2005).

The Calcium Receptor in Hyperparathyroidism

One of the early concerns in developing calcimimetic compounds as treatments for hyperparathyroidism (HPT) was whether the disease process in some way altered the parathyroid calcium receptor so that calcimimetics, although effective in normal animals and humans, were no longer capable of altering plasma levels of PTH in patients with primary or secondary HPT. No mutations, however, have yet been detected in the coding region of the calcium receptor gene in pathological parathyroid glands from patients with primary or secondary HPT (Garrett *et al.*, 1995a; Hosokawa *et al.*, 1995; Cetani *et al.*, 2000). A consistent finding, however, is reduced expression of the calcium receptor in both adenomatous and hyperplastic tissues (Kifor *et al.*, 1996; Farnebo *et al.*, 1997; Gogusev *et al.*, 1997; Kaneko *et al.*, 1999; Taniguchi *et al.*, 2006a). This conclusion is based on semi-quantitative analyses of calcium receptor mRNA and staining intensities of parathyroid tissue using mono- or polyclonal antibodies against the calcium receptor. Like the data for the vitamin D receptor, nodular regions showed a greater loss of staining intensity for the calcium receptor when compared with adjacent areas of diffuse hyperplasia (Rodriguez *et al.*, 2005). In patients with primary HPT, there is a correlation between the level of parathyroid calcium receptor expression, as judged by the intensity of staining of tissues with a polyclonal antibody to the calcium receptor, and the PTH-Ca^{2+} setpoint—the setpoint is higher in those patients with glands having the least staining intensity (Cetani *et al.*, 2000). When parathyroid glands excised from hemodialysis patients with refractory HPT were studied *in vitro*, a similar shift in the setpoint was observed. Moreover, there was an inverse relationship between PTH secretion and levels of calcium receptor expression: those glands with the lowest levels of calcium receptor showed a diminished secretory response to changes in the concentration of extracellular Ca^{2+} (Canadillas *et al.*, 2005). Decreases in calcium receptor expression levels appear to be reversible because recipients of renal allografts show increased staining for the receptor in diffuse (but not nodular) regions of hyperplastic parathyroid glands (Taniguchi *et al.*, 2006b). Thus, in contrast to the altered setpoints in genetic diseases that result from changes in the amino acid sequence of the calcium receptor, those in HPT result largely from changing the density of presumably normal receptors.

Several different lines of evidence suggest that the apparently reduced expression of calcium receptor protein in pathological parathyroid glands does not render such glands insensitive to calcimimetic compounds. First, secretion of PTH from adenomatous or hyperplastic parathyroid glands *in vitro* is inhibited by NPS R-568 (Nemeth, 1996) and by cinacalcet (Kawata *et al.*, 2006). In the latter study, it was shown by immunohistochemistry that staining for the calcium receptor was reduced in all tissue samples; at least in these particular surgical tissue samples, there was enough calcium receptor remaining to respond to cinacalcet. Moreover, the potency of NPS R-568 is similar in normal rats or those with secondary HPT resulting from a 5/6 nephrectomy (Fox *et al.*, 2000). Finally, and most significantly, calcimimetic compounds lower plasma levels of PTH in patients with primary or secondary HPT. In the clinic, there seems to usually be enough calcium receptor expressed for cinacalcet to effectively lower plasma levels of PTH. The only exception might be parathyroid carcinoma.

PRIMARY HYPERPARATHYROIDISM

Primary hyperparathyroidism is characterized by chronically elevated circulating levels of PTH and calcium and usually results from an adenoma of a single parathyroid gland (Heath, 1991). The manifestations of primary hyperparathyroidism may include bone loss, nephrolithiasis and nephrocalcinosis, gastrointestinal distress, and easy fatigability. Although many patients with primary hyperparathyroidism are apparently asymptomatic, it has been argued that these patients will eventually develop specific symptomatology (Fischer, 1993). Moreover, because the incidence of primary hyperparathyroidism increases with age, many of the rather vague symptoms such as muscular fatigue and depressed cognition may be interpreted as part of the normal aging process. Whether these so-called asymptomatic patients should be treated is controversial and at present the most widely followed practice continues to be "watchful waiting" (Bilezikian *et al.*, 2002).

There is somewhat less controversy regarding treatment of moderate and essentially none regarding treatment of severe forms of primary hyperparathyroidism. The treatment of choice is surgical ablation of the offending gland(s) and, when performed by a skilled endocrine surgeon, the success (cure) rate is 95% (Clark, 1995). There are, however, many instances when surgery might not be chosen if effective medical alternatives existed. These include patients who refuse surgery, are elderly, have comorbid conditions, or have undergone previous unsuccessful surgeries. In addition, there are those situations where it would be desirable to delay surgery or to stabilize serum levels of calcium prior to surgery. In these situations, a medicine that safely lowered serum calcium levels would be beneficial.

Vestergaard (2006) provides a comprehensive review of the various pharmacological agents that have been studied as treatments for patients with primary HPT. Of note are a number of recent studies with the bisphosphonate alendronate. Daily dosing with 10 mg/kg alendronate for 48 weeks to two years decreases bone turnover markers and increases bone mineral density (BMD) in patients with mild to moderate primary HPT. Typically, however, there is only a temporary and minor reduction, if any, in serum calcium levels; after 6 months, serum calcium has returned to baseline levels. As noted before (Nemeth, 1996, 2002a), the bisphosphonates treat the hypercalcemia, not the hyperparathyroidism itself. Other approaches using drugs that act directly on parathyroid cells have also been explored but none has proven effective in managing primary HPT (Nemeth, 2002a).

NPS R-568 was the first calcimimetic to be studied in patients with primary HPT. A single oral dose caused a rapid and dose-dependent decrease in circulating levels of PTH and calcium (Silverberg *et al.*, 1997). Two repeat-dosing trials using cinacalcet in patients with primary HPT have now been reported (Shoback *et al.*, 2003; Peacock *et al.*, 2005). Both were randomized, double-blind, placebo-controlled, multisite studies.

The initial study involved 22 male or female patients with mild to moderate degrees of primary HPT as defined by serum levels of PTH ($\geq$45 pg/mL) and calcium ($>$10.3 mg/dL). The first phase of this study examined the efficacy and tolerability of once-daily dosing with 50 mg of cinacalcet. The dose could be increased in 25-mg increments to 100 mg. Plasma PTH levels fell by 55% compared with placebo at 2 hours after dosing but were nearly back to baseline just before the next dose (24 hours later). With this dosing regimen there was an unacceptably high incidence of nausea and dizziness compared with the placebo-treated subjects. To diminish these adverse events and to achieve a more sustained reduction in plasma levels of PTH, a twice-daily dosing regimen was adopted. Subjects were administered 30, 40, or 50 mg of cinacalcet or placebo twice-a-day for 15 days and then followed for an additional 7 days. There was a rapid reduction in plasma levels of PTH that reached a nadir 2 to 4 hours after the first dose and then gradually returned toward baseline levels but was still below baseline 12 hours later, just prior to the second dose. This rapid decrease in plasma levels of PTH correlates precisely with the $C_{\max}$ of cinacalcet following oral administration (Harris *et al.*, 2004). The second dose elicited a pattern of change in plasma PTH levels similar to the first dose: a rapid, 40% to 50% decrease from baseline within 2 to 4 hours that rose to within 15% of baseline levels after 12 hours. The time course and magnitude of change in plasma PTH levels were similar on day 1 and on day 15. Levels of serum calcium fell into the normal range 2 hours after the second dose on day 1 and were maintained within the normal range throughout the 15-day study. Seven days after cessation of dosing, circulating levels of PTH and calcium had returned to baseline levels. There were no consistent changes in levels of plasma PTH and serum calcium compared with baseline in the placebo-treated group. Cinacalcet was well tolerated and no serious adverse events were reported. The most frequent side effect was paresthesia, which reportedly occurred in 33% of the placebo-treated and in 19% of the cinacalcet-treated subjects (Shoback *et al.*, 2003).

The long-term safety and efficacy of cinacalcet was investigated in 78 patients with mild to moderate primary HPT using the same inclusion criteria as in the prior study (Peacock *et al.*, 2005). In this study, subjects were administered 30 mg of cinacalcet twice a day for 1 year. During the first 12 weeks (titration phase), the dose of cinacalcet could be increased to 40 or 50 mg if the patients were still hypercalcemic ($>$10.3 mg/dL) at weeks 4 and 8. Efficacy endpoints were measured after another 12 weeks (maintenance phase) and patients were monitored for another 28 weeks to gather additional safety and efficacy data. During the maintenance phase, there was a significant 7.6% decrease in fasting plasma levels of PTH in the cinacalcet-treated group compared with a 7.7% increase in the placebo-treated group. The magnitude of this effect might seem small but plasma levels of PTH were measured at a time when they would be highest (12 hours after dosing). When studied at week 24, the morning dose of cinacalcet elicited a 37% decrease in plasma levels of PTH at 4 hours compared with predose levels. Plasma levels of PTH in the cinacalcet-treated group fell below those in the placebo-treated group by week 4 and remained so for 1 year. Serum calcium levels had normalized by 2 weeks (the earliest time measured) and remained within the normal range for 1 year. In the cinacalcet-treated group, serum calcium was 10.7 mg/dL at baseline and 9.7 mg/dL at week 52, whereas the placebo-treated group had the same baseline value but completed the study with serum calcium levels of 10.9 mg/dL. Ninety percent of the patients who completed the study remained on 30 mg of cinacalcet twice daily during the maintenance phase, and of those, most achieved normocalcemia with a decrease of 0.5 mg/dL from baseline. In this long-term study, the drug was well tolerated and the most common adverse event in the cinacalcet-treated group was nausea (28% compared with 16% in the placebo-treated group).

A number of secondary endpoints were assessed in both the short- and long-term studies described earlier. In the short-term study, levels of serum phosphorous tended to increase but not significantly so. And there were no significant differences between the cinacalcet- and placebo-treated groups in 24-hour urine calcium excretion, the 24-hour urine calcium-to-creatinine ratio, or the fasting urine calcium-to-creatinine ratio were observed in the 15-day study by Shoback *et al.* (2003). In contrast, treatment with cinacalcet for 1 year resulted in significant increases in serum phosphate levels and decreases in fasting urine calcium-to-creatinine ratio compared with placebo. These changes were accompanied by significant increases and decreases, respectively, in tubular reabsorption of phosphorous and calcium (Peacock *et al.*, 2005). Such changes in mineral metabolism are the expected response to a net decrease in circulating levels of PTH. Predose serum levels of 1,25-dihydroxyvitamin D were not altered so a lowered level of PTH might sufficiently explain these changes in mineral metabolism. At present, however, an action of cinacalcet on renal calcium receptors cannot be excluded.

Treatment with cinacalcet for 1 year increased levels of some bone turnover markers when compared with placebo. Serum levels of bone-specific alkaline phosphatase (BSAP) and N-telopeptide (NTx) and urine NTx were slightly but significantly increased; there was no change in urine deoxypyridinoline levels. BMD at the lumber spine, total femur, and distal one-third of the radius was not affected by 1 year of treatment with cinacalcet when compared with placebo. The failure of cinacalcet to affect BMD in patients with mild to moderate degrees of primary HPT is curious because parathyroidectomy in this patient population often does result in significant gains in BMD within the first year (Silverberg, *et al.*, 1995; Nordenstrom *et al.*, 2004).

Another intriguing and somewhat unexpected observation that emerged from these two studies is that, although daily dosing caused cyclic changes in plasma PTH levels, serum calcium levels remained constant for 8 hours after dosing. It appears that after a couple days of dosing, serum calcium levels achieve a new homeostatic setpoint that no longer responds to rapid changes in plasma levels of PTH.

Cinacalcet has been studied in patients with the rare but far more serious form of primary HPT—parathyroid cancer. In contrast to primary HPT resulting from an adenoma or hyperplasia, that caused by a carcinoma is life-threatening. Patients with parathyroid carcinoma present with the signs and symptoms of severe hypercalcemia. Surgical treatment of this malignant form of primary HPT is considerably more difficult because of metastasis and multiple resections are common (Shane and Bilezikian, 1982). The first calcimimetic was studied in a male patient with severe hypercalcemia resulting from parathyroid carcinoma (Collins *et al.*, 1998). Conventional therapy with calcitonin, furosemide, hydration, or the bisphosphonate pamidronate did not consistently lower plasma Ca^{2+} levels (Fig. 7) and plasma PTH levels were unaffected by these treatments. In contrast, daily oral dosing with NPS R-568 lowered plasma levels of PTH and Ca^{2+} for nearly 2 months. At that time, plasma PTH levels began to increase but plasma Ca^{2+} levels did not. This patient was subsequently maintained on high doses of NPS R-568 (100 mg orally, every 6 hours) for more than 600 days without any instances of hypercalcemic crisis or any adverse effects owing to drug.

The regulatory filing of cinacalcet includes a multicenter, open-label study of 21 patients with parathyroid carcinoma and hypercalcemia (Silverberg *et al.*, 2004). All of the patients studied had at least one prior parathyroidectomy and all presented with at least one symptom of hypercalcemia. Most of the patients (81%) had been treated previously with bisphosphonates. There was an initial dose-titration phase starting with 30 mg of cinacalcet twice daily and increasing to a maximum of 90 mg four times a day. This titration phase lasted until serum calcium levels fell to 10 mg/dL or 16 weeks, whichever came first.

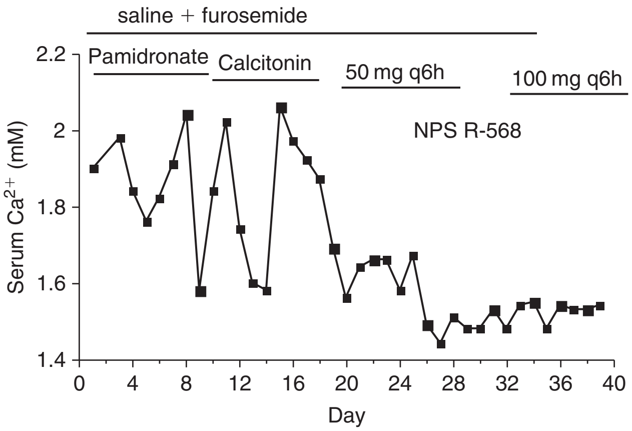

FIGURE 7 NPS R-568 lowers plasma levels of PTH in a male patient with parathyroid carcinoma. (Fig. 1 in Collins *et al.*, 1998).

The mean serum calcium level was 14.5 mg/dL at baseline and by the end of the titration phase it had fallen to 12.4 mg/mL; 71% of the patients achieved a reduction of ≥1 mg/dL. Serum calcium levels were maintained for up to 3 years and there were marked improvements in symptoms of hypercalcemia in all patients.

There was a 15% reduction in mean levels of plasma PTH when measured just before dosing and, as expected, a greater reduction (30%) when measured 2 to 4 hours after dosing. The patients studied were a remarkably heterogeneous group, with plasma PTH values ranging from 232 to 2106 pg/mL and serum calcium ranging from 9.4 to 20.2 mg/dL at entry. These population variances were not diminished by 16 weeks of treatment with cinacalcet.

Treatment of parathyroid carcinoma patients with cinacalcet results in considerably smaller reductions in plasma levels of PTH when compared with patients with HPT resulting from an adenoma or those with secondary HPT (see later). In fact, in a subset of 10 patients that were treated for 1.5 years with cinacalcet, plasma levels of PTH actually increased (by 42%) when compared with baseline, even though serum calcium was maintained at lower level (Rubin *et al.*, 2004). Clearly, parathyroid carcinoma cells display a phenotype quite different from that of adenomatous and hyperplastic parathyroid cells. Secretion of PTH from parathyroid carcinoma cells have long been known to be essentially autonomous and refractory to changes in the concentration of extracellular Ca^{2+}. The meager response of plasma PTH to cinacalcet in patients with parathyroid carcinoma is consistent with this and perhaps results from the loss of calcium receptors to an extreme extent.

Cinacalcet has been used experimentally in three patients with lithium-induced HPT (Gregoor and de Jong, 2007). Daily dosing with cinacalcet lowered serum levels of calcium by about 8% and, despite a 60% drop in plasma levels of PTH, there was no significant increase in serum phosphate levels. It might be noteworthy that these three patients had renal impairment (mean creatinine clearance, 35 μmol/L) and this could have diminished the renal effects of PTH on phosphate reabsorption.

SECONDARY HYPERPARATHYROIDISM

Secondary HPT can result from the normal aging process but it is most often associated with chronic kidney disease (CKD) and most patients with end-stage renal disease (ESRD) suffer from secondary HPT (Coburn and Salusky, 1994). Secondary HPT is characterized by enlargement of all four parathyroid glands, principally through hyperplasia but also involving cellular hypertrophy (Drüeke, 1995). The primary factors that lead to enlargement of the parathyroid glands are hypocalcemia, hyperphosphatemia, and lowered plasma levels of 1,25-dihydroxyvitamin D_3. The mechanisms by which these factors lead to hyperplasia and hypertrophy of the parathyroid glands are not completely understood but each appears capable of acting wholly or partially independent of the others. Plasma levels of PTH increase very early on in the course of CKD and may already be elevated when creatinine clearance is one-half of normal (about 60 mL/min; Fajtova *et al.*, 1995), well before the patient would be started on dialysis. The severity of secondary HPT, as indexed by enlargement of the parathyroid glands and increased circulating levels of PTH, typically increases as the renal disease progresses from mild chronic renal insufficiency (CRI) to ESRD. There is a potentially vicious cycle operating in CKD, because abnormal mineral metabolism and increased circulating levels of PTH seem to increase the rate of progression towards ESRD and dialysis (Ritz *et al.*, 2005; Schwarz *et al.*, 2006)

Chronically elevated levels of circulating PTH and phosphorous and abnormal levels of serum calcium are the major laboratory features of secondary HPT. These blood parameters, in turn, are associated with renal osteodystrophy and soft tissue calcification (London *et al.*, 2005; Ketteler *et al.*, 2005; Goodman, 2006; Moe *et al.*, 2006; Martin and González, 2007). Vascular calcification is most closely associated with hyperphosphatemia and an increase in the calcium-phosphorous product, but increased serum levels of calcium also contribute by augmenting the ability of phosphate to cause vascular calcification. Cardiovascular disease is a major mortality factor in patients on dialysis and it is highly correlated with the degree of vascular calcification (Block and Cunningham, 2006). Disturbances in mineral metabolism are independent risk factors for morbidity and mortality in ESRD patients on dialysis (Block *et al.*, 2004a; Stevens *et al.*, 2004; Melamed *et al.*, 2006). Recognizing this seemingly fundamental relationship between abnormal mineral metabolism and clinical outcomes prompted the National Kidney Foundation/Kidney Disease Outcomes Initiative (NKF-K/DOQI™, 2003) to issue guidelines for circulating levels of PTH (150 to 300 pg/mL), serum calcium (8.4 to 9.5 mg/dL), serum phosphorous (3.5 to 5.5 mg/dL), and the calcium-phosphorous product (less than55 mg^2/dL^2). These target ranges for blood chemistries now define efficacy endpoints for the evaluation of new therapies for secondary HPT in patients with CKD.

The treatment of secondary HPT in CKD essentially involves replacing or removing those serum factors that the kidney can no longer maintain at normal levels. Thus, vitamin D sterols and oral phosphate binders have been the mainstays of medical management (Reichel, 2006; Brown and Slatopolsky, 2007; Torres *et al.*, 2006). Despite their use, it remains difficult to achieve the NKF-K/DOQI™ targets for blood parameters, especially for PTH (Moe and Drueke, 2003; Cunningham, 2004; Wood *et al.*, 2005; Craver *et al.*, 2007). The use of cinacalcet along with vitamin D sterols and phosphate binders increases the proportion of dialysis patients that achieve these targets. The clinical studies that demonstrate this will be discussed after first reviewing the preclinical studies in rodent models of CRI.

Preclinical Studies in Animal Models of Secondary Hyperparathyroidism

The 5/6 nephrectomized rat has been the predominant model used in studies of calcimimetics. This partial nephrectomy model can be combined with diets that differ in their calcium-to-phosphate ratios to produce a range of plasma PTH levels characteristic of mild, moderate, or severe forms of secondary HPT. With minor exceptions, the reported effects of NPS R-568 and cinacalcet on blood parameters and on soft and hard tissues are essentially identical.

Plasma PTH

The oral administration of NPS R-568 caused a dose-dependent decrease in plasma levels of PTH in 5/6 nephrectomized rats with secondary HPT. The magnitude and rate of change of plasma PTH levels following orally administered compound were similar in normal animals or those with secondary HPT (Fox *et al.*, 1999c). Moreover, animals with mild (normal to 150 pg/mL PTH), moderate (150 to 500 pg/mL) or severe secondary HPT (more than 500 pg/mL) all responded similarly to the intra-arterial injection of NPS R-568: the compound reduced plasma PTH levels by 82% to 94% within 20 min of dosing regardless of the initial plasma PTH level (Fox *et al.*, 1999c). Some of these animals had severe hyperphosphatemia and moderate hypocalcemia yet these two stimuli to PTH secretion could not prevent the depressive effect of NPS R-568 on plasma levels of PTH. Thus, activation of the calcium receptor with a calcimimetic markedly lowers circulating levels of PTH irrespective of the severity of the secondary HPT or the magnitude of the hyperphosphatemia. Efficacy independent of disease severity is also observed clinically with cinacalcet (see later).

Despite reports showing a decreased expression of parathyroid calcium receptor mRNA and protein in such animal models of secondary HPT (Mathias *et al.*, 1998; Brown *et al.*, 1999), there is apparently enough calcium receptor expressed to preserve responsivity to calcimimetic compounds. For example, the potencies of NPS R-568 for lowering plasma levels of PTH and Ca^{2+} or for increasing those of calcitonin are the same in normal animals and in those with CRI and moderate to severe secondary HPT (Fox *et al.*, 2000). As noted earlier, this could result if there were a large receptor reserve for the calcium receptor. Another explanation is the recent finding that the phenylalkylamine calcimimetics themselves increase expression of the calcium receptor. In partially nephrectomized rats with severe secondary HPT, daily treatment with NPS R-568 for a week was sufficient to restore normal levels of calcium receptor expression as assessed by mRNA levels and by immunohistochemical staining (Mizobuchi *et al.*, 2004).

The ability of calcimimetics to lower plasma levels of PTH might also involve an inhibitory effect on hormone synthesis. *In vitro* studies using bovine parathyroid cells showed that NPS R-568 also inhibited the synthesis of PTH (Garrett *et al.*, 1995c). As expected, the decreased levels of PTH mRNA were not apparent until 12 hours of exposure to the calcimimetic and reached a maximum within 24 hours. The inhibitory effects of NPS R-568 were stereoselective and they were blocked by the calcilytic compound NPS 89636 and therefore mediated by the calcium receptor. The inhibitory effect of NPS R-568 on the synthesis of PTH also occurs in a rat model of secondary HPT using a diet containing adenine and a high content of phosphorous (Levi *et al.*, 2006). In this animal model, HPT progresses from mild after 7 days to severe (more than 900 pg/mL) after several weeks on the diet. There were corresponding increases in PTH mRNA levels at these times that were totally abrogated at 7 days but only slightly affected after 21 days. It is currently uncertain to what extent the inhibitory effect on synthesis contributes to maintaining a lowered level of plasma PTH.

Bone Quality

Studies in rats with CRI have shown that the decreases in plasma levels of PTH caused by NPS R-568 have beneficial effects on some of the skeletal abnormalities that accompany secondary HPT (Wada *et al.*, 1998). Six weeks following a partial nephrectomy, animals had developed a mild to moderate secondary HPT with profound peritrabecular fibrosis; static and dynamic histomorphometry revealed a high-turnover bone lesion. The daily oral administration of NPS R-568 during the last five weeks completely prevented the development of osteitis fibrosa and tended to normalize histomorphometric parameters. Daily treatment with NPS R-568 additionally restored the decreases in volumetric cortical bone mineral density and in cortical bone stiffness at the femoral midshaft that was observed in vehicle-treated CRI animals. Similar improvements in bone quality were observed when rats were treated with cinacalcet (Wada *et al.*, 2003).

The results obtained by using rodent models of CRI-induced secondary HPT show that calcimimetics reduce plasma levels of PTH over prolonged periods of daily dosing and that there is no development of tolerance. Moreover, the daily cyclic decrease in circulating levels of PTH caused by treatment translates into positive effects on bone quality including the prevention or reversal of osteitis fibrosa.

Parathyroid Gland Hyperplasia

Parathyroid gland hyperplasia begins within days after a partial nephrectomy in the rat (Denda *et al.*, 1996; Wada and Nagano, 2003) and the initial studies examined the effects of NPS R-568 on parathyroid cell proliferation during the first five days of CRI in rats (Wada *et al.*, 1997).

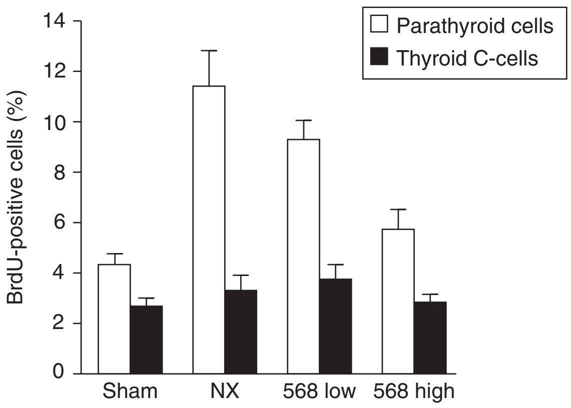

FIGURE 8 NPS R-568 blocks parathyroid cell proliferation in partially nephrectomized rats. NPS R-568 was administered orally twice a day at a dose of either 1.5 (568 low) or 15 mg/kg (568 high). (Fig. 4 in Wada *et al.*, 1997).

Bromodeoxyuridine (BrdU) was infused intraperitoneally throughout and NPS R-568 was administered orally twice a day during the last four days. As shown in Fig. 8, there was a 3-fold increase in cellular labeling with BrdU in partially nephrectomized animals which was blocked in a dose-dependent manner by treatment with NPS R-568. The inhibitory effect of NPS R-568 on BrdU labeling was specific for parathyroid cells and did not affect labeling of C cells, even at the high dose that stimulated calcitonin secretion. These findings suggest that the calcium receptor mediates the effects of hypocalcemia on parathyroid cell proliferation. If the glands responded to the prevailing low calcium levels by a mechanism other than the calcium receptor, then NPS R-568 would have been unlikely to prevent the proliferative response to partial nephrectomy. The mechanisms linking the calcium receptor to the regulation of cellular proliferation are present in parathyroid cells but not in C cells.

These initial findings prompted two long-term studies in rats with CRI. The first assessed whether initiating treatment with NPS R-568 soon after the development of CRI would prevent parathyroid gland hyperplasia (Wada *et al.*, 2000). CRI was induced by ligating the renal arteries and feeding the animals a diet high in phosphate content. Treatment with NPS R-568 was started 6 days after renal artery ligation and continued for 56 days. The vehicle-treated CRI animals developed severe secondary HPT with 10-fold elevations in circulating levels of PTH compared with sham-operated animals. Parathyroid gland volume was 2.8-fold higher in the former group of animals and this resulted mostly from an increase in cell number rather than cell volume. The enlargement and cellular hyperplasia of the parathyroid glands was completely prevented by treatment with NPS R-568.

The second study assessed whether treatment with NPS R-568 could halt the progression of parathyroid gland hyperplasia in animals with CRI (Chin *et al.*, 2000). Secondary HPT was allowed to develop for eleven weeks following a 5/6 nephrectomy before initiating treatment with NPS R-568 for eight weeks. At eleven weeks following surgery, parathyroid gland volume was 1.9-fold larger in CRI animals and the glandular volume continued to increase in these animals another 2-fold by the end of the study. Parathyroid gland enlargement was paralleled by a 5.4-fold increase in plasma levels of PTH in vehicle-treated CRI animals which continued to increase another 2.6-fold during the last eight weeks (Fig. 9). Secondary HPT continued to worsen during the course of this study as indicated by continually rising levels of circulating PTH and progressive enlargement of the parathyroid glands. Daily oral administration of NPS R-568 completely blocked the progression of secondary HPT as indicated by either parameter (see Fig. 9). Moreover, progressive increases in plasma PTH levels and parathyroid gland volume were completely prevented by NPS R-568 even when there was profound hypocalcemia, hyperphosphatemia, and lowered plasma levels of 1,25-dihydroxyvitamin D_3 (Wada *et al.*, 2000b; Chin *et al.*, 2000). Essentially the same results were obtained when cinacalcet was used and proliferating cell nuclear antigen staining was used to quantify cellular proliferation (Colloton *et al.*, 2005). Even in rats with severe secondary HPT, as indexed by plasma PTH levels greater than 3000 pg/mL and a 5-fold increase in parathyroid gland weight, daily treatment with a calcimimetic for just 7 days completely halted parathyroid cell proliferation and lowered plasma levels of PTH (Mizobuchi *et al.*, 2004).

Soft Tissue Calcification and Cardiovascular Function

The effects of calcimimetics on soft tissue and vascular calcification have been examined in two studies (Henley *et al.*, 2005; Lopez *et al.*, 2006). Both used 5/6 nephrectomized rats and included 1,25-dihydroxyvitamin D as a comparator and both began daily dosing one week after partial nephrectomy in a design that tested calcimimetics alone or in combination with 1,25-dihydroxyvitamin D. In many respects, the results of the two studies were similar. Daily treatment with NPS R-568 for 56 days or with cinacalcet for 26 days did not increase vascular calcification as indexed by von Kossa staining or aortic content of calcium and phosphorous. In contrast, 1,25-dihydroxyvitamin D-treated animals showed marked increases in both indices of vascular calcification. Curiously, and seemingly without explanation, the combination of NPS R-568 and 1,25-dihydroxyvitamin D, but not that with cinacalcet, abolished the effects on vascular calcification. Moreover, animals treated with 1,25-dihydroxyvitamin D suffered a very high mortality rate that was lowered by coadministration of NPS R-568 (Lopez *et al.*, 2006); this high rate of mortality was not observed in the shorter-term study using cinacalcet (Henley *et al.*, 2005).

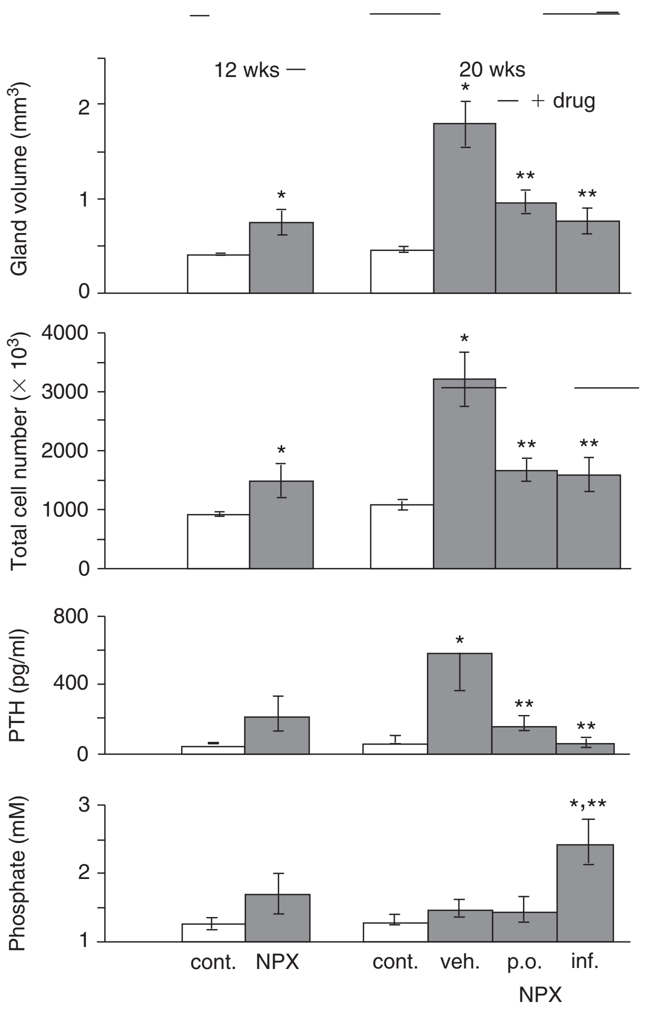

FIGURE 9 NPS R-568 blocks the progression of secondary hyperparathyroidism in CRI rats. NPS R-568 or vehicle was administered orally once a day (p.o.; 10 μmol/kg) or continuously infused (inf.; 20 μmol/kg/day) for 8 weeks beginning 11 weeks after a 5/6 nephrectomy. (Redrawn from data in Fox *et al.*, 2000, Chin *et al.*, 2000).

Cardiovascular and renal functions deteriorate in the partially nephrectomized rat model, as they tend to do in CKD patients whose mineral metabolism is not adequately controlled (Ritz *et al.*, 2005; Schmitt *et al.*, 2006). Using this rodent model, Ogata *et al.* (2003) showed improvements in a number of functional renal and cardiovascular readouts following daily administration of NPS R-568 for four or eights weeks. The rate of progression of renal failure, as assessed by serum creatinine or albumin excretion and by histological examination of the remnant kidney, was reduced by treatment with NPS R-568. Treatment with the calcimimetic also lowered systolic blood pressure and serum levels of high- and low-density lipoprotein and total cholesterol. Cardiac structure at the light microscopic levels was also improved. In general, the treatment effects of the calcimimetic were similar to those achieved by parathyroidectomy.

Beneficial effects of calcimimetics on renal functions have also been noted in other animal models. In a rat model of nephrocalcinosis, treatment with NPS R-467 ameliorated the furosemide-induced increase in kidney calcium content (Pattaragarn *et al.*, 2004). Cinacalcet has also been studied in genetically hypercalciuric rats (Bushinsky *et al.*, 2006). Treatment with cinacalcet did not further augment the hypercalciuria in these rodents.

Cyclic vs. Sustained Decreases in Circulating Levels of PTH

Inherent in the mechanism of action of calcimimetic compounds is their ability to alter plasma PTH levels in a manner that has not been possible previously. The only treatments that directly affect parathyroid cell function act on the synthesis, rather than the secretion of PTH (Naveh-Many and Silver, 1990). This is certainly true for the vitamin D sterols and, if phosphate acts directly on parathyroid cells, this might be how it acts as well. Because of this action, the suppressive effects of vitamin D sterols on plasma levels of PTH, when they do occur, are slow in onset and in recovery. In contrast, because it targets the calcium receptor, a single oral dose of a calcimimetic compound can lower plasma levels of PTH to a nadir within a couple of hours and levels can return to near baseline within 24 hours. Thus, a calcimimetic compound causes a daily cyclic, rather than sustained decrease in circulating levels of PTH (Fig. 10). The overall profile of the change in plasma PTH will vary depending on the dosing regimen and the pharmacokinetic properties of the particular calcimimetic compound being studied.

The catabolic effect of PTH on bone resulting from sustained elevations reverses to an anabolic effect when plasma levels of PTH increase intermittently. It has been proposed that cyclic decreases in abnormally raised levels of plasma PTH might have effects on bone quality similar to those achieved by daily cyclic increases of PTH above normal levels (Nemeth and Bennett, 1998). A treatment for secondary HPT that causes a cyclic decrease in circulating levels of PTH might achieve improvements in bone quality that are superior to those of a treatment that causes a sustained decrease in PTH. There is some evidence to support this hypothesis.

Using a partially nephrectomized rat model of uremia, Schmitt *et al.* (2000) compared the effects of the PTH peptide fragment 1−37 on longitudinal growth when administered intermittently by subcutaneous injection or continuously by infusion. The twice daily administration of PTH fragment 1−37, but not its continuous infusion, improved longitudinal growth in uremic animals.

The effects of cyclic or sustained decreases in plasma levels of endogenous PTH were studied in a rodent model of uremia produced by adriamycin. Rats treated with adriamycin develop glomerular sclerosis, which, after several months, results in a mild secondary HPT. Plasma levels of Ca^{2+} and phosphate are normal but vitamin D sterols are greatly reduced and there is massive proteinuria (Ishii *et al.*, 2000). The renal osteodystrophy in this model is characterized by a low-turnover bone lesion with osteopenia and osteomalacia. Circulating levels of PTH were decreased in a cyclic manner by the daily oral administration of NPS R-568 or they were continuously suppressed by the subcutaneous infusion of this compound. Oral administration, but not continuous infusion of NPS R-568, increased trabecular bone volume and bone mineral density (Fig. 11). Thus, cyclic decreases in plasma levels of PTH have markedly different effects on bone than do sustained decreases and, at least in this animal model of CRI, the daily cyclic decreases in plasma PTH levels had an "anabolic-like" effect on bone.

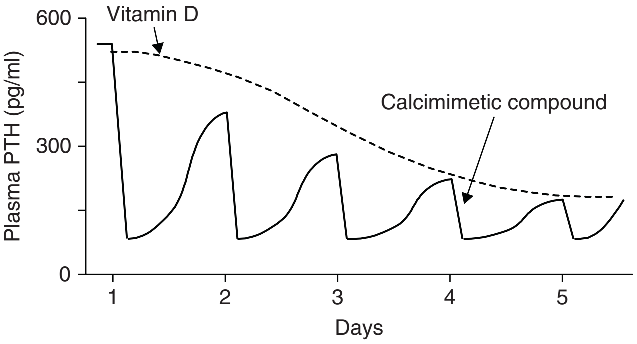

FIGURE 10 Contrasting effects of calcimimetic compounds and vitamin D sterols on the dynamics of changes in circulating levels of PTH. (modified Fig. 1 in Nemeth and Bennett, 1998).

Clinical Studies in Patients with Secondary Hyperparathyroidism

ESRD Patients on Dialysis

The initial clinical investigations of NPS R-568 in renal dialysis patients and the preliminary results obtained with cinacalcet in phase II studies were discussed in the second edition (Nemeth, 2002a). Full reports of these phase II trials have now been published (Goodman *et al.*, 2002; Lindberg *et al.*, 2003; Quarles *et al.*, 2003). Reviewed in this edition are the phase III studies (Block *et al.*, 2004b; Lindberg *et al.*, 2005; Moe *et al.*, 2005a,b) that that formed the basis for regulatory filing of cinacalcet to the Food and Drug Administration. With the exception of the dose-ranging study (Goodman *et al.*, 2002) all were randomized, placebo-controlled, double-blind trials that followed a similar design consisting of an initial titration phase followed by a maintenance phase. Patients on renal dialysis

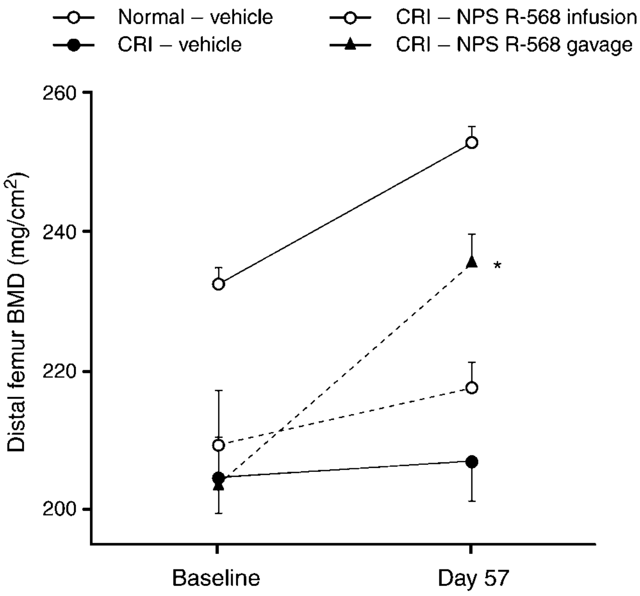

FIGURE 11 Bone mineral density in the distal femur is increased by daily oral administration of NPS R-568 (10 mg/kg) for 56 days in rats with CRI induced by adriamycin. In contrast, the continuous infusion of NPS R-568 (4.5 mg/kg/day) was without effect. (Fig. 3 in Ishii *et al.*, 2000).

with plasma PTH levels higher than 300 pg/mL and serum calcium levels greater than 8.4 or 8.8 mg/mL were eligible for enrollment. Other contemporary reviews of these trials have been published (Goodman, 2004, 2005; Szczech, 2004; Torres, 2004; Gal-Moscovici and Sprague, 2006).

The combined results from two identical trials conducted at 125 sites and involving 741 patients on hemodialysis were reported by Block *et al.* (2004b). There was a 12-week dose-titration phase and a 14-week efficacy-assessment phase and the primary endpoint was the percentage of patients who achieved PTH levels of 250 pg/mL. A 30-mg dose of cinacalcet was administered once daily and could be increased to 180 mg during the dose-titration phase. Treatment with cinacalcet for a total of 26 weeks significantly lowered plasma levels of PTH by 43% compared with baseline levels, whereas PTH levels in the placebo-treated group increased by 9%; 43% of the cinacalcet-treated patients reached the primary endpoint. Patients treated with cinacalcet had significantly lower levels of serum calcium (−6.8%) and phosphorous (−8.4%) compared with those treated with placebo. The calcium-phosphorous product decreased by 14% in the cinacalcet group but remained unchanged in the placebo group, and 89% of those who reached the primary endpoint had a reduction in the calcium-phosphorous product. The most common adverse events in patients treated with cinacalcet were nausea and vomiting and these occurred significantly more often than in those treated with placebo. The frequency of nausea was unrelated to the dose of cinacalcet whereas that of vomiting was. Transient episodes of hypocalcemia (less than 7.5 mg/mL) occurred significantly more often in cinacalcet-treated (5%) than in placebo-treated subjects (1%) but were rarely associated with symptoms and could be managed by adjusting the doses of calcium-containing phosphate binders and/or vitamin D sterols.

All the patients in the two trials reported by Block *et al.* (2004b) were on hemodialysis; a third phase III trial included patients on peritoneal dialysis (Lindberg *et al.*, 2005). The dose-titration phase was 16 weeks and the efficacy-assessment phase was 10 weeks and involved 395 patients of whom 46 were receiving peritoneal dialysis; the primary efficacy endpoint was the proportion of patients achieving a ≥30% reduction from baseline in plasma levels of PTH. The results were essentially the same as in the Block *et al.* (2004b) report. During the efficacy phase, 46% of the cinacalcet-treated patients achieved plasma levels of PTH = 300 pg/mL compared with 9% in the placebo-treated group. The proportion of patients with a ≥30% reduction from baseline in plasma PTH levels was 65% for patients treated with cinacalcet but only 13% for those treated with placebo. Serum levels of calcium and phosphorous decreased by 6.5% and 7.2%, respectively, and the calcium-phosphorous product decreased by nearly 13% compared with baseline in cinacalcet-treated subjects but remained unchanged in those treated with placebo. The results obtained in patients on peritoneal dialysis were similar to those on hemodialysis with 50% of the former achieving a plasma PTH level of 300 pg/mL and similar reductions in serum calcium and phosphorous levels and the calcium-phosphorous product. The most frequent adverse events were nausea and vomiting. Transient episodes of hypocalcemia occurred more frequently in cinacalcet-treated subjects but they were not associated with symptoms and could be readily managed by changing dialysate calcium concentration and/or comedications (Lindberg *et al.*, 2005).

The data from all the phase III studies were combined to assess the overall efficacy of cinacalcet relative to the guidelines established by the NKF-K/DOQI™ (Moe *et al.*, 2005a). At baseline, the median plasma PTH levels of cinacalcet-treated and control subjects were similar (596 and 564 pg/mL, respectively). After treatment for 6 months with cinacalcet, 56% of the subjects achieved mean plasma PTH levels at or below the upper limit of the NKF-K/DOQI™ target (less than 300 pg/mL) compared with 10% of control subjects (Fig. 12). Serum levels of calcium and phosphorous were within the NKF-K/DOQI™ target range in 49% and 46% of cinacalcet-treated subjects compared with 24% and 33%, respectively, in placebo-treated subjects. The target value for the calcium-phosphorous product was achieved in 65% of patients treated with cinacalcet compared with 36% of those treated with placebo (Fig. 13). All of these between-group differences were highly significant. The differences resulting from treatment with cinacalcet are larger than these comparisons suggest because about one-third of all subjects were already in the NKF-K/DOQI™ range for these serum parameters at baseline. Perhaps the most impressive index of efficacy is the percentage of patients achieving the targets for both serum PTH levels and calcium-phosphorous product, which none of the subjects met at baseline. After 6 months, this endpoint was achieved in 41% of cinacalcet-treated subjects but only 6% of placebo-treated subjects (Moe *et al.*, 2005a).

The long-term safety and efficacy of cinacalcet was assessed in a 2-year open-label extension study (Moe *et al.*, 2005b). Subjects who had completed one of the four phase II studies were enrolled and all (59 patients) were treated with cinacalcet. After 2 years of daily dosing with cinacalcet, 59% of subjects had plasma PTH levels of 300 pg/mL. At baseline, 39% of patients treated previously with cinacalcet and 19% of patients treated previously with placebo had plasma PTH levels of 300 pg/mL. After 2 years of treatment with cinacalcet, 66% of subjects had achieved a ≥30% reduction in plasma levels of PTH. The proportion of patients with serum levels of calcium and phosphorous and the calcium-phosphorous product within the NKF-K/DOQI™ target range did not change over 2 years when compared with baseline values at entry. Sixteen patients had participated in a 1-year phase II and therefore received cinacalcet daily for 3 years. After 3 years of treatment with cinacalcet, 70% of subjects achieved a ≥30% reduction in

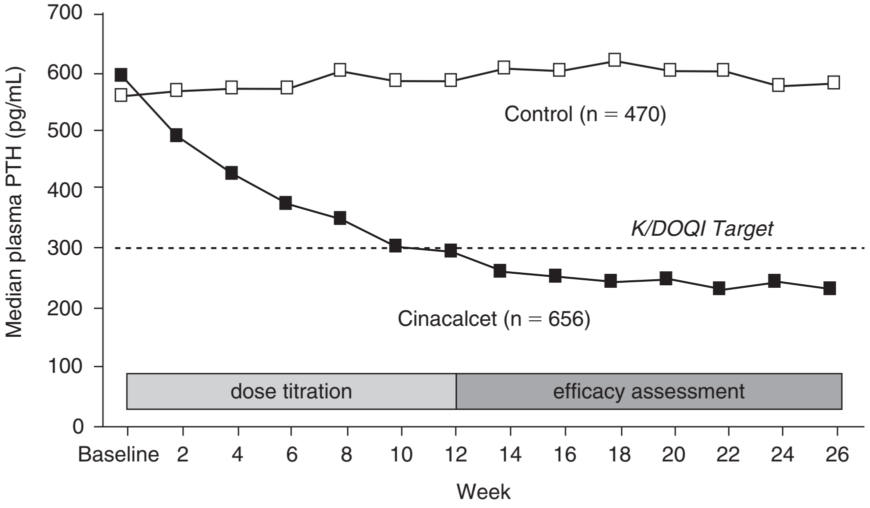

FIGURE 12 Once daily dosing with cinacalcet reduces plasma levels of PTH in patients with secondary HPT on dialysis. (Fig. 2A in Moe *et al.*, 2005).

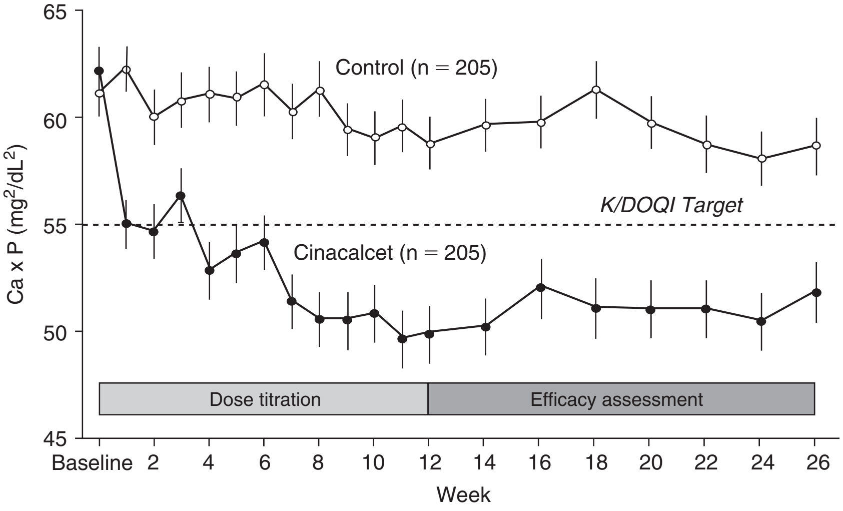

FIGURE 13 Once daily dosing with cinacalcet reduces the calcium-phosphorous product in patients with secondary HPT on dialysis. (Modified Fig. 2 in Moe *et al.*, 2005).

plasma levels of PTH. Adverse events were mild to moderate in severity and the most common were nausea and vomiting, both of which resolved spontaneously in about a third of the patients (Moe *et al.*, 2005b).

Clinical studies with cinacalcet were underway when it was realized that commercial assays for PTH were detecting some large C-terminal peptide fragments of PTH in addition to the intact hormone. This discovery led to the development of improved immunometric assays that detect solely the intact hormone (D'Amour, 2006). A head-to-head comparison of plasma PTH levels using a first-generation ("intact-PTH") and a second-generation immunometric assay ("bio-intact-PTH") was performed in 410 patients (Martin *et al.*, 2005). Treatment with cinacalcet for 26 weeks lowered plasma levels of PTH in the same percentage of subjects (56%) and by the same magnitude (38%) using either assay. In the control group, plasma PTH levels increased compared with baseline and the percent increase was significantly greater (23%) with a second-generation than with a first-generation assay. The bioactive PTH/intact PTH ratio remained constant at 56% throughout the study. Thus, the absolute level of plasma PTH is assay-dependent but the magnitude percent decrease from baseline and the proportion of patients responding to cinacalcet is independent of the assay used to measure PTH.

The collective results of all these prospective clinical trials are consistent and demonstrate the efficacy and safety of cinacalcet in the treatment of secondary HPT in patients in on dialysis. A meta-analysis of these studies confirms that treatment with cinacalcet improves biochemical parameters and the proportion of patients that achieve the NKF-K/DOQI™ targets (Strippoli *et al.*, 2006). Similar improvements were obtained in everyday practice by using a treatment algorithm that includes cinacalcet (Spiegel *et al.*, 2006).

Several points deserve mention when considering the efficacy of cinacalcet in the treatment of secondary HPT. First, in all of the studies with cinacalcet, plasma PTH levels were measured 24 hours after the last dose of drug but prior to the dose on the following day. Yet the maximal decrease in plasma levels of PTH occurs 2 to 4 hours after dosing with cinacalcet. Thus, the levels of PTH reported in all these studies are an underestimate of the average levels over a 24-hour period. A much higher percentage of patients would have achieved the primary endpoint if plasma PTH levels were measured at 2 to 4 hours after dosing. These studies thus provide a rather conservative estimate of efficacy. Second, most of the patients studied were already receiving medications to manage secondary HPT. For example, in the study of Block *et al.* (2004b), 67% of the total patient population was already taking vitamin D sterols and 93% were taking phosphate binders. So nearly all the patients' labeled "control" in Figures 12 and 13 were receiving what was then standard of care. The fact that all these patients had plasma PTH levels greater than 300 pg/mL highlights the difficulty of lowering circulating levels of PTH prior to the introduction of cinacalcet. Third, more than 20% of all patients studied had severe HPT (greater than 800 pg/mL) yet most of those treated with cinacalcet achieved the NKF-K/DOQI™ target for serum PTH or a 30% reduction from baseline levels. Thus, like the results obtained in rodent models, the degree of severity of secondary HPT does not impact the efficacy of calcimimetics in lowering circulating levels of PTH. In patients with secondary HPT, the efficacy of cinacalcet is the same regardless of the severity of the disease and the length of time on dialysis, at least within the first 6 months of treatment.

At present, there are only limited data describing the effect of cinacalcet treatment on skeletal parameters relevant to renal osteodystrophy. BMD of the proximal femur and the lumbar spine was assessed in a small number of patients on dialysis or with stage 4 chronic kidney disease (Lien *et al.*, 2005). Treatment of dialysis patients with cinacalcet for 26 weeks lowered plasma levels of PTH by 44% compared with baseline. Plasma PTH levels in predialysis patients were reduced by 73% following treatment with cinacalcet for 6 weeks. When analyzed collectively, treatment with cinacalcet significantly increased BMD and *T* score of the proximal femur but did not affect either parameter in the lumbar spine. There was a positive correlation between femur BMD and absolute decrease in plasma level of PTH. In the subset of dialysis patients, treatment with cinacalcet resulted in a 2.2% increase in proximal femur BMD, whereas BMD decreased by 1.9% in patients treated with placebo.

Bone biopsy samples were collected from 32 patients at baseline and after 1 year of treatment with cinacalcet (19 patients) or placebo (13 patients; Mallauche *et al.*, 2004). At baseline, 84% of patients in each group had high bone turnover as assessed by the numbers of osteoblasts and osteoclasts and the activation frequency. After 1 year of treatment with cinacalcet, there were reductions in activation frequency and bone cell numbers; these parameters were also reduced in the placebo group but to a lesser extent. Treatment with cinacalcet did improve marrow fibrosis compared with the placebo-treated group. Three patients treated with cinacalcet developed adynamic bone disease but in two of these, plasma levels of PTH were suppressed below 100 pg/mL. It is difficult to draw any conclusions from the limited data available but there are some trends toward improvements in skeletal parameters following treatment with cinacalcet.

The ability of cinacalcet to lower plasma levels of PTH and the calcium-phosphorous product, and the association of these blood biochemistries with vascular calcification and renal osteodystrophy, offers some reason to suppose that the drug will reduce morbidity and mortality in patients with secondary HPT. The cumulative safety data from all the trials with cinacalcet support this. The combined data, based on 1184 subjects (697 cinacalcet-treated subjects) showed that randomization to cinacalcet significantly reduced the relative risk of parathyroidectomy, fracture, and cardiovascular hospitalization compared with placebo (Cunningham *et al.*, 2005). There were also some significant improvements in quality of life, such as self-reported physical function and diminished bodily pain.

Pre-dialysis Patients

The results of one prospective study in patients with chronic kidney disease (CKD) and secondary HPT but not yet on dialysis have been reported (Charytan *et al.*, 2005). This randomized, double-blind, placebo-controlled study assessed the safety and efficacy of cinacalcet in 54 subjects with secondary HPT as indicated by plasma levels of PTH greater than 130 pg/mL. Cinacalcet was administered daily during a 12-week dose-titration phase followed by a 6-week phase to determine efficacy. The primary endpoint was again a 30% fall in plasma levels of PTH when measured 24 hours after the last dose. The baseline level of plasma PTH was 240 pg/mL and 28% and 43% of the patients were on vitamin D sterols and phosphate binders, respectively. In cinacalcet-treated subjects, plasma levels of PTH fell by 33% after 2 weeks and remained at 30% to 40% of baseline throughout the study, whereas patients receiving placebo maintained PTH levels at about baseline. Most patients treated with cinacalcet (56%) reached the primary endpoint. There was a decrease in serum levels of calcium and an increase in the serum levels of phosphorous that occurred within one week and persisted throughout the study. Twenty-four hour urinary calcium increased whereas that of phosphorous decreased. All these changes in serum and urine parameters might be explained by a decrease in plasma levels of PTH in the setting of a mostly functional kidney, but an action of cinacalcet on renal calcium receptors cannot be excluded.

Renal Transplant Patients

Kidney transplant in ESRD patients is typically successful and over time the secondary HPT spontaneously resolves. There remain, however, a variable percentage of patients (anywhere from 2% to 20%) that present with persistent HPT after kidney transplantation (Lewin and Olgaard, 2006). These patients are logical candidates for treatment with cinacalcet. Several independent studies, each comprising a small number of transplant patients, have been reported (Kruse *et al.*, 2005; Serra *et al.*, 2005; Apostolou *et al.*, 2006a, b; Leca *et al.*, 2006; Srinivas *et al.*, 2006; Szwarc *et al.*, 2006; Dorsch, 2007; El-Amm *et al.*, 2007). A total of 83 renal allograft recipients with persistent HPT have been treated with cinacalcet daily for 10 weeks to 6 months. Treatment with cinacalcet lowered serum calcium levels in every patient studied and returned into the normal range in most patients within 2 weeks. All but two reports (Kruse *et al.*, 2005; Srinivas et al, 2006) observed a fall in plasma levels of PTH after treatment with cinacalcet. Serum phosphorous levels did not change in five of the studies and slightly increased in the other three. Overall, the results of these small observational studies suggest that cinacalcet might be useful in managing persistent secondary HPT following renal transplantation. Appropriately designed clinical trials are required to establish the efficacy and safety of cinacalcet in this patient population.

Calciphylaxis

This rare but serious condition is characterized by cutaneous ischemia and necrosis, vascular calcification, and refractory infections; it is typically fatal (Wilmer and Magro, 2002). There have been two case reports of renal dialysis patients who developed calciphylaxis and were successfully treated with cinacalcet (Velasco *et al.*, 2006; Robinson *et al.*, 2007). In both instances, treatment with cinacalcet lowered plasma levels of PTH and normalized serum levels of calcium and phosphorous; skin ulcerations healed following several months of treatment.

Cinacalcet Indications

Cinacalcet•HCl is marketed by Amgen as Sensipar® in North America and Mimpara® in the European Union. Sensipar® is approved for the treatment of secondary HPT in patients with CKD on dialysis. It is also approved for the treatment of hypercalcemia in patients with parathyroid carcinoma. At present, it is approved for the treatment of other forms of primary HPT only in Switzerland. Cinacalcet has recently been approved in Japan.

OSTEOPOROSIS

PTH increases bone turnover and the resulting overall effect on the skeleton is highly dependent on the temporal changes in the circulating levels of PTH. Thus, sustained elevations in plasma PTH levels, such as occur in HPT, lead to increased bone resorption and a net decrease in bone mass, especially at cortical sites. In contrast, temporary increases in plasma levels of PTH achieved by daily (or near daily) injection, stimulate new bone formation in animal models of osteopenia (Dempster *et al.*, 1993) and in postmenopausal osteoporotic women (Rittmaster *et al.*, 2000). The results of these studies using intermittent administration demonstrate that PTH is an effective anabolic agent.

The effects of parathyroid hormone (PTH) on the skeleton are mediated by a G protein-coupled receptor, the PTH-R1. This receptor is also activated by N-terminal-intact peptide fragments of PTH or parathyroid hormone-related peptide (PTHrP) and such peptide fragments likewise stimulate new bone formation. The 1–34 fragment of PTH, teriparatide (Forteo®), is the first anabolic therapy for osteoporosis to reach the market; the intact hormone (Preotact®) is approved for use in Europe. Other anabolic peptides in clinical development are PTHrP(1–36) and the cyclic peptide analog Ostabolin C™ (Fox, 2002). However, the therapeutic use of all these peptides is compromised by the need for systemic administration of a costly biological product. Calcilytic compounds might provide an alternative approach to systemic administration of PTH peptides by increasing the circulating levels of endogenous PTH (Nemeth, 2002c).

Preclinical studies using the calcilytic compound NPS 2143 in ovariectomized (OVX) rat models of osteopenia have generated results consistent with this hypothesis and were discussed in the second edition (Nemeth, 2002a). Daily oral administration of NPS 2143 for five weeks increased circulating levels of PTH and increased bone turnover in osteopenic OVX rats (Gowen *et al.*, 2000). When administered together with 17β-estradiol, this compound increased new bone formation in the proximal tibial metaphysis and increased bone mass in the distal femur. An anabolic effect of NPS 2143, however, required coadministration of 17β-estradiol and it did not increase (or decrease) BMD when administered alone; although it stimulated new bone formation, it also stimulated bone resorption so no net gain in BMD was achieved. The failure to achieve a net anabolic effect with NPS 2143 probably results from the pharmacokinetic profile of this compound: although orally active, it has a very long plasma $t_{1/2}$ and causes sustained increases in circulating levels of PTH. Despite the long plasma $t_{1/2}$, daily dosing of OVX rats with NPS 2143 for five weeks did not increase parathyroid cell proliferation, either by itself or when administered together with estradiol (Gowen *et al.*, 2000). These initial studies provided proof of concept that an antagonist of the calcium receptor could increase circulating endogenous PTH to levels sufficient to stimulate new bone formation and do so without causing parathyroid gland hyperplasia.

To be useful therapeutically, a calcilytic compound must possess some challenging pharmacokinetic properties. The compound must be orally bioavailable and it must rapidly reach a C_{max} yet have a short plasma $t_{1/2}$; there can be no active metabolites that linger in the circulation. These are the pharmacokinetic properties that will facilitate patient compliance and achieve a rapid and transient increase in plasma levels of PTH. Calcilytic compounds with at least some of these properties have now been obtained.

Compound 1 (see Fig. 5) has improved pharmacokinetic properties when compared with NPS 2143. Compound 1 inhibits extracellular Ca^{2+}-induced increases in $[Ca^{2+}]_i$ and inositol phosphates in HEK 293 cells expressing the human calcium receptor with potencies of 64 and 229 n*M*, respectively (Arey *et al.*, 2005). It is orally bioavailable and reaches a C_{max} of about 2 μ*M* at 1 hour following a single oral dose (30 mg/kg). At this dose, there was a 2- to 3-fold increase in plasma levels of PTH that coincided with the C_{max} of the compound and returned to baseline levels within 3 hours. The effects of compound 1 in an animal model of osteoporosis have yet to be reported but the magnitude and time course of change in plasma levels of PTH are appropriate to increase net bone formation.

Structural modifications to NPS 2143 have yielded several analogues with improved pharmacokinetic profiles, similar to those of compound 1. One of these analogues has been studied in the OVX rat model. Aged female rats were OVX and osteopenia was allowed to develop for 6 weeks before treatment. Daily oral dosing with the calcilytic was for 12 weeks either alone or together with estradiol. The single oral administration of the calcilytic caused a 2- to 3-fold increase in plasma PTH levels that peaked within 20 minutes and returned to baseline levels by 2 hours. After 3 months of daily dosing, there was an increase in bone formation rate at the lumbar spine. Treatment with estradiol alone did not increase bone formation rate nor did it diminish the stimulatory effect of the calcilytic. Significantly, treatment with the calcilytic alone increased BMD at the lumbar spine and increased vertebral bone strength as assessed by compression force. These findings in the OVX rat model provide proof of concept that a calcilytic compound can, by itself, increase BMD when orally administered daily.

In postmenopausal women, the oral administration of another amino alcohol chemotype, SB-751689 (see Fig. 5), caused a dose-dependent, rapid and transient increase in serum levels of PTH. After 28 days, there were increases in serum levels of the bone formation markers procollagen type I amino-terminal peptide, BSAP, and osteocalcin that were similar in magnitude to those produced by 28 days of daily dosing with teriparatide or PTH. Serum levels of the bone resorption marker C-terminal telopeptide of type I collagen were not different from baseline following one month of daily dosing with the calcilytic. A phase II clinical trial of SB-751689 in postmenopausal osteoporotic women has recently been initiated.

In the aggregate, the preclinical and clinical data suggest that enough PTH can be released from the parathyroid glands by a calcilytic compound to stimulate new bone formation. Calcilytics therefore have the potential to become an orally active, cost-effective anabolic therapy for osteoporosis.

CONCLUSION

It has always been difficult to separate the effects of calcium, phosphorous, and vitamin D on parathyroid gland function because their homeostatic mechanisms are so interwoven. Calcimimetic and calcilytic compounds permit the study of parathyroid physiology without changing the level of any of these serum factors and allow circulating levels of endogenous PTH to be altered at will. The use of these compounds has shown that the calcium receptor regulates a number of distinct parathyroid cell functions beyond secretion and including the synthesis of PTH and parathyroid cell proliferation. All of these functions were either known or suspected to be regulated by the calcium receptor, and altering its activity pharmacologically reinforces or solidifies our understanding of parathyroid physiology. What calcimimetics and calcilytics reveal uniquely is the principal role of the calcium receptor in parathyroid physiology—cellular functions coupled to this receptor will dominate those mediated by phosphate and/or vitamin D.

The most significant use of calcimimetics and calcilytics, of course, is therapeutically. Cinacalcet is a welcome addition to the armamentarium used to manage secondary HPT in patients on dialysis. Dialysis patients treated with cinacalcet can achieve NKF-K/DOQI™ targets even when other therapies fail. Cinacalcet is also the only medical treatment for parathyroid cancer. Yet the drug is not without its limitations and it has yet to be approved for treating patients not on dialysis or those with primary HPT not owing to carcinoma. What might be needed in these patient populations is a calcimimetic that maximally activates calcium receptors on parathyroid cells without activating those in the kidney and gastrointestinal tract. Cinacalcet does selectively target parathyroid cells when compared with C cells, but its relative potency on calcium receptors in the parathyroid gland, the kidney, and the gut is still unknown. Nonetheless, cinacalcet can still claim several “firsts.” It is the first drug for parathyroid carcinoma. It is also the first drug to act on the calcium receptor. Significantly, cinacalcet is the first drug to act allosterically on any G protein-coupled receptor. As such, it defines an entirely new class of pharmaceutical for this large family of drug targets.

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Chapter 81

Bisphosphonates

Mechanisms of Action

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We dedicate this chapter to the memories of Herbert Fleisch and Gideon Rodan. Herbie and Gideon played major roles in the development of the bisphosphonates, and were authors of the chapter that appeared in earlier versions of this book. They are both sadly missed by their many friends and colleagues, and we hope they would have been pleased to see the continued progress being made in the field they helped to create.

INTRODUCTION

The bisphosphonates are a class of drugs developed since the late 1960s for use in various diseases of calcium metabolism. Known to chemists since the mid-nineteenth century, they were first synthesized in Germany (Menschutkin, 1865) and were used mainly in the textile, fertilizer, and oil industries to prevent scaling because of their property to inhibit calcium carbonate precipitation (Blomen, 1995). This chapter will cover the history of the development, chemistry, biological actions, and molecular mechanisms of action of bisphosphonates. Emphasis will be given to newer developments, but we will not deal with details of clinical efficacy and therapeutics. The reader is encouraged to consult other recent reviews for additional information (Rogers, 2004; Roelofs *et al.*, 2006a; Coxon *et al.*, 2006; Kimmel, 2007; Russell *et al.*, 2007, 2008).

HISTORY OF BISPHOSPHONATE DEVELOPMENT FOR USE IN BONE DISEASES

Our knowledge of the biological characteristics of bisphosphonates dates back about 40 years, the first report appearing in 1968 (Fleisch *et al.*, 1968). The concept of using bisphosphonates was derived from earlier studies on inorganic pyrophosphate (PPi), which was found to prevent both the formation (Fleisch and Neuman, 1961) and dissolution of calcium phosphate *in vitro* (Fleisch *et al.*, 1966). Because pyrophosphate was subsequently shown to prevent ectopic calcification *in vivo* and to be present in urine and plasma (Fleisch and Bisaz, 1962), it was suggested that PPi was a physiological regulator of calcification and perhaps also of decalcification *in vivo* (Fleisch *et al.*, 1966). Owing to its rapid hydrolysis, pyrophosphate found therapeutic use only in scintigraphy and in toothpaste, added to prevent dental calculus. This prompted the search for analogues with similar physicochemical properties, but that resisted enzymatic hydrolysis and metabolism. The bisphosphonates fulfilled these criteria.

CHEMISTRY AND GENERAL CHARACTERISTICS

Bisphosphonates are compounds characterized by two C–P bonds. When the two C–P bonds share a single carbon atom (P–C–P), they are deemed to be analogues of pyrophosphate (P–O–P) and are called geminal bisphosphonates (so-called because the carbon is at the central or geminal position) (Fig. 1). The P–C–P bonds of the geminal bisphosphonate are stable to heat and most chemical

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reagents and are completely resistant to enzymatic hydrolysis. Only geminal bisphosphonates seem to have a strong activity on the skeleton. For the sake of simplicity they are generally called bisphosphonates.

A key pharmacological feature of bisphosphonates is their ability, like pyrophosphate, to bind to bone mineral. Binding of bisphosphonates to bone mineral can be bidentate through the two phosphonates, as is the case for clodronate, or it can be tridentate (Barnett and Strickland, 1979) through a third moiety, such as a hydroxyl or a nitrogen attached to the carbon atom, as is the case for most of the bisphosphonates in current clinical use. Tridentate binding increases the affinity for mineral.

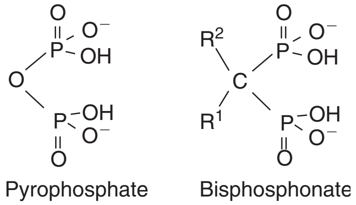
Pyrophosphate
Bisphosphonate

FIGURE 1 The structure of pyrophosphate and a geminal bisphosphonate.

The P–C–P structure allows a great number of possible variations by changing the two side chains on the carbon atom (Fig. 2). Many bisphosphonates have been studied in animals. It is becoming more apparent that each bisphosphonate has its own physicochemical and biological profile, which implies that one cannot automatically extrapolate the findings from one compound to another, with respect to its actions.

PHARMACOKINETICS

Bisphosphonates are synthetic compounds, not found to occur naturally in animals or humans. No enzymes capable of cleaving the P–C–P bond have been discovered. The bisphosphonates studied and reported so far, including alendronate, clodronate, etidronate, pamidronate, and several others, appear to be absorbed, stored, and excreted from the body unaltered, except for the incorporation of

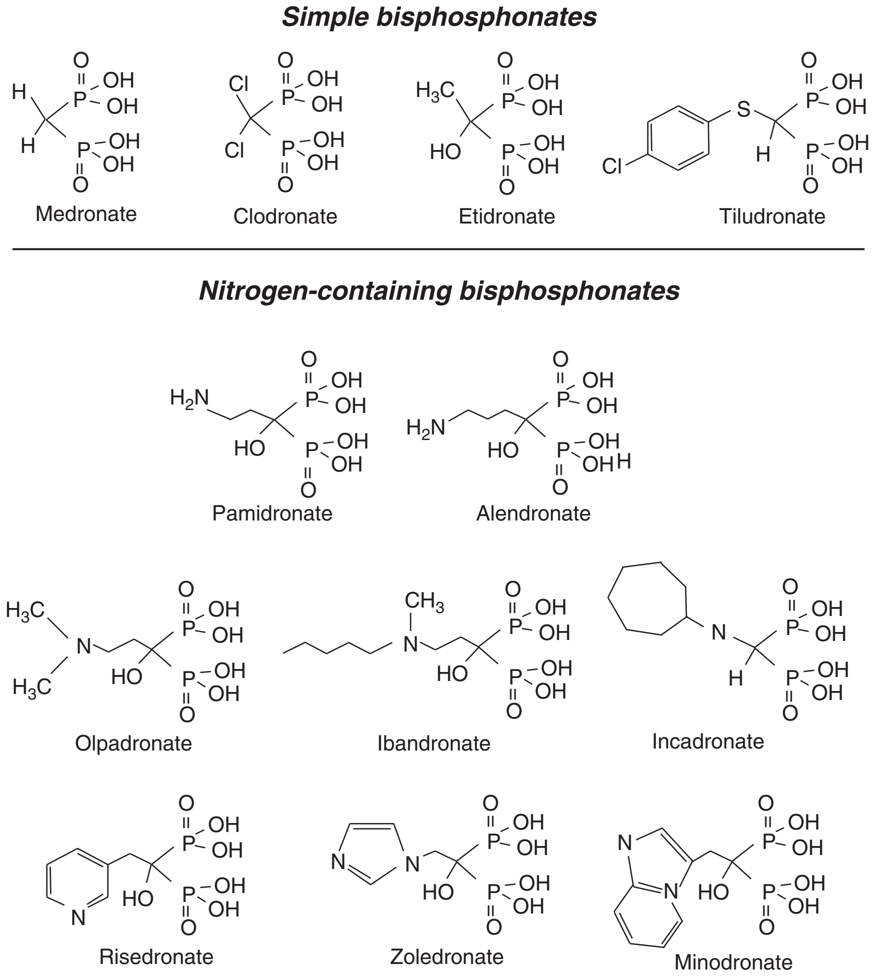
Simple bisphosphonates
Medronate
Clodronate
Etidronate
Tiludronate
Nitrogen-containing bisphosphonates
Pamidronate
Alendronate
Olpadronate
Ibandronate
Incadronate
Risedronate
Zoledronate
Minodronate

FIGURE 2 Structures of bisphosphonates (shown as the protonated, bisphosphonic acid forms).

etidronate, clodronate, and tiludronate into ATP inside the osteoclast, described later (for reviews, see Papapoulos, 1995; Lin, 1996; Porras *et al.*, 1999).

Intestinal Absorption

The oral bioavailability both in animals and in humans is low, probably because of low lipophilicity and high negative charge. It ranges from less than 1% to 10% of an oral dose, is generally higher in the young, sometimes higher at higher doses, and shows some inter- and intraspecies variation (Michael *et al.*, 1972; Recker and Saville, 1973; Yakatan *et al.*, 1982). Absorption occurs primarily in the small intestine and appears to occur by passive diffusion, possibly through a paracellular pathway (Boulenc *et al.*, 1993). It is diminished when the drug is given with meals, in the presence of calcium, and, interestingly, also even when given with beverages such as coffee, tea, or orange juice (Gertz *et al.*, 1995).

Circulating Half-Life

The half-life of circulating bisphosphonates is short, in the order of minutes in the rat (Bisaz *et al.*, 1978) and approximately 0.5 to 2 hours in humans. While in the circulation, variable amounts of different bisphosphonates are ultrafiltrable. The values are strongly species dependent. The remainder is either bound to proteins, especially albumin (Lin *et al.*, 1994), or present in very small aggregates (Wiedmer *et al.*, 1983).

Renal Clearance

Between 30% and 70% of absorbed bisphosphonates are taken up by bone, the remainder being rapidly excreted in the urine (Michael *et al.*, 1972; Yakatan *et al.*, 1982). The renal clearance of bisphosphonates is high. When taking into account that they are only partially ultrafiltrable, the renal clearance can exceed glomerular filtration rate, suggesting that renal secretion can occur (Troehler *et al.*, 1975; Lin *et al.*, 1992). Urinary excretion is decreased in renal failure and their removal by peritoneal dialysis is poor, which should be taken into account when the compounds are administered to patients with renal insufficiency.

Uptake and Retention in the Skeleton

The rate of uptake by bone is very fast, bone clearance being compatible with complete first-passage extraction from the circulation by the skeleton (Bisaz *et al.*, 1978). Skeletal uptake might therefore be determined to a large extent by bone vascularization. Soft tissues are thus exposed to these compounds for short periods only, which explains their bone-specific effects.

The uptake of bisphosphonate by the skeleton varies with species, gender, and age and can differ among compounds. Sometimes bisphosphonates can deposit in other organs such as the stomach, liver, and spleen. Some of this extraosseous deposition appears to be caused by the formation of complexes, occurring when large amounts are infused rapidly. If given at therapeutic doses and infused slowly, extraosseous deposition seems to be negligible.

When bisphosphonates are given at clinical doses to humans, there seems to be no saturation of the total skeletal uptake, at least not within periods as long as years or decades. In contrast, the antiresorbing activity rapidly reaches a maximum value, both in animals (Reitsma *et al.*, 1980) and in humans (Garnero *et al.*, 1994). The fact that a plateau of activity is reached, despite continuous bisphosphonate accumulation in the skeleton, is consistent with entombment of the compound, where it is inactive because it is not accessible to the osteoclasts on the bone surface.

The bisphosphonates are probably liberated from the skeleton by both passive and active processes; release by physicochemical mechanisms, such as desorption, outward diffusion, and ion exchange, may occur to a lesser extent than release when the bone in which they are deposited is resorbed (Kasting and Francis, 1992). The notion of skeletal retention being described in terms of "half-life" of retention in the body has led to some confusion, because calculated half-lives depend on the experimental design and duration of studies, in addition to any inherent differences between compounds (Russell *et al.*, 2008). Skeletal retention also depends on the rate of bone turnover, which, in turn, is influenced by bisphosphonates themselves. The drugs can therefore prolong their own survival in the skeleton. Retention times and terminal half-lives have been estimated to be up to 1 year in mice or rats (Mönkkönen, *et al.*, 1990), and even longer, up to 10 years, in humans (Kasting and Francis, 1992; Lin 1996). It is possible that some of the administered bisphosphonate can stay buried in the skeleton for life, although in inactive form. This is also true for other "bone-seeking" substances such as tetracyclines, heavy metals, and fluoride.

BINDING AFFINITIES FOR BONE

Recent studies have measured and compared the relative affinities of various clinically used bisphosphonates for hydroxyapatite or human bone (Nancollas *et al.*, 2006; Leu *et al.*, 2006).

Binding to Hydroxyapatite

The binding of bisphosphonates to human bone restricts their tissue distribution almost entirely to the skeleton. In doing so, the molecular mechanisms of action (discussed later) are limited to the bone microenvironment. Hydroxyapatite in pure form is related to hydroxyapatite in bone, although it lacks both the carbonate content and

TABLE I Binding affinities of BPs for natural bone and hydroxyapatite

Bisphosphonate	Relative affinity for natural bone (μM)*	Estimated affinity for hydroxyapatite (μM)†
Clodronate	806	1.39
Etidronate	90.7	0.84
Risedronate	84.6	0.46
Ibandronate	116	0.42
Alendronate	60.9	0.34
Zoledronate	80.8	0.29
Pamidronate	82.7	nd
Tiludronate	173	nd

*Based on Leu et al., 2006.

†Based on Nancollas et al., 2006

associated protein components. Binding affinities of bisphosphonates for the pure chemical components of hydroxyapatite have been calculated through constant composition kinetic studies of crystal growth (Nancollas *et al.*, 2006). These studies suggest a narrow range for binding affinities (0.3 to 1.4 μM, as shown in Table I). Notably and curiously, the affinities in these studies are approximately two orders of magnitude greater than have been reported for human bone (see later). The ranking order for affinity of the various bisphosphonates for hydroxyapatite showed clodronate to bind with weakest affinity, whereas risedronate and ibandronate fell into the middle of the range, and alendronate and zoledronate bound with highest affinity.

Binding to Human Bone

Binding constants representing bisphosphonate affinity for human bone have been calculated by using several different methodologies. The direct binding affinity of alendronate for human bone was measured by Scatchard analysis, with a measured K_d of 110 μM (Leu *et al.*, 2006). The difference with the reported affinity for pure hydroxyapatite (0.34 μM) is potentially attributable to differences in methodology and/or the composition of the binding surface (hydroxyapatite versus bone). For example, a more recent study demonstrated that the more soluble carbonated apatite gave a similar rank order of affinities, but with somewhat less variation (Henneman *et al.*, 2008). Independent measurements of the on-rate (69/M/min) and the off-rate (0.033/min) for binding to human bone yielded a K_d of 470 μM, which was about 4-fold higher than the observed K_d of 110 μM and about 1000-fold higher than that seen with hydroxyapatite. The measurement of an off-rate for alendronate demonstrated that it does not irreversibly bind to the bone surface. Once released, the bisphosphonate can engage in any of three activities: (1) reattachment, (2) redistribution to other sites on bone, or (3) excretion in the urine. The ability of bisphosphonate to rebind to newly resorbed surfaces during osteoclastic bone resorption has recently been visualized *in vitro* by using fluorescently labeled alendronate (Coxon *et al.*, 2008). This suggests that the long relative retention of bisphosphonates on bone is (in part) attributable to their ability to rebind after being released. Thus, bone affinity may influence not only the initial distribution to bone, but also the redistribution within the skeleton.

The affinity of different bisphosphonates for bone varies based on the chemical groups at the R^1 and R^2 positions. In an effort to make comparisons between the various bisphosphonates, competition binding assays were used to assess relative affinity for human bone (Leu *et al.*, 2006). In these studies, the ability of nonradiolabeled bisphosphonates to compete with radiolabeled alendronate for binding sites was used as a measure of affinity. This showed that bisphosphonates bearing a hydroxyl (OH) at R^1 (see Table I) bind in a relatively narrow (60 to 120 μM) range. Etidronate, alendronate, ibandronate, pamidronate, risedronate, and zoledronate thus all bound within an approximate twofold affinity range. Others have measured similar affinities toward mouse long bones (Van Beek *et al.*, 1994, 1996, 1998). Tiludronate and clodronate, both of which lack an R^1 hydroxyl group, displayed significantly weaker binding affinities to human bone (about 170 μM and about 800 μM, respectively). An additional study on the relative binding of bisphosphonates to human bone was recently reported (Mukherjee *et al.*, 2008). In this study, binding affinity was compared by utilizing nuclear magnetic resonance spectroscopy where binding was found to be well-described by Langmuir-like isotherms. Pamidronate, zoledronate, and alendronate were found to bind more strongly than risedronate and compelling arguments were made that this difference was strongly influenced by the different charge states at the nitrogen moiety at physiological pH.

Clinical Relevance of Differences in Affinity for Bone

The clinical relevance of differences in bone affinity between bisphosphonates continues to be debated (Leu *et al.*, 2006; Nancollas *et al.*, 2006; Papapoulos, 2006; Russell *et al.*, 2008), because variations in binding affinity for bone may have an impact on the speed of onset and duration of action on the skeleton (reviewed by Russell *et al.*, 2008).

One head-to-head clinical study investigated the uptake and release of a single dose of ^{14}C-labeled risedronate and alendronate during ongoing administration of weekly doses of the corresponding drugs (Christiansen *et al.*, 2003). After 72 hours, a significantly greater percentage (approximately 20%) of the ^{14}C-labeled alendronate was retained versus risedronate as measured by urinary excretion, consistent with the *in vitro* affinity studies described earlier. However, even the relatively lower-affinity bisphosphonates such as risedronate (as measured in some of these models) have demonstrated extended BMD maintenance and antifracture protection for up to a year despite a resolution of turnover markers within that time frame. This indicates that the primary determinant of antiresorptive potency for the bisphosphonates is more likely to be the biochemical and cellular effects of bisphosphonates on bone cells, as illustrated by correlations between potency for effects at the molecular/cellular level and antiresorptive potency in animal models (Dunford *et al.*, 2001; Lundy *et al.*, 2007). These cellular and molecular mechanisms of action of the bisphosphonates will be discussed in the following sections.

PHYSICOCHEMICAL EFFECTS

As anticipated, the physicochemical effects of most bisphosphonates are very similar to those of pyrophosphate. Thus, most inhibit the formation and aggregation of calcium phosphate crystals (Francis, 1969; Francis *et al.*, 1969; Fleisch *et al.*, 1970) and slow down their dissolution (Fleisch *et al.*, 1969; Russell *et al.*, 1970). These effects are related to the marked affinity of these compounds for the surface of solid-phase calcium phosphate (Jung *et al.*, 1973), where they interfere with both crystal growth and dissolution.

Like pyrophosphate, bisphosphonates can inhibit calcification *in vivo*. Thus, they prevent experimentally induced calcification of many soft tissues when given parenterally or orally (Fleisch *et al.*, 1970), as well as ectopic ossification. Topical administration leads to a decreased formation of dental calculus (Briner *et al.* 1971).

If administered in sufficiently high doses, bisphosphonates can also impair the mineralization of normal calcified tissues such as bone, cartilage, dentine, and enamel (King *et al.*, 1971; Schenk *et al.*, 1973). The amount required to produce these effects varies somewhat according to the bisphosphonate used, the animal species, and the length of treatment. However, in contrast to bone resorption where the different compounds vary greatly in their activity, they inhibit mineralization at similar doses. For most species that dose is in the order of 1 to 10 mg of compound phosphorus per kilogram per day parenterally. There is a close relationship between the ability of a bisphosphonate to inhibit calcium phosphate precipitation *in vitro* and its effectiveness at inhibiting calcification *in vivo* (Fleisch *et al.*, 1970; Trechsel *et al.*, 1977; van Beek *et al.*, 1994). Therefore, the mechanism for this effect is likely to be a physicochemical one. It is possible that other cellular effects, for example, changes in the bone matrix, are involved, but this remains unproven. The inhibition of mineralization can lead to fractures (Flora *et al.*, 1980) and to impaired healing of the fractures (Fleisch, 2001). Although the inhibition is eventually reversed after discontinuation of the drug, the propensity to inhibit the calcification of normal bone has hampered the therapeutic use of bisphosphonates for ectopic calcification. However, this is not the case for their use to inhibit bone resorption, because several bisphosphonates can inhibit resorption at doses 1000 times lower than those inhibiting mineralization. The doses used to inhibit resorption do not impair fracture healing either, when assessed by estimating biomechanical properties (Fleisch, 2001). Indeed, bisphosphonates may actually enhance fracture repair by stabilizing the fracture callus (Little *et al.*, 2005).

Although the ability of bisphosphonates to prevent bone loss was initially ascribed to their physicochemical properties, it has become clear over the years that bisphosphonates act by inhibiting osteoclastic bone resorption.

ANTIRESORPTIVE EFFECTS IN PRECLINICAL MODELS

Bisphosphonates have been shown to be very powerful inhibitors of bone resorption when tested in a variety of preclinical *in vitro* and *in vivo* models.

In vitro

Bisphosphonates block bone resorption induced by various means in organ and cell culture. They decrease the destruction of bone in embryonic long bones and in neonatal calvaria (Fleisch *et al.*, 1969; Russell *et al.*, 1970; Reynolds *et al.*, 1972). In cell culture, the bisphosphonates inhibit the formation of pits by isolated osteoclasts cultured on mineralized substrata (Flanagan and Chambers, 1989; Sato and Grasser, 1990). With few exceptions, the correlation between bisphosphonate potency *in vitro* and that *in vivo* was rather poor (Green *et al.*, 1994). As described earlier, this may be because the relative affinity for bone has not generally been considered in *in vitro* models (Leu *et al.*, 2006).

Normal Animals

In growing rats, bisphosphonates block degradation of both bone and cartilage, thus suppressing the remodeling of the metaphysis which becomes club-shaped and radiologically denser than normal (Schenk *et al.*, 1973). This effect is used as a model, the “Schenk assay,” to study the potency of bisphosphonates (Schenk *et al.*, 1986). The inhibition of endogenous bone resorption has also been documented by ^{45}Ca kinetic studies and by markers of bone resorption (Gasser *et al.*, 1972). Effects occur within 24 to 48 hours and are therefore somewhat slower than that of calcitonin (Mühlbauer and Fleisch, 1990).

The decrease in resorption caused by bisphosphonates is accompanied by a positive calcium balance (Gasser *et al.*, 1972) and an increase in mineral content of bone. This is possible because of an increase in intestinal absorption of calcium consequent to an elevation of 1,25$(OH)_2$ vitamin D. Bone formation also decreases after a certain time, attributed to "coupling" between formation and resorption. The main effect is, therefore, a reduction in bone turnover and some increase in bone mineral content. Less is known about the effect in normal adult animals. In dogs and minipigs, long-term administration of alendronate did not lead to an increase in bone mass (Peter *et al.*, 1996). This might be explained by the physiological biomechanical homeostasis of bone structure, which would eliminate an unnecessary excess of bone. A 3-year clinical study of alendronate showed normal bone quality in transiliac bone biopsies (Chavassieux *et al.*, 1997), whereas a 7-year study showed safe and continuous increases in bone mineral density (Tonino *et al.*, 2000). These and similar studies (reviewed in Russell *et al.*, 2008) suggest that concerns about dangers of long-term use of therapeutic doses may not be warranted.

Models of Hyperresorptive Diseases

Bisphosphonates can prevent experimentally induced increases in bone resorption. They impair resorption induced by agents such as parathyroid hormone, 1,25$(OH)_2$ vitamin D, and retinoids (Fleisch *et al.*, 1969; Russell *et al.*, 1970). The effect on retinoid-induced hypercalcemia was developed into a rapid screening assay for new compounds (Trechsel *et al.*, 1987).

Osteoporosis Models

The first experimental model in which bisphosphonates prevented bone loss was limb immobilization by sciatic nerve section (Michael *et al.*, 1971; Mühlbauer *et al.*, 1971). Frequently used models also involve ovariectomy (Shiota, 1985; Thompson *et al.*, 1992) or orchidectomy (Wink *et al.*, 1985) in various species. However, it should be noted that many of these experiments were performed in growing animals, in which it is often difficult to assess to what extent the effect on bone density or bone mass is owing to inhibition of sex steroid deficiency-induced bone loss, or of the bone resorption associated with modeling and remodeling during growth. Bisphosphonates were also effective in preventing bone loss in animals treated with corticosteroids (Jee *et al.*, 1981) or thyroid hormone (Yamamoto *et al.*, 1993).

Tumor-Induced Bone Resorption

Bisphosphonates very effectively inhibit tumor-induced bone resorption, both *in vitro* and *in vivo*, when osteolysis is induced by circulating humoral factors as well as by local tumor invasion. When added *in vitro*, bisphosphonates inhibit the bone-resorbing effect of supernatants of various cancers in mice calvaria (Galasko *et al.*, 1980). Inhibition was also seen when the bisphosphonates were injected into the mice before explantation of the calvaria (Jung *et al.*, 1981). Many types of tumor cells have been used in various *in vivo* models, such as bladder tumors, rat mammary adenocarcinoma, prostate adenocarcinoma, myeloma, and melanoma. Perhaps the best host is the nude mouse (Nemoto *et al.*, 1990; Sasaki *et al.*, 1995, 1998, 1999; Hiraga *et al.*, 1996a,b; Yoneda *et al.*, 2000). The first such studies performed showed that the humoral hypercalcemia induced by subcutaneously implanted Walker 256 carcinoma cells or by implanted Leydig tumor cells (Martodam *et al.*, 1983; Jung *et al.*, 1984; Guaitani *et al.*, 1985) could be partially prevented.

Arthritis Models

Several bisphosphonates inhibit local cartilage resorption, preserve the joint architecture, and prevent local and systemic bone loss in several types of experimental arthritis, e.g., induced by Freund's adjuvant, carrageenin, or collagen (Francis *et al.*, 1972, 1989; Dunn *et al.*, 1993; Österman *et al.*, 1994; Zao *et al.*, 2006; Herrak *et al.*, 2004; Sims *et al.*, 2004; Matsuo *et al.*, 2003), although effects on the inflammatory component in these disease models is inconsistent. Although zoledronate was found to preserve joint architecture in a recent clinical trial (Jarrett *et al.*, 2006), the effectiveness of other bisphosphonates at preventing joint destruction in other clinical studies in patients with rheumatoid arthritis have been less convincing, perhaps because of insufficient dosing (Breuill *et al.*, 2006; Ritchlin *et al.*, 2004; Maksymowych *et al.*, 2003; Eggelmeijer *et al.*, 1996).

Other Models of Hyperresorptive Diseases

Of interest in the dental field is the fact that bisphosphonates slow down periodontal bone destruction in animal models of spontaneous and induced periodontitis (Shoji *et al.*, 1995; Reddy *et al.*, 1995; O'Uchi *et al.*, 1998). Furthermore, they inhibit tooth movement and alveolar bone resorption induced by orthodontic procedures, and these effects can be achieved when the compounds are administered topically (Adachi *et al.*, 1994; Yaffe *et al.*, 1997; Kaynak *et al.*, 2000). Finally, in a model developed to mimic osteolysis and aseptic loosening around total hip arthroplasty, alendronate also inhibited bone destruction (Shanbhag *et al.*, 1997).

Combination Therapy with Anabolic Agents

Treatments such as prostaglandins, IGF-1, and parathyroid hormone (PTH), which increase bone formation, remain effective when coadministered with bisphosphonates in animal models, sometimes resulting in additive effects on bone mass (Jee *et al.*, 1993; Takano *et al.*, 1996). However, several clinical studies have shown that prior or concurrent treatment with alendronate appears to interfere with the anabolic response to PTH (Finkelstein *et al.*, 2006; Black *et al.*, 2003), although the exact mechanism underlying this effect remains unknown. Recent clinical studies suggest that prior treatment with risedronate may impair the subsequent response to PTH less than alendronate (Delmas *et al.*, 2007). Experimental studies in animals suggest that the action of PTH may be less compromised after single, rather than repeated, doses of bisphosphonates (Gasser *et al.*, 2000).

ANTIFRACTURE MECHANISM IN OSTEOPOROSIS

The antifracture effect of bisphosphonates in osteoporosis may not be caused solely by an increase in bone mass, as was once thought, but also by an improvement in microarchitecture and probably a reduction in bone remodeling. Numerous preclinical studies have shown that, when not given in excess, bisphosphonates produce an improvement in bone biomechanical properties, including torsional torque, ultimate bending strength, stiffness, maximum elastic strength, Young's modulus of elasticity, and others, both in normal animals and in experimental models of osteoporosis. This is the case for a variety of bisphosphonates and was seen in various animals, although most experiments have been performed in the rat (Geusens *et al.*, 1992; Toolan *et al.*, 1992; Ammann *et al.*, 1993; Balena *et al.*, 1993; Guy *et al.* 1993; Ferretti *et al.*, 1993; Motoie *et al.*, 1995; for review, see Ferretti, 1995). These positive effects on bone quality in preclinical studies have since been confirmed in the clinical setting (Chavassieux *et al.*, 1997; Recker *et al.*, 2004; Ericksen *et al.*, 2002). Furthermore, although several studies have shown that high doses in animals may prevent naturally occurring microscopic cracks in bone from healing and thus increase microdamage, which may reduce bone toughness, this may be offset by an increase in bone strength (Mashiba *et al.*, 2005; Komatsubara *et al.*, 2004).

The higher number of bone-remodeling sites that occurs in osteoporotic bone leads to the development of areas of stress concentration and hence to increased fracture risk. Because bisphosphonates decrease remodeling they serve as a means of reducing this effect, thus reducing the incidence of new fractures. The prevention of bone loss is probably explained to a large extent by the decrease in bone turnover. The initial rise in bone mass is because of "filling of the remodeling space"; in other words, bone formation continues to rebuild basic multicellular units (BMUs) initiated prior to bisphosphonate treatment, whereas fewer new BMUs are initiated. In addition, some bone can be lost at each BMU, because a greater amount of bone is resorbed than formed. The decrease in turnover would therefore slow down total bone loss. Furthermore, bisphosphonates also act at individual BMUs by decreasing the depth of resorption (Balena *et al.*, 1993; Boyce *et al.*, 1995). Both effects will lead to a decreased number of trabecular perforations, thus reducing the decrease in bone strength and the occurrence of fractures.

Another mechanism may be related to the increase in mineralization associated with lower bone turnover (Meunier and Boivin, 1997; Boivin *et al.*, 2000). A lower turnover will lengthen the life span of the BMU, thus permitting it to mineralize more completely, which will increase mineral content, measured as BMD, independent of effects on bone mass. This has been described in alendronate-treated baboons (Meunier and Boivin, 1997) and more recently in osteoporotic women (Boivin *et al.*, 2000).

RELATIVE POTENCY OF BISPHOSPHONATES FOR INHIBITION OF RESORPTION

The activity of bisphosphonates on bone resorption in the models described earlier varies greatly from compound to compound (Shinoda *et al.*, 1983), which is not the case for the inhibition of mineralization. For etidronate, one of the first bisphosphonates to be investigated, the dose required to inhibit resorption is relatively high, greater than 1 mg/kg/day parenterally. Because this dose is similar to that which impairs normal mineralization, one of the early aims of bisphosphonate research was to develop compounds with a more powerful antiresorptive activity and less effect on inhibition of mineralization. The first of these, clodronate, was more potent than etidronate (Fleisch *et al.*, 1969; Russell *et al.*, 1970) and less active in inhibiting normal mineralization (Schenk *et al.*, 1973). Later, pamidronate was found to be still more active (Lemkes *et al.*, 1978), and several compounds have subsequently been developed that are up to 10,000 times more powerful than etidronate in inhibiting bone resorption in animals (reviewed in Fleisch, 2000). Perhaps the best way to compare potency in humans, bypassing the differences in oral bioavailability, is to examine maximally effective intravenous doses in hypercalcemia of malignancy, a condition produced by massive bone resorption. Bisphosphonate treatment is typically given in one or two intravenous doses. As a result, urinary and serum markers for resorption are typically normalized within 48 hours and resorption control can last up to about a month. Maximally effective doses of alendronate (15 mg), ibandronate (6 mg), pamidronate (90 mg), and zoledronate (2-4 mg) result in normalization of serum calcium in 77% to 100% of patients, depending on the study (Nussbaum *et al.*, 1993a,b; Ralston *et al.*, 1997; Body *et al.*, 1999). This suggests an overall spread in potency of about 40-fold, 6-fold if pamidronate is excluded. In animal studies the spread in potency is approximately 1000-fold for these bisphosphonates (Fleisch, 2000). Therefore, whereas rank ordering for potency in animals is predictive, the actual potency range is narrower in patients and has to be determined in clinical studies.

STRUCTURE–ACTIVITY RELATIONSHIP FOR INHIBITION OF BONE RESORPTION

Prior to elucidation of the mechanisms of action of bisphosphonates, it was difficult to establish a detailed structure–activity relationship. The length of the aliphatic carbon was found to be important and adding a hydroxyl group to the carbon atom at position 1 increased potency and binding to bone (Shinoda *et al.*, 1983; van Beek *et al.*, 1996). Derivatives with an amino group at the end of the side chain are very active, the highest activity being found with a backbone of four carbons, as present in alendronate (Schenk *et al.*, 1986). A primary amine is not necessary for this activity, and in fact dimethylation of the nitrogen of pamidronate, as seen in olpadronate, increases potency (Boonekamp *et al.*, 1987). Potency is still further increased when other groups are added to the nitrogen, as seen in ibandronate (Mühlbauer *et al.*, 1991). Cyclic geminal bisphosphonates are also very potent, especially those containing a nitrogen atom in a heterocyclic ring, such as risedronate (Sietsema *et al.*, 1989). The most potent compounds described so far, zoledronate (Green *et al.*, 1994) and minodronate, also contain nitrogen atoms in heterocyclic ring structures.

CELLULAR EFFECTS OF BISPHOSPHONATES ON BONE CELLS

Our understanding of the mode of action of the bisphosphonates has made great progress in the past two decades. There is no doubt that the action *in vivo* is mediated mostly, if not completely, through mechanisms other than the physicochemical inhibition of crystal dissolution, initially postulated. Many of these mechanisms have been unraveled and more than one mechanism may be operating simultaneously.

The Osteoclast Is the Main Target Cell Type of Bisphosphonates

Although many studies have demonstrated that bisphosphonates (BPs) are capable of affecting a wide variety of cell types, such as macrophages (Rogers *et al.*, 1996; Coxon *et al.*, 1998), endothelial cells (Fournier *et al.*, 2002; Wood *et al.*, 2002), and a wide variety of tumor cells (reviewed in Green, 2004) *in vitro*, these compounds have a high selectivity for bone-resorbing osteoclasts *in vivo*, which is owing to a combination of factors. First of all, their high affinity for calcium-rich bone ensures the efficient adsorption and retention of bisphosphonates in the skeleton, resulting in a low bioavailability of these drugs outside the skeleton (Russell *et al.*, 2006). In addition, BPs have been shown to preferentially bind to exposed bone mineral at surfaces undergoing osteoclastic bone resorption (Sato *et al.*, 1991; Azuma *et al.*, 1995; Masarachia *et al.*, 1996), although the relative deposition on bone forming and bone resorbing areas depends on the amount of bisphosphonate administered (Masarachia *et al.*, 1996). Furthermore, osteoclasts acidify the resorption lacuna underneath the cell by secreting protons, which aids in the digestion of matrix proteins (Baron, 2003). This is believed to facilitate the release of bisphosphonates from the bone mineral surface owing to protonation of the phosphate groups and the resulting decrease in ability to chelate Ca^{2+} ions, which could give rise to locally high concentrations of bisphosphonates in solution in the osteoclast resorption lacunae (Ebetino *et al.*, 1998). It was calculated that the concentration of alendronate achieved in the resorption lacunae underneath osteoclasts could be as high as 0.1 to 1 mM (Sato *et al.*, 1991). Finally, it has recently become clear that the cellular uptake of the negatively charged bisphosphonates primarily occurs via fluid-phase endocytosis followed by acidification of intracellular vesicles and release of bisphosphonate into the cytosol (Thompson *et al.*, 2006). This is likely to contribute to the selective targeting of bisphosphonates to highly endocytic bone-resorbing osteoclasts.

The efficient uptake of bone-bound bisphosphonate by osteoclasts has recently been demonstrated by using fluorescently labeled bisphosphonate to visualize mineral binding and cellular uptake of bisphosphonate *in vitro*. Although nonosteoclast cell types can internalize bisphosphonate from solution (to various extents depending on endocytic activity), only osteoclasts are able to efficiently internalize bisphosphonate from the bone surface (Coxon *et al.*, 2008). Intracellular uptake of bisphosphonate by osteoclasts *in vivo* has been shown using radiolabeled bisphosphonate, whereas intracellular levels in osteoblasts were below the limit of detection (Sato *et al.*, 1991; Masarachia *et al.*, 1996).

Cellular Effects on Osteoclasts

Bisphosphonates have been shown to induce apoptosis in osteoclasts *in vitro* (Hughes *et al.*, 1995; Hiroi-Furuya *et al.*, 1999; Benford *et al.*, 2001) and *in vivo* (Hughes *et al.*, 1995; Ito *et al.*, 1999). In addition to causing apoptosis, bisphosphonates induce more subtle ultrastructural changes in mature osteoclasts. It was reported long ago that, after bisphosphonate administration, osteoclasts show changes in morphology and appear inactive (Schenk *et al.*, 1973). The changes are numerous (Sato and Grasser, 1990), but perhaps the most striking feature is the shrinkage, or complete loss, of the ruffled border (Schenk *et al.*, 1973; Sato *et al.*, 1991), which is associated with disruption of the cytoskeleton and loss of F-actin rings (Murakami *et al.*, 1995; Hiroi-Furuya *et al.*, 1999). Following these observations, the decrease in bone resorption in response to bisphosphonate treatment has been mainly attributed to the detrimental effects of these compounds on mature resorbing osteoclasts, although inhibition of osteoclastogenesis may also contribute to the antiresorptive effects (Boonekamp *et al.*, 1987; Löwik *et al.*, 1988; Hughes *et al.*, 1989; Van Beek *et al.*, 2002). It should be noted that apoptosis appears to be the primary mechanism of the inhibition of bone resorption by clodronate and etidronate, but is not required for the inhibition of resorption by the nitrogen-containing bisphosphonates (discussed later) (Halasy-Nagy *et al.*, 2001).

Potential Effects on Osteocytes

The effects of bisphosphonates on osteocytes have only recently been studied. Plotkin *et al.*, 1999) found that very low concentrations of bisphosphonates prevented apoptosis of osteocyte-like cells induced by etoposide, TNFα or dexamethasone *in vitro*, and treatment of mice with alendronate *in vivo* prevented the increase in apoptosis of osteocytes and osteoblasts following prednisolone administration. More recently, bisphosphonates were shown to also prevent the apoptotic effects of glucocorticoids or cyclic mechanical loading in experimental animal models (Follet *et al.*, 2007). The antiapoptotic effect of bisphosphonates is in stark contrast to their proapoptotic effects on osteoclasts and appears to involve the rapid activation of extracellular signal-regulated kinases (ERKs) following opening of hexameric connexin-43 hemichannels and calcium influx (Plotkin *et al.*, 2002, 2005), suggesting that these effects may be mediated through extracellular effects and do not require intracellular uptake of bisphosphonate. The potency of individual bisphosphonates to prevent osteocyte apoptosis is independent of their ranking as inhibitors of osteoclastic activity, and, in particular, those with little antiresorptive activity are also able to increase osteocyte survival (Plotkin *et al.*, 2006; Kogianni *et al.*, 2004), supporting the view that the effects of bisphosphonates on osteocytes are mediated via a separate mechanism. The importance of these effects *in vivo* remains to be determined, but these findings raise the possibility that potential interactions of bisphosphonates with osteocytes *in vivo* may contribute to antifracture efficacy independently of the better known classical actions via osteoclasts.

Potential Effects on Osteoblasts

Several *in vitro* studies have shown that bisphosphonates can affect bone-forming osteoblasts. They have been shown to stimulate the proliferation of osteoblast-like cells at very low concentrations (Fast *et al.*, 1978; Endo *et al.*, 1996). As with the antiapoptotic effects of bisphosphonates on osteocytes, the proliferative effect on osteoblasts has been attributed to opening of calcium channels and activation of ERKs (Mathov *et al.*, 2001). Furthermore, alendronate can stimulate the expression of collagenase-3 by rat calvarial osteoblasts (Varghese *et al.*, 2000), whereas pamidronate and zoledronate can stimulate the differentiation and bone mineral-forming ability of osteoblasts *in vitro* (Reinholz *et al.*, 2000). In addition, several studies reported that osteoblast-like cells inhibited osteoclastic bone resorption when exposed to low concentrations of bisphosphonate *in vitro*, believed to involve the release of an osteoclast inhibitory factor (Sahni *et al.*, 1993; Yu *et al.*, 1996; Nishikawa *et al.*, 1996, Vitté *et al.*, 1996), although the exact mechanism involved has not been elucidated. However, despite many studies, direct effects of bisphosphonates on osteoblasts *in vivo* have been hard to demonstrate using clinically relevant concentrations of bisphosphonates. Most of the clinical effects on osteoblast function (e.g., suppression of osteoblast-derived biochemical markers) can be attributed to indirect effects on the remodeling cycle mediated through reduction of bone resorption.

MOLECULAR MECHANISMS OF ACTION OF BISPHOSPHONATES

Studies over the past two decades examining the mechanisms of action of bisphosphonates have uncovered the primary sites of inhibition that account for most of the potency of clinically used bisphosphonates. These studies paint a compelling picture pointing to two general classes of bisphosphonates: those that are metabolized within the cell to form toxic analogues of ATP (the "simple" bisphosphonates clodronate, etidronate, and tiludronate) and those that inhibit farnesyl diphosphate synthase (alendronate, ibandronate, incadronate, olpadronate, pamidronate, risedronate, and zoledronate). The properties that segregate bisphosphonates into these two classes appear to be a function of the moieties attached to the geminal carbon at R^2, which can vary in size and complexity (Fig. 2). Without regard to size or shape, the prevailing determinant for the mechanism of action of the bisphosphonates used in the clinic is the presence or absence of a nitrogen atom located three to five positions away from the geminal carbon of the P–C–P backbone in the R^2 group. Bisphosphonates that contain nitrogen, hereafter referred to as N-BPs, inhibit farnesyl diphosphate (FPP) synthase with IC_{50}s in the nanomolar range, whereas the simple BPs that lack a nitrogen are metabolized to cytotoxic ATP analogues. Throughout the remainder of this section, the salient features of these two classes of bisphosphonate are discussed.

Mechanism of Action of Simple Bisphosphonates

Simple Bisphosphonates are Metabolized into ATP

Analogues

Studies in the 1970s suggested that clodronate and etidronate could affect a variety of intracellular metabolic processes, including glycolysis, lactate production, fatty acid oxidation, adenylate cyclase, and phosphohydrolases (Fast *et al.*, 1978; Felix *et al.*, 1976, 1981). Because bisphosphonates are close structural analogues of inorganic pyrophosphate, it is perhaps not surprising that they can affect a wide variety of enzymes and metabolic pathways. However, none of these effects satisfactorily explain the ability of clodronate and etidronate to inhibit osteoclast function and cause osteoclast apoptosis.

The first clue to the likely molecular mechanism of action of clodronate and etidronate arose from studies using methylenebisphosphonate (medronate) as a marker to study intracellular pH in single-celled amebae of the slime mold *Dictyostelium discoideum* (Klein *et al.*, 1989; Rogers, 2004). Using ^{31}P NMR to study the pH-dependent δ-shift of the phosphonate groups in medronate, new peaks were identified in the ^{31}P NMR spectrum of medronate-treated amebae. These peaks were identified as nonhydrolyzable, methylene-containing analogues of adenosine triphosphate (ATP) and diadenosine tetraphosphate (Ap4A). The metabolites, AppCH2p and App-CH2ppA, contained the P–C–P moiety of medronate in place of a P–O–P moiety (Fig. 3), and were thus resistant to hydrolysis. FPLC and UV absorbance, as well as ^{31}P NMR analysis, showed that clodronate and certain other bisphosphonates (etidronate, fluoromethylene bisphosphonate, difluoromethylene bisphosphonate, and hydroxymethylene bisphosphonate) are metabolized to methylene-containing (AppCp-type) analogues of ATP, but not to analogues of Ap4A (Rogers *et al.*, 1992, 1994; Pelorgeas *et al.*, 1992). Interestingly, bisphosphonates with larger R^2 side chains (such as the amino-alkyl bisphosphonates and heterocycle-containing bisphosphonates), which poorly resemble pyrophosphate, are not metabolized (with the exception of tiludronate). Similar results were obtained using cell-free extracts of human HL60 cells as well as other cell lines, in particular J774 macrophages which (like osteoclasts and *Dictyostelium* amebae) are highly endocytic and may internalize bisphosphonates more efficiently than most other cell types (Rogers *et al.*, 1996, 1997; Frith *et al.*, 1997; Benford *et al.*, 1999). Liposome-encapsulated bisphosphonates are internalized even more effectively by phagocytic cells, leading to greater accumulation of intracellular AppCp-type metabolites (Frith *et al.*, 1997). Up to 50% of clodronate internalized intracellularly is metabolized by macrophages *in vitro* to AppCCl2p, which may reach an intracellular concentration as high as 1 mM (Mönkkönen *et al.*, 2001). The metabolism of clodronate, etidronate, and tiludronate (see Fig. 3), but not alendronate, pamidronate, or ibandronate, has been unequivocally demonstrated using a combination of ion-pairing HPLC and electrospray ionization mass spectrometry (Auriola *et al.*, 1997; Benford *et al.*, 1999; Mönkkönen *et al.*, 2000).

Mechanism of Formation of Bisphosphonate Metabolites

The incorporation of bisphosphonates into AppCp-type nucleotides is probably achieved through interactions with the family of type II aminoacyl-tRNA synthetases (which utilize the amino acids Asn, Asp, Gly, His, Lys, Phe, and Ser) (Rogers *et al.*, 1994, 1996). These enzymes play an essential role in protein synthesis (Fig. 4), because they catalyze the condensation of an amino acid with ATP (Appp) to form an aminoacyl-adenylate (amino acid-AMP), releasing pyrophosphate (P–O–P) in a reversible reaction (reaction I). The aminoacyl-adenylate then condenses with a molecule of tRNA to form aminoacyl-tRNA, utilized for ribosome-dependent translation of mRNA into protein (reaction II). It appears that tiludronate and bisphosphonates with short side chains (but not more potent bisphosphonates that contain a nitrogen in the R^2 side chain) can replace P–O–P in the active site of type II aminoacyl-tRNA synthetases, but not type I enzymes. Because bisphosphonates (P–C–P) resemble pyrophosphate (P–O–P) in structure, the reverse reaction of (I) can occur with P–C–P in place of P–O–P, to form an analog of ATP (AppCp) containing the bisphosphonate (reaction III), a reaction demonstrated more than 30 years ago (Zamecnik and Stephenson, 1968). For medronate, difluoromethylenebisphosphonate, and hydroxymethylenebisphosphonate (but not clodronate or etidronate), AppCp can be further metabolized (to AppC-ppA, an analogue of AppppA), by the condensation of ATP with AppCp to form AppCppA + AMP (reaction IV) (Rogers *et al.*, 1994; Zamecnik, 1983). It should be noted that a recent report also described the ability of T4 RNA ligase to catalyze the synthesis of ATP analogues of methylenebisphosphonate, clodronate, and etidronate (Sillero *et al.* 2006).

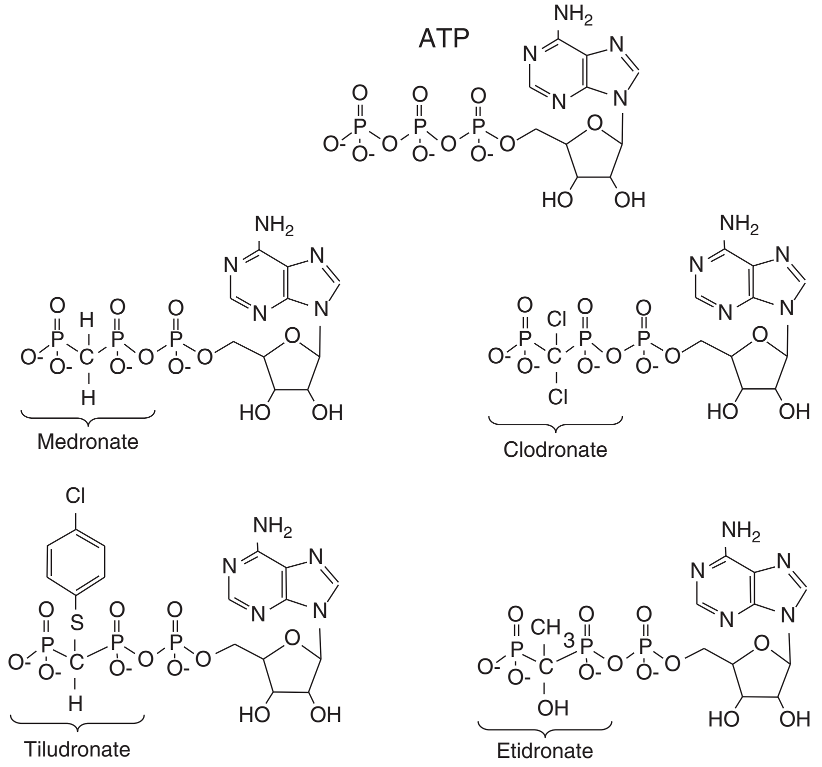
ATP
NH2
N
N
N
N
O
O
P
O-
O-
HO
OH
H
C
Cl
S
CH3
Medronate
Clodronate
Tiludronate
Etidronate

FIGURE 3 The structure of ATP and the AppCp-type metabolites of medronate, clodronate, etidronate, and tiludronate.

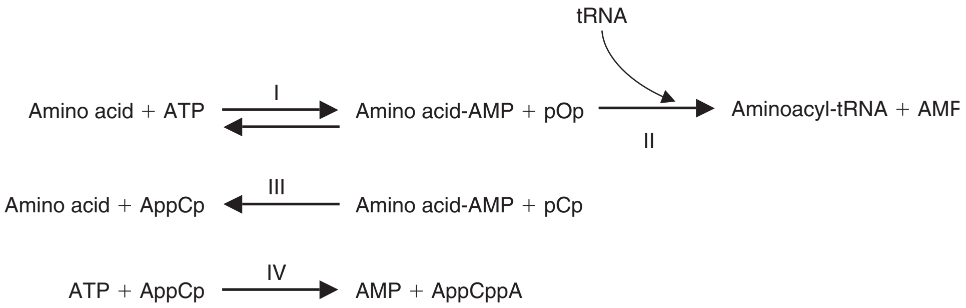
tRNA
Amino acid + ATP
I
Amino acid-AMP + pOp
II
Aminoacyl-tRNA + AMF
Amino acid + AppCp
III
Amino acid-AMP + pCp
ATP + AppCp
IV
AMP + AppCppA

FIGURE 4 The mechanism by which aminoacyl-tRNA synthetase enzymes catalyze the formation of AppCp-type metabolites of bisphosphonates. An amino acid condenses with ATP (Appp) to form an aminoacyl-adenylate (amino acid-AMP), releasing pyrophosphate (pOp) in a reversible reaction (I). The aminoacyl-adenylate then condenses with a molecule of tRNA to form aminoacyl-tRNA (reaction II). Because bisphosphonates (pCp) resemble pyrophosphate in structure, the reverse reaction of (I) can occur with pCp in place of pOp, to form an analogue of ATP (AppCp) containing the bisphosphonate. AppCH2p, the metabolite of medronate, can condense with another molecule of ATP (reaction IV) to form an AppCppA-type nucleotide (i.e., AppCH2ppA).

Metabolism of Simple Bisphosphonates in Osteoclasts in Vivo

Although for many years bisphosphonates were considered to be metabolically inert, the discovery that some could be metabolized intracellularly to AppCp-type nucleotides by cells *in vitro* suggested the possibility that their antiresorptive effect may be caused by the accumulation of these metabolites in osteoclasts *in vivo* (Frith *et al.*, 1997). The ability of osteoclasts to metabolize clodronate and etidronate *in vitro* has been confirmed using HPLC-electrospray mass spectrometry to identify AppCCl2p in extracts of purified rabbit osteoclasts. Furthermore, the AppCCl2p metabolite of clodronate was found in extracts of osteoclasts purified by magnetic bead separation following injection of rabbits with clodronate, confirming that osteoclasts can metabolize clodronate *in vivo* (Frith *et al.*, 2001).

Metabolites of Simple Bisphosphonates Induce Osteoclast Apoptosis

The functional significance of the conversion of bisphosphonates into nonhydrolyzable analogues of ATP was demonstrated by showing that treatment of osteoclasts with AppCCl2p induced osteoclast apoptosis and inhibited bone resorption (Fig. 5), effects that were indistinguishable from the effects of clodronate treatment (Frith *et al.*, 2001). It has been suggested that these ATP analogues may induce toxicity by inhibiting the activity of ATP-dependent intracellular enzymes such as the mitochondrial adenine nucleotide translocase (ANT). ANT is thought to be involved in controlling apoptosis by regulating the permeability transition pore in the mitochondrial membrane. It is suggested that by inhibiting ANT AppCCl2p may cause opening of the permeability transition pore (Lehenkari *et al.*, 2002), which would lead to disruption of the mitochondrial membrane potential and the release of Apaf-1 and cytochrome C, causing the subsequent activation of proapoptotic caspases (Cohen, 1997; Crompton *et al.*, 1999; Nicholson, 1999; Benford *et al.*, 2001). Induction of osteoclast apoptosis appears to be the primary mechanism by which the simple, non-N-BPs inhibit bone resorption, because the ability of clodronate and etidronate to inhibit resorption *in vitro* is blocked when osteoclast apoptosis is prevented by using a caspase inhibitor (Halasy-Nagy *et al.*, 2001).

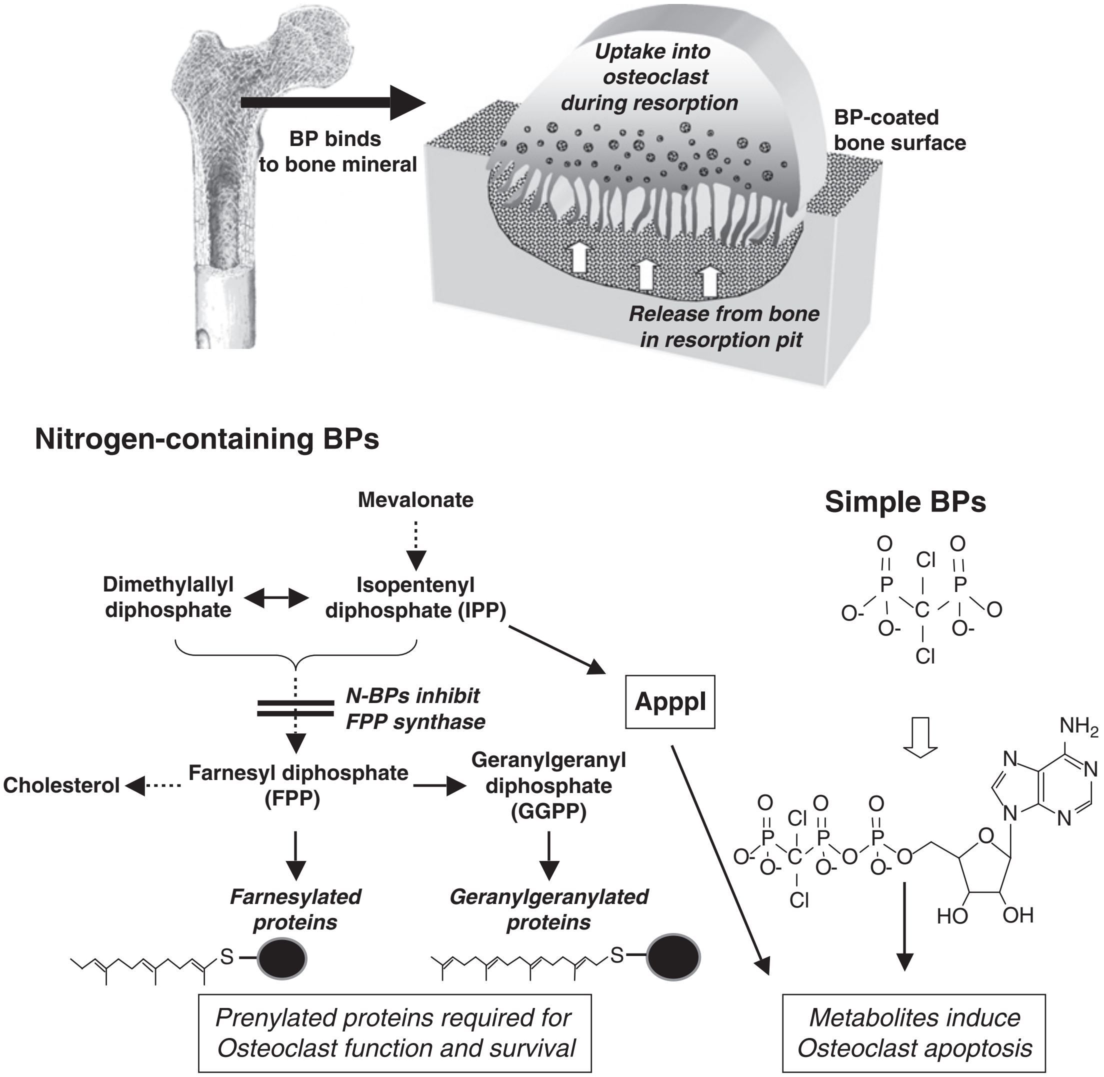
BP binds
to bone mineral
Uptake into
osteoclast
during resorption
BP-coated
bone surface
Release from bone
in resorption pit
Nitrogen-containing BPs
Mevalonate
Dimethylallyl diphosphate
Isopentenyl diphosphate (IPP)
N-BPs inhibit
FPP synthase
Cholesterol
Farnesyl diphosphate (FPP)
Geranylgeranyl diphosphate (GGPP)
Farnesylated proteins
Geranylgeranylated proteins
S
ApppI
Simple BPs
O
Cl
P
O-
NH2
N
HO
OH
Prenylated proteins required for Osteoclast function and survival
Metabolites induce Osteoclast apoptosis

FIGURE 5 Mechanisms of action of bisphosphonates on osteoclasts. After binding to bone mineral, the drugs are internalized into bone-resorbing osteoclasts by endocytosis. Simple bisphosphonates (e.g., clodronate, etidronate) are metabolized in the osteoclast cytosol to ATP analogues that induce osteoclast apoptosis. Nitrogen-containing bisphosphonates inhibit FPP synthase, thereby preventing the prenylation of small GTPase proteins essential for the function and survival of osteoclasts. Inhibition of FPP synthase also causes the accumulation of IPP, which is incorporated into ApppI (an analogue of ATP capable of inducing osteoclast apoptosis).

Mechanism of Action of Nitrogen-Containing Bisphosphonates

Inhibition of the Mevalonate Pathway by N-BPs

Studies seeking to identify squalene synthase inhibitors first led to the finding that the N-BPs incadronate (YM175), ibandronate, pamidronate, and alendronate inhibit the mevalonate/cholesterol biosynthetic pathway (Amin *et al.*, 1992, 1996). Etidronate and clodronate, which lack nitrogen in the R^2 side chain, were inactive in the same assays. Whereas incadronate and ibandronate were shown to potently inhibit squalene synthase, alendronate and pamidronate were inactive, yet nonetheless inhibited sterol biosynthesis with IC_{50}s of 170 and 420 nM, respectively. This suggested the presence of a target upstream in the mevalonate pathway (see Fig. 5).

FPP Synthase as the Molecular Target of the N-BPs

The upstream enzyme inhibited by all of the N-BPs examined to date was identified as FPP synthase (Table II; Van Beek *et al.*, 1999a,b; Bergstrom *et al.*, 2000; Dunford *et al.*, 2001). The most potent antiresorptive N-BPs such as zoledronate and minodronate (YM-529) are extremely potent inhibitors of FPP synthase (IC_{50} 3nM with respect to recombinant human FPP synthase; Dunford *et al.*, 2001). The discovery of these inhibitory activities revealed several interesting findings: (1) Although alendronate is a specific inhibitor of FPP synthase, some other N-BPs inhibit other enzymes of the mevalonate pathway, including squalene synthase (Amin *et al.*, 1992; van Beek *et al.*, 1999b; Bergstrom *et al.*, 2000; Dunford *et al.*, 2001) and geranylgeranyl diphosphate (GGPP) synthase, albeit with lower potency than their potency for inhibiting FPP synthase (Guo *et al.*, 2007). (2) In enzymatic assays risedronate is 90-fold more potent than alendronate for inhibition of purified FPP synthase (Bergstrom *et al.*, 2000, Dunford *et al.*, 2008), but only 5-fold more potent in crude enzyme preparations (Van Beek *et al.*, 1999, Dunford *et al.*, 2001), although it is now clear that the time-dependent nature of the slow-tight inhibition (Dunford *et al.*, 2008) by N-BPs may result in different values of IC_{50} depending on the conditions of the assay. Nonetheless, because these two bisphosphonates appear to be approximately equipotent for increasing bone mineral density after oral administration (5 mg p.o. q.d., 2 years) in the clinic (Bone *et al.*, 1997; Fogelman *et al.*, 2000), this suggests that differences in pharmacokinetics, pharmacodynamics, binding to bone (van Beek *et al.*, 1994, 1998), osteoclast targeting, cell permeability, or intracellular protein binding between these mechanistically similar bisphosphonates contribute significantly to their ultimate potency *in vivo*. (3) Although risedronate is a potent inhibitor of FPP synthase, modifications (e.g., addition of a methyl group) to the structure of the R^2 side chain that influence the position of the nitrogen group in relation to the phosphonate groups give rise to analogues that are markedly less potent inhibitors of FPP synthase and less effective inhibitors of bone resorption *in vivo*. Furthermore, for a wide range of N-BPs, there is a significant correlation between potency for inhibition of FPP synthase and antiresorptive potency *in vivo*, pointing to FPP synthase as the major molecular target of the N-BPs

TABLE II Inhibition of FPP synthase by various bisphosphonates

Bisphosphonate	IC_{50} (nM) human*[a]	IC_{50} (nM) human†[b]	IC_{50} (nM) murine endogenous‡[c]	Isomerization constant§
Alendronate	340	50	500	6.3
Ibandronate		20	310	53
Incadronate		30		
Minodronate		3		
Pamidronate	500	200	850	
Risedronate	3.9	10	100	226
Zoledronate		3	20	1244
Clodronate	NA ≤ 350,000	NA ≤ 100,000	NA ≤ 100,000	
Etidronate	80,000	NA ≤ 100,000	NA ≤ 100,000	

Analyses of FPP synthase inhibition were performed using purified (NA, *not active/negligible inhibition*):

**Recombinant human enzyme* (Bergstrom *et al.*, 2000),

†*Recombinant human enzyme in* E. coli *lysate* (Dunford *et al.*, 2001), *or*

‡*Endogenous murine enzyme in J774 macrophage lysate* (Dunford *et al.*, 2001).

§*The isomerization constant* (Russell *et al.*, 2007; Dunford *et al.*, 2008) *reflects the reversibility of enzyme inhibition* (*the greater the value, the less reversible the inhibition*).

(Dunford *et al.*, 2001, 2008). (4) Consistent with the initial findings (Amin *et al.*, 1992), neither clodronate nor etidronate showed any significant inhibitory activity against FPP synthase (van Beek *et al.*, 1999b; Bergstrom *et al.*, 2000; Dunford *et al.*, 2001).

Mechanism of FPP Synthase Inhibition

Initially, computer modeling was used to try to understand how N-BPs inhibit FPP synthase, which suggested that N-BPs may inhibit the enzyme by acting as isoprenoid transition-state analogues (Martin *et al.*, 1999). More recently, the generation of x-ray crystal structures of the human FPP synthase enzyme, cocrystallized with various N-BPs (Rondeau *et al.*, 2006; Kavanagh *et al.*, 2006), revealed that N-BPs bind in one of the two isoprenoid lipid-binding pockets in the enzyme-active site, namely the dimethylallyl pyrophosphate (DMAPP)/geranyl pyrophosphate (GPP) binding site of the enzyme, with the phosphonate groups bound to a cluster of three magnesium ions. The R^2 side chain is positioned in the hydrophobic cleft that normally accommodates an isoprenoid lipid, with stabilizing interactions occurring between the nitrogen moiety of the N-BP and a conserved threonine and lysine residue in the enzyme (Fig. 6). Crystal structures of bacterial FPP synthase (Hosfield *et al.*, 2004) and FPP synthase from *Trypanosoma cruzi* (Gabelli *et al.*, 2006) in complex with N-BPs showed a similar mode of binding. Enzyme kinetic analysis with human FPP synthase indicated that the interaction with N-BPs is highly complex and characteristic of "slow, tight binding" inhibition (Kavanagh *et al.*, 2006). Initially, N-BPs appear to compete directly with DMAPP or GPP for binding to the DMAPP/GPP-binding pocket. This is followed by more complex interactions that promote binding of isopentenyl diphosphate (IPP) in the second isoprenoid binding site, causing conformational changes in the enzyme that stabilize the final ternary complex of enzyme + N-BP + IPP (Rondeau *et al.*, 2006; Kavanagh *et al.*, 2006). The determination of an isomerization constant that reflects these changes in enzyme conformation following binding of N-BP gives a more accurate indication of the extent of inhibition of the enzyme by different N-BPs (Russell *et al.*, 2007; Dunford *et al.*, 2008), because this illustrates the extent to which N-P binding and the changes in enzyme conformation are reversible (see Table II). From these values it is apparent that inhibition of FPP synthase by zoledronate and risedronate is virtually irreversible, but that inhibition by ibandronate and alendronate is more reversible in nature. These differences probably relate to the strength of the interaction between the N-BP side chain and amino acids in the GPP pocket (for example, between the nitrogen moiety and the Lys200 and Thr201 residues), which appear to be similar for zoledronate and risedronate but weaker for ibandronate, alendronate, and pamidronate (Russell *et al.*, 2007; Dunford *et al.*, 2008).

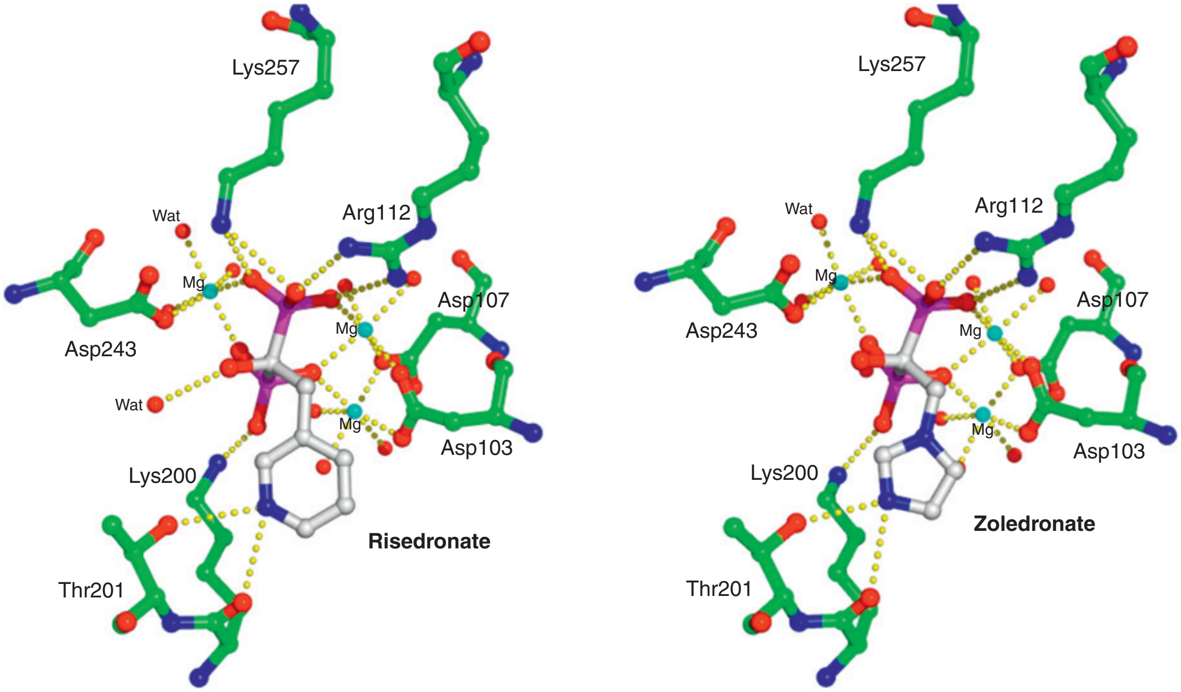

FIGURE 6 Binding of risedronate and zoledronate in the GPP pocket of FPP synthase. Interactions between the nitrogen (blue) of the bisphosphonate side chain and a conserved Lys200 and Thr201 help to stabilize the binding, hence explaining why the position and orientation of the nitrogen atom plays a crucial role in determining antiresorptive potency. (See plate section)

This detailed understanding of exactly how the N-BPs interact with FPP synthase helps to explain the inhibitory potency of some N-BPs toward this enzyme. The interaction of the phosphonate groups with magnesium ions in the aspartate-rich diphosphate-binding site of the enzyme explains why modifications to either or both of the phosphonate groups (such as methylation) prevents these compounds from inhibiting protein isoprenylation (discussed later) and reduces antiresorptive potency (Ebetino and Jamieson, 1990; Luckman *et al.*, 1998a). This confirms the earlier view that the two phosphonate groups have a dual purpose (Rogers *et al.*, 1995; Luckman *et al.*, 1998a), being required for the molecular mechanism of action as well as for targeting to bone mineral (as discussed previously). Furthermore, the length and orientation of the bisphosphonate R^2 side chain affects the interaction of the nitrogen in the side chain with the threonine and lysine residues in the active site cleft (Russell *et al.*, 2007), hence explaining why minor changes to the structure or conformation of the side chain also affect the ability to inhibit protein isoprenylation (Luckman *et al.*, 1998b) and markedly influence antiresorptive potency (Shinoda *et al.*, 1983; Schenk *et al.*, 1986; Sietsema *et al.*, 1989; van Beek *et al.*, 1994; Rogers *et al.*, 1995).

Inhibition of FPP Synthase Blocks Protein Isoprenylation and Sterol Synthesis

The mevalonate pathway is not only responsible for the production of cholesterol but also isoprenoid lipids such as IPP (five carbon), FPP (15 carbon), and GGPP (20 carbon), the latter two being substrates for protein isoprenylation (see Fig. 5). Isoprenylation involves the transfer of a farnesyl or geranylgeranyl lipid group onto a cysteine residue in characteristic carboxy-terminal (e.g., CAAX) motifs (Zhang and Casey, 1996; Sinensky, 2000), giving rise to farnesylated and geranylgeranylated proteins. Most of the isoprenylated proteins identified to date are small GTPases (the majority of which are geranylgeranylated) (Zhang and Casey, 1996), which are important signaling proteins that regulate a variety of cell processes important for osteoclast function, including cell morphology, integrin signaling, membrane ruffling, trafficking of endosomes, and apoptosis (Ridley *et al.*, 1992; Ridley and Hall, 1992; Zhang *et al.*, 1995; Clark *et al.*, 1998; reviewed in Coxon *et al.*, 2003). Isoprenylation is required for the correct function of these proteins, because the lipid isoprenyl group serves to anchor the proteins in cell membranes and may also participate in protein–protein interactions (Zhang and Casey, 1996).

The ability of N-BPs to inhibit protein isoprenylation was first directly demonstrated by using J774 macrophages, where the N-BPs risedronate, incadronate, ibandronate, and alendronate were found to prevent the incorporation of [^{14}C]mevalonate into isoprenylated proteins (both farnesylated and geranylgeranylated proteins), whereas the bisphosphonates that lack a nitrogen in the R^2 side chain (clodronate and etidronate) had no effect (Benford *et al.*, 1999; Luckman *et al.*, 1998b). Risedronate almost completely inhibited protein isoprenylation at a concentration of 10^{-5} M, which is similar to the concentration that affects osteoclast viability *in vitro* (Sato and Grasser, 1990; Carano *et al.*, 1990; Breuil *et al.*, 1998) and, based on findings for alendronate, could be achieved within the osteoclast resorption lacuna (Sato *et al.*, 1991). N-BPs (zoledronate, risedronate, ibandronate, alendronate, and pamidronate) also inhibit protein isoprenylation in osteoclasts *in vitro*. Alendronate has been shown to inhibit incorporation of [^{14}C]mevalonate into either isoprenylated proteins or sterols (nonsaponifiable lipid) in purified murine osteoclasts (Bergstrom *et al.*, 2000), whereas another study found that the N-BPs alendronate, ibandronate, pamidronate, risedronate, and zoledronate, but not the non-N-BPs clodronate, etidronate, or tiludronate, prevented incorporation of mevalonate into isoprenylated proteins in purified rabbit osteoclasts (Coxon *et al.*, 2000). Inhibition of protein isoprenylation in osteoclasts was dose-dependent, with alendronate inhibition at $\geq 15\,\mu$M, whereas zoledronate was effective at $\geq 10\,\mu$M (Bergstrom *et al.*, 2000; Coxon *et al.*, 2000).

Evidence of Inhibition of the Mevalonate Pathway in Vivo

The molecular actions of the N-BPs described earlier, have been confirmed in osteoclasts *in vivo* (Fisher *et al.*, 2000; Frith *et al.*, 2001). Direct demonstration of inhibition of FPP synthase *in vivo* is not possible, thus necessitating the use of surrogate markers for measuring this effect. In one study, the previously documented feedback regulation of hydroxymethyl glutaryl-CoA (HMG-CoA) reductase expression by mevalonate pathway metabolites, illustrated by the induction of HMG-CoA reductase in the liver of lovastatin-treated rats (Singer *et al.*, 1988), was examined (Fisher *et al.*, 2000). Rats were therefore administered bisphosphonate and expression of HMG-CoA reductase was examined in the proximal tibia. The N-BPs alendronate, ibandronate, and risedronate, but not clodronate or etidronate (which lack nitrogen), suppressed expression of HMG-CoA reductase in the osteoclast., but no changes were seen in other bone- or marrow-associated cells. This decrease in HMG-CoA reductase expression appeared to be mediated, at least in part, by the accumulation of metabolites upstream of FPP synthase, because coadministration of simvastatin along with alendronate partially blocked the effect. In other studies (Frith *et al.*, 2001; Coxon *et al.*, 2005), geranylgeranylation of the small GTPase Rap1A or of Rab GTPases was measured as a surrogate marker for inhibition of FPP synthase in osteoclasts isolated and purified from rabbits that had been administered N-BPs *in vivo*. Alendronate and risedronate suppressed protein geranylgeranylation in osteoclasts, but not other marrow- and bone-derived cells.

Cellular effects of N-BPs are primarily due to inhibition of FPP synthase

Inhibition of isoprenylation of small GTPases such as Rho, Rac, cdc42, and Rab accounts for many or all of the various effects on osteoclast function, including loss of the ruffled border and disruption of the actin cytoskeleton (Sato *et al.*, 1991; Sato and Grasser, 1991; Selander *et al.*, 1994), altered trafficking of membranes and intracellular proteins such as the osteoclast proton ATPase (Carano *et al.*, 1990; Zimolo *et al.*, 1995), disrupted intracellular signaling by integrins (Fong and Ingber, 1996), and the induction of osteoclast apoptosis (Hughes *et al.*, 1995; Ito *et al.*, 1998, 1999; Reszka *et al.*, 1999).

Several lines of evidence support the current view that the primary mechanism of action of N-BPs is inhibition of FPP synthase and the subsequent prevention of post-translational prenylation of proteins (see Fig. 5). First, the inhibitory effect of N-BPs on osteoclast formation and function, and the induction of osteoclast apoptosis, can be overcome by bypassing the inhibitory effect on the mevalonate pathway and geranylgeranylation of proteins by supplementing cells with geranylgeraniol (Van Beek *et al.*, 1999a; Fisher *et al.*, 1999; Reszka *et al.*, 1999). Geranylgeraniol is intracellularly metabolized to geranylgeranylpyrophosphate (Crick *et al.*, 1997), which serves as substrate for protein prenylation, thus bypassing the inhibition of FPP synthase by N-BPs. Addition of farnesol, mevalonate, or squalene had little or no effect in these studies (Van Beek *et al.*, 1999a; Fisher *et al.*, 1999; Reszka *et al.*, 1999). Second, structure–activity relationships demonstrated a clear correlation between the potency of different N-BPs to inhibit protein prenylation (Luckman *et al.*, 1998a), and inhibit FPP synthase (Dunford *et al.*, 2001) *in vitro*, and the potency to inhibit bone resorption *in vivo*. Third, the effect of N-BPs can be mimicked *in vitro* by other inhibitors of the mevalonate pathway. Lovastatin and mevastatin, which inhibit the upstream enzyme HMG-CoA reductase, also inhibit bone resorption, prevent osteoclast formation in bone marrow cultures, and disrupt the actin cytoskeleton of osteoclasts, and these effects can also be completely blocked by addition of geranylgeraniol, but not farnesol or squalene, to osteoclast cultures (Fisher *et al.*, 1999; Woo *et al.*, 2000). Furthermore, selective inhibition of protein geranylgeranylation by an inhibitor of geranylgeranyl transferase I (GGTI-298) also inhibits bone resorption *in vitro* (Coxon *et al.*, 2000). Taken together, these observations strongly suggest that the ability to inhibit bone resorption is a consequence of inhibition of protein geranylgeranylation.

Effects on Downstream Signaling Pathways

It was noted that N-BP- and statin-induced macrophage apoptosis occurs after a lag period of 15 to 24 hours, whereas in the osteoclast apoptosis is observed at ≥12 hours, although other inducers can act within 2 to 4 hours (Reszka *et al.*, 1999). This seems to be because of the requirement of protein synthesis for the induction of apoptosis by N-BPs and statins (which also prevent isoprenylation) (Coxon *et al.*, 1998). This raised the possibility that apoptosis, and possibly other cellular effects of N-BPs, are the result of the accumulation of unprenylated proteins rather than the loss of normal prenylated ones.

Interestingly, it has been demonstrated recently that the unprenylated forms of Rho family GTPases that accumulate after treatment with N-BPs are in the active GTP-bound form, most likely owing to their inability to interact with regulatory proteins such as Rho-GAP (Dunford *et al.*, 2006). Other studies have shown that inhibition of protein prenylation by statins also results in increased levels of unprenylated, GTP-bound Ras and Rac (Vecchione and Brandes 2002; Cordle *et al.*, 2005). These unprenylated proteins could have dominant negative effects on normal cellular function by inappropriate activation of downstream signaling pathways. In support of this, N-BPs were found to cause sustained activation of p38 in macrophages and breast cancer cells, which was found to be downstream of Rac in macrophages (Merrell *et al.*, 2003; Dunford *et al.*, 2006). However, p38 activation in these cells acted as a survival factor, rather than mediating the cytotoxic and growth inhibitory effects of N-BPs. Nevertheless, these findings raise the possibility that mevalonate pathway inhibitors such as N-BPs may exert their effects through inappropriate stimulation, rather than inhibition, of small GTPase-mediated signaling pathways (Coxon *et al.*, 2006).

It should be noted that an alternative mechanism by which inhibition of FPP synthase can lead to induction of apoptosis, independent from inhibition of prenylation, has recently been proposed. Inhibition of FPP synthase causes intracellular accumulation of upstream metabolites in the mevalonate pathway, including IPP (Bergstrom *et al.*, 2000). This IPP can be intracellularly converted into a toxic ATP analogue called ApppI (see Fig. 5) in osteoclasts and macrophages *in vitro* (Mönkkönen *et al.*, 2006), and accumulation of IPP and ApppI was detected in peritoneal macrophages *in vivo* following an i.p. injection of zoledronate (Mönkkönen *et al.*, 2007). Although ApppI is capable of inducing apoptosis in osteoclasts (Mönkkönen *et al.*, 2006), its functional significance in the induction of apoptosis *in vivo* following N-BP treatment is presently unclear.

ADDITIONAL MOLECULAR TARGETS

Although the primary molecular mechanisms of action of bisphosphonates are now well understood (as discussed earlier), it is likely that (some) bisphosphonates inhibit additional molecular targets that may contribute to (some of) their effects on osteoclasts or other cell types. This is supported by a recent study showing that, in contrast to other N-BPs, the antiresorptive effect of pamidronate could only partially be overcome by replenishing cells with geranylgeraniol (Van Beek *et al.*, 2003), suggesting that mechanisms distinct from inhibition of protein geranylgeranylation may contribute to the effects of this bisphosphonate on osteoclasts.

Prior to the identification of FPP synthase as the major molecular target of N-BPs, bisphosphonates were shown to inhibit protein tyrosine phosphatases (Schmidt *et al.*, 1996; Endo *et al.*, 1996; Murakami *et al.*, 1997; Opas *et al.*, 1997; Skorey *et al.*, 1997). However, the IC_{50}s for inhibition of several protein tyrosine phosphatases by the bisphosphonates tested correlated poorly with their potency for inhibition of bone resorption, suggesting this was not the major antiresorptive mechanism. In another study, tiludronate was shown to inhibit the osteoclast vacuolar H^+-ATPase at micromolar concentrations (David *et al.*, 1996).

In addition, it has become clear in recent years that some N-BPs and N-BP analogues are capable of inhibiting enzymes other than FPP synthase in the mevalonate pathway. Prior to the discovery that N-BPs are potent inhibitors of FPP synthase, it was demonstrated that some N-BPs, most notably incadronate, are inhibitors of squalene synthase (Amin *et al.*, 1992). More recently, a bisphosphonate was discovered that, in addition to inhibiting FPP synthase, is also a weak inhibitor of IPP isomerase (Thompson *et al.*, 2002), and replacement of one of the phosphonate groups of risedronate by a carboxylate group gives rise to a compound (3-PEHPC, also referred to as NE10790) that specifically inhibits Rab geranylgeranyl transferase (Coxon *et al.*, 2001, 2005; Roelofs *et al.*, 2006b). Even more recently, it was demonstrated that some N-BPs, most notably zoledronate and minodronate, are also inhibitors of GGPP synthase, albeit with lower potency than their potency for inhibiting FPP synthase in enzyme activity assays (Guo *et al.*, 2007).

The exact relevance of these various additional molecular targets is presently unclear, but it is worth considering the possibility that the primary mechanism of action for a given bisphosphonate (i.e., inhibition of FPP synthase by N-BPs or metabolism of non-N-BPs to cytotoxic analogues of ATP) may occur in addition to background inhibition of other enzymes. Furthermore, as intracellular concentrations of bisphosphonates increase, inhibition of secondary enzyme(s) may assume greater prominence in their overall effect.

ANTITUMOR EFFECTS OF BISPHOSPHONATES

In addition to the well-characterized antiresorptive effects of bisphosphonates on osteoclasts, these agents also have antitumor effects in a variety of *in vitro* and *in vivo* models.

In Vitro

Bisphosphonates exert antitumor effects on a wide variety of cancer cells *in vitro*. It has been demonstrated that BPs inhibit proliferation and induce apoptosis in human myeloma cells (Shipman *et al.*, 1997, 1998; Aparichio *et al.*, 1998) and osteosarcoma cells *in vitro* (Mackie *et al.*, 2001; Sonneman *et al.*, 2001). Furthermore, BPs exert cytostatic and proapoptotic effects on a variety of other tumor cell types that often metastasize to bone, including breast (Fromigue *et al.*, 2000; Senaratne *et al.*, 2000), pancreatic (Tassone *et al.*, 2003), and melanoma cancer cells (Riebeling *et al.*, 2002 ; Forsea *et al.*, 2004). Whereas tumor cell proliferation and survival are affected at relatively high concentrations (generally $>10\,\mu M$) of bisphosphonate, which may not be attainable *in vivo*, bisphosphonates inhibit invasion of breast and prostate cancer cells *in vitro* at much lower (nanomolar or even picomolar) concentrations (Boissier *et al.*, 2000; Virtanen *et al.*, 2002). In addition, they inhibit adhesion of breast and prostate cancer cells to bone and unmineralized matrices *in vitro* at low micromolar concentrations (Van der Pluijm *et al.*, 1996; Boissier *et al.*, 1997; Virtanen *et al.*, 2002).

In Vivo

Multiple Myeloma

Several studies using animal models of multiple myeloma reported that bisphosphonates are effective in reducing tumor burden *in vivo* (Sasaki *et al.*, 1995; Yaccoby *et al.*, 2002; Croucher *et al.*, 2003), and one study showed that survival of 5T2MM mice receiving zoledronate treatment was increased (Croucher *et al.*, 2003). In addition, some studies demonstrated an increase in apoptosis of tumor cells *in vivo* following BP treatment (Hiraga *et al.*, 2001; Yaccoby *et al.*, 2002). In contrast, other studies failed to demonstrate an increase in the proportion of plasma cells undergoing apoptosis in 5T2MM mice receiving treatment with either ibandronate (Shipman *et al.*, 2000) or zoledronate (Croucher *et al.*, 2003), or a reduction in tumor burden in a murine model of human myeloma following treatment with ibandronate (Dallas *et al.*, 1999; Cruz *et al.*, 2001). It is unclear whether the differences reflect the use of different bisphosphonates, or differences in animal models used.

Bone Metastases

Bisphosphonates have been demonstrated to exert antitumor activity in various animal models of bone metastasis (reviewed in Green, 2004; Clézardin *et al.*, 2005; Stresing *et al.*, 2007). In most studies, bisphosphonate administration was either started on the day of tumor cell inoculation (preventative protocol), or was initiated after tumors were established in bone (treatment protocol). Zoledronate inhibited the progression of bone metastasis in the B02 metastatic breast cancer model (Peyruchaud *et al.*, 2001). Using intracardiac injection of MDA-MB-231 breast cancer cells in severe combined immunodeficient (SCID) mice to simulate human metastatic breast cancer, Yoneda and colleagues showed that risedronate, ibandronate, and zoledronate inhibited the development of new bone metastases as well as progression of established bone metastases (Yoneda *et al.*, 2000). In a more recent study, using a similar animal model, N-BP treatment was most effective in the preventative setting, and was much less effective in reducing tumor burden already established (Van der Pluijm *et al.*, 2005). Bisphosphonates also prevented development of bone metastasis in a mouse model of breast cancer in which 4T1 mouse breast cancer cells are injected into the mammary fat pad of syngeneic mice. Tumor burden in bone was decreased and an increase in apoptosis of metastatic 4T1 tumor cells was observed (Yoneda *et al.*, 2000). Bisphosphonates have also been shown to inhibit the development and/or progression of bone metastases secondary to other types of cancer, such as small-cell lung (Yano *et al.*, 2003) and prostate cancer (Corey *et al.*, 2003).

Soft-Tissue Tumors

Although some studies showed no effect on visceral metastases (Kostenuik *et al.*, 1993; Sasaki *et al.*, 1995; Michigami *et al.*, 2002; Yano *et al.*, 2003), several more recent studies reported an inhibitory effect of bisphosphonate therapy on the development and progression of soft-tissue tumors (Hiraga *et al.*, 2004; Hashimoto *et al.*, 2005; Ory *et al.*, 2005).

Patients

Despite studies in preclinical models that have clearly shown the potential of bisphosphonates as antitumor agents, the current dosing regimens of bisphosphonates in humans fail to show any convincing antitumor effects. This may in part be because of the relatively high total cumulative doses of bisphosphonate administered in many animal studies, far exceeding the current recommended dose for humans. In this respect, it is interesting to note that results from a recent study in a mouse model of breast cancer metastasis suggest that, while maintaining the same total cumulative dose, treatment with frequent (i.e., daily or twice weekly) low-dose zoledronate may be more effective in decreasing tumor burden than the currently used once monthly treatment regimen in cancer patients (Daubine *et al.*, 2007). This suggests that altering the dosing regimen to a more frequent administration may improve efficacy in cancer patients.

MECHANISMS OF ANTITUMOR EFFECTS

The exact mechanisms for the observed antitumor effects in preclinical models are presently unclear. Bisphosphonates could exert antitumor effects *in vivo* via direct effects of the bisphosphonate on the tumor cells, potentially resulting in inhibition of tumor cell adhesion, invasion, migration, survival, and proliferation, as described preciously. Alternatively, the effects of bisphosphonates on skeletal metastasis or tumor burden in bone could result from inhibition of bone resorption, by reducing the levels of tumor growth and survival factors in the bone microenvironment. Bisphosphonates have also been suggested to exert indirect antitumor effects via inhibition of angiogenesis and/or immunomodulatory effects (reviewed in Roelofs *et al.*, 2006a; Stresing *et al.*, 2007). Owing to the rapid accumulation of bisphosphonate in bone, the brief serum half-life, and low serum concentrations (nM), effects of bisphosphonates are produced primarily by the bisphosphonate on the bone surface. The degree to which tumor cells or any other nonosteoclast cells are exposed to bisphosphonate *in vivo*, therefore, remains to be established.

Indirect Antitumor Effects via Inhibition of Bone Resorption

In the bone marrow microenvironment, the interaction between the tumor cells and the osteoclasts, via cell-to-cell contact and/or secretion of various growth factors and cytokines, leads to expansion of the tumor burden on the one hand, and exacerbates the osteolytic bone disease on the other. Local decreases in the liberation or production of tumor-stimulating cytokines such as transforming growth factor (TGF-β) and insulin-like growth factors (IGFs) following a reduction in osteolysis would interfere with the "seed and soil" interactions between tumor cells and the bone microenvironment (Martin *et al.*, 2005; Matsumoto and Abe, 2005; Guise *et al.*, 2006;). This raises the possibility that the reduction in tumor burden observed in some animal models could be accounted for by inhibition of osteoclast activity. In support of this, a recent study demonstrated that treatment with the osteoclast inhibitor osteoprotegerin resulted in similar decreases in tumor burden, increases in tumor cell apoptosis, and decreases in tumor cell proliferation in a mouse model of breast cancer metastasis as that observed following treatment with ibandronate (Zheng *et al.*, 2007). However, conclusive evidence for either a direct or indirect antitumor activity of bisphosphonates is currently lacking.

Antiangiogenic Effects

Results from *in vitro* and *in vivo* studies have indicated that bisphosphonates have antiangiogenic properties. In *in vitro* experiments, bisphosphonates have been shown to inhibit endothelial cell proliferation, adhesion of endothelial cells to matrix proteins, and capillary-like tube formation (Fournier *et al.*, 2002; Wood *et al.*, 2002; Bezzi *et al.*, 2003). In addition, Fournier *et al.,* 2002) demonstrated that bisphosphonates such as ibandronate and zoledronate reduced revascularization of the prostate gland in a testosterone-stimulated angiogenesis model in castrated rats, and Wood *et al.,* 2002) demonstrated antiangiogenic effects of ZOL in the rat aortic ring assay and the chicken egg chorioallantoic membrane assay. Recently, zoledronate treatment of 5T2MM-bearing mice has been shown to result in a decrease in microvessel density in areas of tumor cell infiltration (Croucher *et al.*, 2003). Zoledronate also decreased vessel density and increased apoptosis of endothelial cells in tumors in a mouse model of cervical carcinoma (Giraudo *et al.*, 2004). Similarly, minodronate treatment has been shown to decrease tumor blood vessel density in a mouse model of melanoma (Yamagishi *et al.*, 2004). In all animal models, this was associated with decreases in tumor burden. In patients with solid tumors, pamidronate and zoledronate treatment has been shown to result in lower circulating levels of proangiogenic vascular endothelial growth factor (VEGF) and platelet-derived growth factor (Santini *et al.*, 2002, 2003; Vincenzi *et al.*, 2005). It is presently unclear whether bisphosphonates directly affect endothelial cells *in vivo* to cause antiangiogenic effects and subsequent decreases in tumor growth and survival, or whether the antiangiogenic effects observed *in vivo* are the indirect result of a decrease in secretion of angiogenic factors such as VEGF by the tumor cells and/or other cells affected by bisphosphonates.

Immunomodulatory Effects

Another way in which N-BPs could exert indirect antitumor effects is by activating γδ T cells. Specifically, bisphosphonates are able to activate Vγ9 Vδ2 T cells, the major γδ T cell subset in humans. Activation of these γδ T cells is believed to be the initiating factor in causing the acute-phase response to an intravenous infusion with bisphosphonates (Fig. 7; see also later). These Vγ9 Vδ2 T cells are, however, also important players in tumor surveillance by the innate immune system (Kunzmann *et al.*, 2005), and activation of this subset of T cells by bisphosphonates may have antitumor effects. Activated Vγ9 Vδ2 T cells are capable of directly recognizing and killing tumor cells, or they may exert antitumor effects indirectly via release of interferon-γ, which has inhibitory effects on tumor growth and angiogenesis (reviewed in Thompson and Rogers, 2006). Activation and expansion of γδ T cells has been observed *in vivo* in some patients with lymphoid malignancies receiving pamidronate therapy in combination with IL-2 (to support γδ T cell expansion), which correlated with response to therapy in terms of tumor regression (Wilhelm *et al.*, 2003). A further study by Dieli *et al.*, 2003) in cancer patients treated with 4 mg of zoledronic acid (without IL-2) every 3 weeks reported increased IFNγ production from peripheral blood mononuclear cells (PBMCs) *in vitro.* Tumor cells are able to directly stimulate Vγ9 Vδ2 T cells following uptake of bisphosphonate *in vitro* (Kunzmann *et al.*, 2000; Gober *et al.*, 2003). However, whether tumor cells take up sufficient amounts of bisphosphonate *in vivo* to directly stimulate these γδ T cells, or whether activation of γδ T cells is mediated indirectly by other cells is presently unclear.

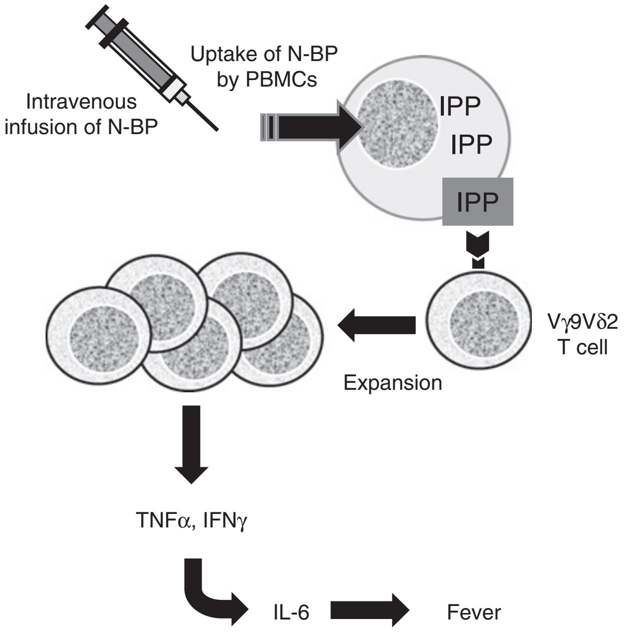

FIGURE 7 The acute phase reaction to N-BPs. After IV infusion, transient uptake of nitrogen-containing bisphosphonates by peripheral blood mononuclear cells (PBMCs) causes inhibition of FPP synthase and thus the accumulation of IPP. Recognition of IPP by γ-δ T cells triggers their activation and expansion and causes release of pro-inflammatory cytokines, resulting in a flu-like response.

SAFETY AND ADVERSE EFFECTS

Toxicological animal studies have been published for several bisphosphonates. Acute, subacute, and chronic administration in several animal species have, in general, revealed little toxicity. Teratogenicity, mitogenicity, and carcinogenicity tests have been negative. When bisphosphonates are administered subcutaneously, local toxicity can occur, with local necrosis. Mild hypocalcemia owing to inhibition of bone resorption has been observed at high doses. Acute systemic toxicity and hypocalcemia can also be caused following intravenous administration of high doses of less potent bisphosphonates by the formation of complexes with calcium.

The chronic toxicity of the bisphosphonates is extremely low. This probably owes, in part, to their strong affinity for bone, which allows them to be cleared rapidly from blood. The toxic effects occur, in general, when doses substantially larger than those that inhibit bone resorption are used. As for polyphosphates and phosphate itself, the first alterations are usually seen in the kidney (Alden *et al.*, 1989; Cal and Daley-Yates, 1990). The liver, as well as the testis, the epididymis, the prostate, and possibly the lung, can in some cases also show changes.

The most relevant toxicity associated with the low-potency bisphosphonate etidronate is the inhibition of bone and cartilage calcification, as described earlier (King *et al.*, 1971; Schenk *et al.*, 1973). This effect starts to occur in animals at parenteral doses of approximately 5 to 10 mg P/kg daily. The radiological appearance resembles rickets or osteomalacia, although there are some histological differences. Fractures can occur after long-term administration of high doses and are probably the result of defective mineralization (Flora *et al.*, 1980). Developmental disturbances of enamel can also appear at high systemic doses. This problem is not observed with the more potent bisphosphonates in current use, as these are administered at far lower doses.

Some bisphosphonates, such as etidronate and pamidronate, cross the placenta and can affect the fetus. Very large doses can lead to a decrease in the number of live pups, to abnormalities of the fetal skeleton and the skin, and to malformations and hemorrhages (Eguchi *et al.*, 1982; Graepel *et al.*, 1992; Okazaki *et al.*, 1995). In view of these results, bisphosphonates should not be administered to pregnant women.

Gastrointestinal Toxicity

Following the appearance in humans of gastrointestinal adverse events after oral administration of N-containing bisphosphonates (De Groen *et al.*, 1996), the effects of oral bisphosphonates were studied in animals. Alendronate, given orally to rats at suprapharmacological doses, has been reported to occasionally induce gastric and esophageal erosions and ulcerations and delay healing of indomethacin-induced gastric erosions. These effects are not attributable to changes in gastric acid secretion, or prostaglandin synthesis, but are thought to be caused by a topical irritant effect. Similar effects were reported with etidronate, risedronate, and tiludronate when given at pharmacologically equivalent doses. These effects were obtained at doses equivalent to (Peter *et al.*, 1998a) or much larger than the ones given in humans (Blank *et al.*, 1997; Elliott *et al.*, 1998; Peter *et al.*, 1998b).

Recent studies have examined potential mechanisms by using *in vitro* models for the esophageal stratified epithelium and the large intestine (Suri *et al.*, 2001; Reszka *et al.*, 2001). N-BPs were shown to induce apoptosis in CACO-2 intestinal epithelium cells and in Ch1.Es esophageal fibroblasts, and to decrease proliferation in normal human epidermal keratinocytes and CACO-2 cells (Suri *et al.*, 2001; Reszka *et al.*, 2001). These effects were associated with an inhibition of protein prenylation and could be prevented by geranylgeraniol, suggesting that N-BP inhibition of protein geranylgeranylation was instrumental in the antiproliferative and apoptotic response. These *in vitro* studies thus suggest that the N-BP-induced gastrointestinal irritation and/or delayed repair of irritation may be mediated by inhibition of FPP synthase in the affected tissues. These findings await *in vivo* validation. It has also been shown that N-BPs compromise the surface hydrophobic phospholipid barrier of gastrointestinal tissue in rats, which may cause mucosal injury and ulceration (Lichtenberger *et al.*, 2000).

Acute-Phase Reaction

Intravenous administration of N-BPs is commonly associated with a flu-like syndrome known as the acute-phase reaction, which occurs in roughly one-third of patients receiving some N-BPs for the first time (Adami *et al.*, 1987; Schweitzer *et al.*, 1995). The molecular mechanism underlying this response was first revealed by Kunzmann *et al.* in 1999, who reported that patients that suffered an acute-phase reaction to pamidronate had increased circulating levels of $\gamma\delta$ T cells up to 28 days after the infusion (Kunzmann *et al.*, 1999). It is now clear that activation and proliferation of $\gamma\delta$ T cells (Kunzmann *et al.*, 1999, 2000) and the release of pro-inflammatory cytokines such as TNFα, and IFNγ (Schweitzer *et al.*, 1995; Sauty *et al.*, 1996; Thiebaud *et al.*, 1997) underlie the acute-phase reaction (see Fig. 7).

N-BPs were previously thought to directly activate Vγ9 Vδ2 T cells by acting as antigens for the Vγ9 Vδ2 T cell receptor (Das *et al.*, 2001). However, more recent evidence suggests that N-BPs indirectly activate Vγ9 Vδ2 T cells through inhibition of FPP synthase in PBMCs, and the resulting intracellular accumulation of upstream metabolites such as IPP and DMAPP (see Fig. 7; Gober *et al.*, 2003; Thompson *et al.*, 2004; Hewitt *et al.*, 2005; Thompson *et al.*, 2006b), which are known antigens for the Vγ9 Vδ2 T cell receptor (Tanaka *et al.*, 1995). This hypothesis is supported by the finding that N-BPs must be internalized in order to activate Vγ9 Vδ2 T cells *in vitro* (Gober *et al.*, 2003), that the ability to activate Vγ9 Vδ2 T cells closely matches the ability to inhibit FPP synthase (Thompson *et al.*, 2004), and that statins, which inhibit the upstream enzyme HMG-CoA reductase, can prevent Vγ9 Vδ2 T cell activation *in vitro* most likely by preventing the intracellular accumulation of IPP, DMAPP, and possibly other mevalonate pathway metabolites (Gober *et al.*, 2003; Thompson *et al.*, 2004; Hewitt *et al.*, 2005).

Osteonecrosis of the Jaw

Recently, osteonecrosis of the jaw (ONJ) has emerged as a potential complication, particularly with long-term, high-dose intravenous bisphosphonate therapy in malignant diseases (Maerevoet *et al.*, 2005). Although many of the clinical and pathological features are debated, the current definition is of nonhealing lesions involving exposed bone in the mandible or maxilla. Most reported ONJ cases have been in cancer patients, especially those with myeloma or breast cancer metastases, who received i.v. bisphosphonates (pamidronate and zoledronate), although some cases of ONJ have also been reported in patients on oral bisphosphonate therapy for benign disorders. ONJ appears to be frequently triggered by dental interventions, such as tooth extraction. There are no prospective properly controlled trials that show an excess of ONJ cases associated with bisphosphonate therapy (Grbic *et al.*, 2008). The etiology of osteonecrosis of the jaw is at present unknown, and relevant prospective clinical trials to study pathogenic mechanisms are currently not available. Many cancer patients presenting with ONJ had also received other drugs, including glucocorticoids or chemotherapeutic agents, making the potential pathogenic mechanisms complex. Recent reviews and guidelines emphasize that any causal role for bisphosphonates remains unproven (Van den Wijngaerten *et al.*, 2006, 2007; Khosla *et al.*, 2007; Glick, 2008).

SUMMARY

Great progress has been made over the past two decades in understanding the mechanism of action of the bisphosphonates. This class of drugs is widely used in the treatment of osteoporosis, Paget's disease, tumor-associated bone disease, with potential uses in several other skeletal conditions. Bisphosphonates target to the skeleton owing to their bone-binding characteristics, where they primarily act by inhibiting osteoclastic bone resorption, although it is presently still debated whether they might directly affect other cell types such as osteoblasts, osteocytes, and tumor cells *in vivo*. The simple bisphosphonates, clodronate, etidronate and tiludronate, are intracellularly metabolized to cytotoxic analogues of ATP, whereas the more potent, nitrogen-containing bisphosphonates act by inhibiting the enzyme FPP synthase, thereby preventing the prenylation of small GTPases that are necessary for the normal function and survival of osteoclasts. With emerging differences between bisphosphonates in bone affinity and enzyme inhibitory potency, it is becoming apparent that each bisphosphonate may have a unique pharmacological profile. Unraveling the exact molecular mechanisms underlying differences in efficacy and adverse effects may help to expand the utility of bisphosphonates and to ensure their overall safe use in the treatment of a variety of bone diseases characterized by excessive osteoclastic bone resorption.

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Chapter 82

The Pharmacology of Estrogens in Osteoporosis

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INTRODUCTION

Control of bone remodeling is intimately linked to estrogen status in the human female and perhaps also in the human male. Loss of estrogen production by the ovary, at the time of menopause, produces and an alteration in the equilibrium between bone resorption and formation (Lindsay, 1995). The consequence is loss of bone tissue with accompanying microarchitectural damage that leads to increased bone fragility, commonly called osteoporosis (Consensus Development Conference, 1993). Estrogen intervention restores bone remodeling to its premenopausal equilibrium and prevents bone loss. Observational and some clinical trial data suggest that estrogens also reduce the risk of fractures (Weiss *et al.*, 1979; Hutchinsen *et al.*, 1979; Paganini-Hill *et al.*, 1981; Kreiger *et al.*, 1982; Kiel *et al.*, 1987; Lindsay, 1995; Lindsay, 1980; Komulainen, 1998; Herrington, 2000 add), the only clinical consequence of osteoporosis. The precise mechanism by which estrogens control bone remodeling is still relatively poorly understood, although significant advances in our understanding have been made in recent years. In this chapter, we will review the current status of our knowledge of the actions of sex steroids in both prevention and treatment of osteoporosis in postmenopausal women.

RESPONSES TO ESTROGEN ADMINISTRATION

Biochemistry

When estrogens are administered to estrogen-deficient women, there is a reduction in bone remodeling that can be monitored biochemically (Siebel *et al.*, 1993). During the initial weeks of therapy, there is a gradual decline in biochemical markers of bone resorption aminoterminal and carboxyterminal telopeptides such as the pyridinoline cross-linking molecules [NTX or CTX] (Delmas, 1995). There is a similar but more delayed decline in biochemical measures of bone formation, such as osteocalcin and bone-specific alkaline phosphatase. The timing of these biochemical changes is presumed to represent an initial rapid reduction in activation of new remodeling sites, and a reduction in the work rate of active osteoclasts. Because resorption is the initial event in remodeling, a decline in the rate at which new remodeling sites are coming "on-line," initiated at the start of treatment, would produce a reduced supply of the products of resorption into the circulation. Formation follows resorption and is a more prolonged process. Consequently, formation would be expected to continue unabated in those remodeling sites activated before treatment until the osteoblast teams complete their work. These modifications in skeletal remodeling may occur through direct effects of estrogens on bone cells and in part indirectly by increased resistance to the bone-resorbing effects of parathyroid hormone (PTH). Accompanying these changes in skeletal metabolism are modifications in calcium homeostasis, including reduced renal excretion of calcium, improved hydroxylation of 25(OH)D to $1,25(OH)_2D$, and improved calcium absorption across the intestine. In some studies, declining remodeling within the skeleton is also associated with a small fall in serum calcium and a rise in PTH that might affect the renal and intestinal calcium handling.

Bone Density

The consequence of these alterations in skeletal homeostasis is preservation of bone mass, which has been detailed in many controlled studies (Lindsay, *et al.,* 1976, 1978a, 1980, 1984; Horsmann *et al.*, 1977; Christiansen and Rodbro, 1983 add PEPI(Postmenopausal Estrogen Progestin Intervention

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Study) and WHI). Most studies in which estrogens are given during active bone loss provide evidence of a gradual increase in measured bone mass or density that occurs primarily during the first 1 to 2 years of treatment. It has been assumed that this phenomenon results from the reduced activation of remodeling, which would mean that at any time point after initiation of therapy, there would be a smaller proportion of the skeleton undergoing the remodeling process. The consequence would be an apparent gradual increase in bone mass, without the necessity of an anabolic effect of estrogen. This increment would be time dependent during the early months of treatment as completing remodeling sites gradually filled with new bone tissue, and bone mass would be expected to be relatively stable thereafter (Delmas, 1995). Indeed, all agents that act by reducing bone remodeling produce the same effect during the first 1 to 2 years of treatment. The magnitude of the effects vary depending upon the site measured (the spine usually shows greater increases) and the magnitude of the reduction in remodeling (bisphosphonates and estrogen are most potent of the currently available agents but RANK-ligand antibody (denosumate) may be even more potent). Additionally, with slower rates of remodeling there may be a longer period of secondary mineralization that would also appear as an increase in bone mineral density (BMD) by DXA(Dual X ray absorptiometry).

In long-term studies with estrogens, there are somewhat divergent results, possibly owing to a variety of issues. First, it is clear that the long-term compliance with estrogen is less than perfect, and consequently bone loss, which is known to restart when treatment is stopped, might be expected to be seen among some individuals who are not compliant with treatment (Lindsay *et al.*, 1978; Christiansen and Christiansen, 1981). Second, the response of the skeleton may be variable and dependent upon the bone measured. For example, the vertebral bodies contain the highest proportion of cancellous bone and produce the greatest initial response to estrogen. Some data suggest that there is very little subsequent loss in the vertebrae, whereas the hip may be more sensitive to other factors, especially physical activity (Lindsay, 1995; Orwoll *et al.*, 1996). Thus, although initial cross-sectional data suggested very little loss of bone from any skeletal sites, more recent longitudinal data have confirmed some slow loss (~0.5% per year) from the hip in patients who have been on long-term estrogen (more than 5 years).

When estrogen therapy is discontinued, there is an immediate increase in bone remodeling that is reminiscent of ovariectomy (Lindsay *et al.*, 1978; Christiansen and Christiansen, 1981). The consequence of this is an increase in the rate of bone loss (Lindsay *et al.*, 1978; Greenspan *et al.*, 1999). The longer the process of bone loss continues, the more the treated population will begin to resemble an untreated population in terms of bone density. More than 20 years ago, we demonstrated that the effects of 4 years of estrogen intervention were virtually lost 4 years after estrogens had been discontinued (Lindsay *et al.*, 1978).

Effects on Fracture

The consequence of reduced bone turnover and prevention of bone loss is a reduction in the risk of fracture. Numerous epidemiological studies have shown that estrogen exposure is associated with a reduction in the risk of fractures of the hip and wrist (Lindsay *et al.*, 1976, 1978a; 1978b; 1980a; 1984; Horsman *et al.*, 1977; Christiansen and Christiansen, 1981; Christiansen and Rodbro, 1983). In general, there appears to be about a 50% reduction in hip fracture risk. More recent data indicate that the estrogen effect is greatest among those currently taking treatment, and those who began earliest (Cauley *et al.*, 1995). These epidemiological data are compatible with the prospective effects on bone mass and emphasize the importance of continued therapy to obtain the maximum effects on fracture. Because menopause occurs at the average age of 50, and hip fractures affect mostly women over 70, this may mean 20 to 30 years of treatment, or even treatment for life may be necessary. Controlled clinical trial data suggest a reduction of 50% to 80% in the risk of vertebral fractures, when estrogens are used for prevention (Lindsay *et al.*, 1980a) or treatment (Lufkin, 1992). One recent clinical trial has confirmed the effects of hormone replacement therapy (HRT) on clinical nonvertebral fractures in a recently menopausal population (Komulainen, 1998). However, in one study of HRT's effects on cardiovascular disease (the largest outcome study completed as yet using HRT), there was an equal number of fractures in the HRT and placebo arms, casting doubt on fracture efficacy (Hulley *et al.*, 1998). It is important to emphasize that this particular study recruited women based upon cardiac history without regard to skeletal status, which was largely unknown. Moreover, there was a high incidence of obesity in that population, which might have reduced the likelihood of a significant estrogen effect on fractures. There was no comprehensive evaluation of vertebral compression fracture because spine radiographs were not routinely performed. However, there did not appear to be a reduction in height loss. In another smaller study in which cardiac outcomes were also assessed by cardiac catherization, although there was no effect on atheroma, there was a reduction in the number of clinical fractures. Thus, despite one negative study, most data support a fracture benefit for estrogens.

These data are supported by the results from the Womens Health Initiative (WHI). This study comprises the two largest clinical trials completed with hormonal therapy. The results have been published in detail (Writing Group for the Women's Health Initiative Investigators, 2002). Briefly, roughly 16,000 asymptomatic women between ages 50 and 79 who had transitioned through a natural menopause were randomized to conjugated equine estrogens (CEE; 0.625 mg/day) plus medroxyprogesterone acetate (MPA; 5 mg/day) or placebo. The outcomes were cardiovascular disease, breast and other cancers, and fractures. The second WHI study randomized 10,000 hysterectomized women to CEE 0.625 mg/day or placebo, with the same outcome measures (Writing Group for the Women's Health Initiative Investigators, 2004). Both studies were discontinued prior to completion, the former because of an increase in the "global index" that suggested overall harm to the population, and the second because of an increase in the risk of stroke. In both studies there were significant reductions in the risk of all fractures, and particularly fractures of the hip and spine (Cauley *et al.*, 2003; Jackson *et al.*, 2006). More recent data from WHI suggest that much of the risk occurs in the older population (Roussow *et al.*, 2007) and CEE alone was not associated with an increased risk of breast cancer. BMD was performed in only a small segment of the overall groups and a small minority had osteoporosis by BMD. WHI, therefore, provides the most robust data for fracture reduction in individuals with relatively normal BMD. Although it is clear from WHI that giving hormone therapy (HT) or estrogen therapy (ET) to older individuals cannot be recommended, the studies suggest that in selected younger women who are close to menopause ET/HT remains a viable option for prevention of bone loss and osteoporosis-related fractures.

Route of Administration and Dosing

The evidence suggests that the route of estrogen administration required to obtain a skeletal effect is irrelevant and that if adequate doses are given, reduced bone remodeling results (Christiansen and Lindsay, 1991). Thus, data obtained from transdermal, percutaneous, and subcutaneous as well as oral administrations all confirm skeletal conservation. For transdermal estrogens, the 50-μg patch, which results in estradiol levels in circulation of ~50 pg/ml, appears sufficient (Lufkin *et al.*, 1992) Prestuad *et al.* have shown that 0.25mg micronized estradiol increases BMD with minimal estrogen target organ effects. In contrast, vaginal estrogen administration does not appear to result in high enough estrogen levels in circulation to exert an effect on skeletal remodeling.

After WHI, there was renewed interest into whether doses of estrogen lower than 0.625 mg CEE or its equivalent could provide skeletal benefit. Although no fracture data exist for these lower doses, BMD and turnover data clearly demonstrate skeletal activity. For CEE, 0.3 mg is the lowest dose examined in clinical trials (Lindsay, 2002; Lindsay *et al.*, 2005). For transdermal estrogen, a dose of 0.014 mg/day appears to have at least some activity (Ettinger *et al.*, 2004).

Presently, data demonstrate that all estrogens used in the postmenopausal population produce skeletal effects. For some, such as CEEs, the data include dose-response and fracture outcomes. For others, more limited data on bone mass are available, often without good dose-response data. Synthetic estrogens such as those used in the oral contraceptive also produce skeletal effects in estrogen-deficient individuals (Lindsay *et al.*, 1976). Indeed ethinyl-estradiol in doses between 5 and 10 μg per day prevent bone loss. In addition, at the 5-μg dose there appears to be a modest increase when norethindrone (1 mg/day) is combined with the estrogen (Rowan *et al.*, 2006).

However, the use of oral contraceptives, with doses of ethinyl-estradiol between 20 and 30 μg per day, probably inhibits skeletal maturation in growing teenagers. Whether this results in long-term effects is not clear. Whether the use of oral contraceptives among premenopausal but skeletally mature women produces much in the way of alterations in skeletal homeostasis is not clear but seems unlikely.

Reduction in skeletal remodeling is an effect that can be produced with other steroids. Progestins given to postmenopausal women also produce reductions in bone remodeling and prevention of bone loss (Lindsay *et al.*, 1978). The required doses are greater than those normally used in estrogen-deficient women when given with estrogen to protect the uterus (20 mg/day for medroxyprogesterone acetate [MPA] and 5 mg/day for norethindrone) (Lindsay *et al.*, 1978; Gallagher and Kable, 1991). The addition of a C-21 progestin in more conventional doses to estrogen therapy does not modify the estrogen response significantly (PEPI). However, there may be a modest additive effect when doses lower than 0.625 mg are supplemeneted with an MPA (Lindsay, 2001). As noted in earlier, there are some data suggesting an additive effect of the 19-nortestosterone derivative norethindrone when given in combination with 5 μg of ethinyl-estradiol (Speroff, 1996). On the other hand, evidence suggests that the progestins, when used by themselves for contraceptive purposes (Depo-MPA) in premenopausal estrogen-replete women, will actually be detrimental to skeletal health, presumably by reducing endogenous production of estrogen by the ovary (Mark, 1994). Although there is clear evidence of bone loss in that situation, no fracture data exist. There is no evidence that progestin creams can result in high enough circulating levels of progesterone to produce skeletal effects.

The activity of progestins on bone is also evident in the data published on tibolone (Lindsay *et al.*, 1980; Milner *et al.*, 2000; Castelo-Branco, 2000). This steroid is a pro-drug related to norethindrone and thus is a 19-nortestosterone derivative. It has androgenic, progestogenic, and weak estrogenic activity. In doses that do not appear to stimulate the endometrium, it produces estrogen-like effects in bone. Thus, tibolone is a unique tissue-selective compound, that produces some effects related to its androgenicity and progestin activity rather than its estrogen action.

Estrogens are potent hormones with effects on multiple tissues. Some of these effects confer significant benefit and some significant risk to the postmenopausal women. One clear-cut benefit of HRT is the suppression of menopausal symptoms, such as hot flashes and vaginal dryness. Many of the other potential benefits of HRT, however, have not been proven. WHI results suggest that estrogens do not decrease the risk of cardiovascular disease, although this may differ in younger women close to menopause (Clarkson, 2007). A large body of clinical trial data indicates that estrogen improves intermediate markers of cardiovascular health, including cholesterol and other lipoproteins, and the vasodilatory coronary artery response to acetylcholine (Hodis *et al.,* 2003; Manson *et al.,* 2003; Writing Group for PEPI, 1995). In addition to WHI, two other studies found a lack of efficacy against clinical heart disease outcomes in patients with active cardiovascular disease (HERS) Health and Estrogen Replacement Study (Hulley *et al.*, 1998) and a lack of regression of coronary artery plaque (Herrington *et al.*, 2000) in those women assigned to estrogen or HRT. Clarkson has developed the interesting hypothesis based on data from nonhuman primates that suggests that there is a window of opportunity for cardiovascular protection that may close a few years after menopause (Clarkson, 2007). The reanalyses of WHI seem in part to support this hypothesis because the majority of the risk occurred among the older population.

Women's Health Initiative Mental Status Study (WHIMS), a substudy of WHI, suggested that HT may be associated with a worsening of cognitive function. However, WHIMS was a study of women over age 65, and if the Clarkson hypothesis applies to the brain also (for which we have no evidence) then a positive result would not be expected. HT appears also to worsen urogenital symptoms such as incontinence in some studies. HRT increases the risk of venous thromboembolic disease threefold and the risk of gallbladder disease twofold.

Estrogens also cause endometrial hyperplasia and increase the risk of endometrial malignancy (Jick *et al.*, 1979; Shapiro *et al.*, 1985). Protection of the endometrium is the only rationale for the prescription of a progestin along with estrogen to postmenopausal women (Woodruff and Pickar, 1994). Consequently, there has been considerable interest in the idea that estrogen analogs (Selective Estrogen Receptor Modulators – SERMs) might be found that would provide the beneficial effects of estrogen on bone, brain, and heart, without endometrial or breast stimulation. There are at least two forms of the estrogen receptor (alpha and beta) with dimerization required for gene activation, and heterodimerization possible and thus separation of estrogen effects by tissue might be possible. In addition, multiple coactivator and corepressors exist with different tissue distribution, further emphasizing how estrogens and estrogen-like drugs might act differently in different tissues.

ESTROGEN ACTIONS

The molecular actions of estrogens have been reviewed in detail elsewhere in this book. Estrogen receptors (ER) are members of the steroid receptor family that include thyroid hormone, vitamin D, and retinoids among their ligands as well as sex steroids and adrenal steroids (Auchus and Fugue, 1994). In cells that express estrogen receptors, estrogens produce a potential cascade of gene activation that is dependent on a variety of intracellular factors. Generally responsive cells contain 10,000 to 100,000 receptors, and clearly the concentration of receptors is one variable that might affect response (in general, lower levels appear in nonreproductive cells) (Brown, 1994). After ligand binding the receptor complex dimerizes and complex binds to DNA with high affinity that is dependent on the integrity of the amino acid sequence related to the base of the first zinc finger on the C region of the receptor (Picard *et al.*, 1990). In addition, ligand binding alters the conformation of the receptor, exposing the DNA-binding domain. In addition, while ligand binding is usual for dimerization, DNA binding, and subsequent transcription activity, the ERs can activate genes in the absence of ligand. DNA binding and transcription is also modulated by a variety of cellular proteins (coactivators and corepressors plus other transcription activators that may modulate the responsivity of the genome to ER even in the presence of ligand) (Ronies and Spelsberg, 1989; Brown, 1994).

Given the complexity of the system, it is not difficult to suggest how estrogen responsivity might be modulated. Abnormal receptors, or abnormal conformational changes in the receptor upon ligand binding, the absence of cofactors, or the alteration in the response elements of target genes all might modify the cellular responses to estrogen (Auchus and Fugue, 1994). Tissue selectivity of estrogen response could be obtained by differences in receptor number or the expression of factors within the cells of that tissue influencing the cascade, for example, the capability to phosphorylate the ER ligand complex.

Several genes are targets for estrogen actions including nuclear proto-oncogenes (c-*fos*, c-*myc*, and c-*jun*), and the estrogen responses include expression of several genes including growth factors and cytokines (Murphy and Murphy, 1994).

The estrogen target that is responsible for the effects of these agents in bone is still not clearly understood. Osteoblasts have been shown to have ERs (Eriksen *et al.*, 1988; Komm *et al.*, 1988), but very variable responses to estrogen in osteoblasts or osteoblast cell lines have been published (Turner *et al.*, 1994). Indeed some evidence suggests that only cells transfected with a functional ER will respond to estrogens (Ernst *et al.*, 1991). Some data suggest that osteoclasts also respond directly to estrogens (Oursler *et al.*, 1991). The strongest evidence of ERs in osteoclasts comes from studies of avian osteoclasts. Rat osteoclasts probably do not respond to estrogens, and there is almost no information on human osteoclasts (Arnett *et al.*, 1996). The most likely cell targets for estrogen appear to be other constituent cells of bone marrow. Release of second messengers, such as TGF-β (transforming growth factor-beta), or reduction in secretion of cytokines, such as IL-1 or IL-6, could produce estrogen effects by reducing the recruitment and maturation of osteoclasts (Oursler *et al.*, 1991; Turner *et al.*, 1994). Estrogen has also been reported to cause osteoclast apoptosis, although not in all systems (Arnett *et al.*, 1996). Finally, estrogens also have effects on calcium homeostasis. Estrogens reduce urinary calcium loss and, at the renal level, cause an increase in PTH activity. Estrogens, however, increase skeletal resistance to the resorptive stimulatory effects of PTH without appearing to interfere with the anabolic effects of PTH (Cosman *et al.*, 1993). Estrogens also stimulate intestinal absorption of calcium, perhaps directly or by modulating the effects of PTH on the renal 1α-hydroxylase, thereby increasing the supply of 1,25-dihydroxyvitamin D. Whether these effects of estrogen on the calcium homeostatic system are primary or secondary to the skeletal effects of estrogens is not clear.

The knowledge that estrogen responsivity may vary from tissue to tissue has clearly heightened interest in the concept of the ideal estrogen. Such a compound would produce the beneficial effects of estrogen on bone, cardiovascular system, brain, and urogenital tissues without stimulating endometrial growth or producing effects on mammary tissue that might increase the risk of breast cancer. Several compounds are under investigation for such activities. The first noted is tibolone, which clearly produces bone effects at doses that do not appear to produce much endometrial stimulation. It is not clear, however, whether tibolone can reduce the risk of ischemic heart disease. Because the effects of estrogen on the cardiovascular system probably include multiple effects on lipid metabolism and on the physiology of the circulatory system, further investigation of this compound would seem to be warranted, even though it does not produce an estrogen-like response in terms of circulating lipoproteins. The realization that tamoxifen exerted estrogen-like effects in some cell systems stimulated evaluation of its effects in women who were being given tamoxifen as adjuvant treatment for breast cancer (Cosman and Lindsay, 1999). Surprisingly, these women were not losing bone when compared with postmenopausal controls. Formal controlled clinical trials confirmed that tamoxifen does appear to conserve bone mass and produce estrogen-like effects on lipoprotein metabolism (Cosman and Lindsay, 1999). Tamoxifen also stimulates the endometrium, albeit weakly, and endometrial cancer is a recognized risk of long-term tamoxifen treatment. Knowledge of these effects awakened interest in the pharmaceutical industry in compounds that might have tamoxifen-like effects on breast cancer but would produce estrogen-like effects on bone and cardiovascular diseases. Raloxifene, a benzothiaphene, is now available for prevention and treatment of osteoporosis. Raloxifene and tamoxifen produce similar estrogen-like effects on bone in ovariectomized rats (Black *et al.*, 1994). However, raloxifene appears to have little stimulatory effects on rat uterus. Raloxifene reduces bone remodeling and prevents bone loss in postmenopausal women. Raloxifene reduces the risk of vertebral fractures, but did not have a statistically significant effect on nonvertebral fractures (Ettinger *et al.*, 1999). The use of raloxifene in clinical practice is strongly influenced by its capacity to reduce the clinical appearance of ER-positive breast cancer over a 4-year period. Raloxifene also lowers circulating low-density lipoprotein similar to estrogen, although no increase in high-density lipoprotein was seen as is usually the case with oral estrogens (Draper *et al.*, 1996). Raloxifene increases the risk of deep vein thrombosis and pulmonary embolism to the same degree as HRT. Clearly, compounds such as raloxifene will be powerful tools in the dissection of the mechanisms of estrogen action at the molecular level. The realization that different cells respond to estrogen-like molecules in different ways also raises the intriguing question of whether compounds can be found that reduce the recruitment and activity of osteoclasts, like estrogen, and produce lesser effects on bone formation. Tamoxifen, for example, appears to have more potent effects on bone resorption *in vitro* than estrogens, perhaps by being a more potent stimulator of osteoclast apoptosis. Clomiphene also increases trabecular thickness in rats, an effect not usually associated with estrogens.

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Chapter 83

Vitamin D and Analogues

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INTRODUCTION

Vitamin D, its metabolites and analogues constitute a valuable group of compounds that can be used to modulate many aspects of osteoblast and osteoclast biology. The parent vitamin (or UV light that substitutes for any vitamin D pharmaceutical preparation as a source of the parent vitamin) has been used as a treatment for rickets and osteomalacia since its discovery in the 1920s. The first analogue of vitamin D, dihydrotachysterol, was developed for use in metabolic bone disease in the 1930s before the elucidation of the metabolism of vitamin D. In fact, it was not until the discovery of the principal metabolites: 25-hydroxyvitamin D_3 (25-OH-D_3) and 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$), in the early 1970s that further generations of vitamin D analogues were developed (DeLuca, 1988; Jones and Calverley, 1993; Jones *et al.*, 1998). With the understanding of the molecular action of the hormonal form, 1α,25-$(OH)_2D_3$, has come an appreciation that it is not only a *calcemic agent*, regulating calcium and phosphate transport, but also a *cell-differentiating agent*, promoting the terminal development of a number of cell types, including the osteoclast, the enterocyte, and keratinocyte (Miyaura *et al.*, 1981). Thus, pharmaceutical companies have striven hard over the past three decades to separate these two properties and thereby develop synthetic vitamin D analogues with specialized "calcemic" and "non-calcemic" (cell-differentiating) uses (Calverley and Jones, 1992; Bouillon *et al.*, 1995). From this type of research has come several "low-calcemic" agents in recent years in the form of calcipotriol, OCT, 19-nor-1α,25-$(OH)_2D_2$, and 1α-OH-D_2, which have found widespread use in dermatology and the treatment of secondary hyperparathyroidism. Not only do newer analogues include specialized selective vitamin D receptor (VDR) agonists but also VDR antagonists and compounds that target CYP24, a component of the calcitriol metabolism machinery that extends the life of calcitriol within the target cell. These other analogues are thus under development for use in metabolic bone diseases, osteoporosis, and cancer (Jones *et al.*, 1998; Masuda and Jones, 2006). This chapter will review the spectrum of compounds available, possible uses of these compounds, and their potential mechanisms of action.

PHARMACOLOGICALLY IMPORTANT VITAMIN D COMPOUNDS

Vitamin D compounds can be subdivided into three major groups, listed in Tables I through III and described below.

Vitamin D and Its Natural Metabolites

Table I shows the structures of vitamin D_3 and some of its important metabolites. Ironically, vitamin D_3, the natural form of vitamin D, is not approved for use as a drug in the United States, whereas it is available as a pharmaceutical agent or as an over-the-counter supplement in virtually every other country in the world.

During the late 1960s and early 1970s, most of the principal vitamin D metabolites were first isolated and identified by GC-MS and then their exact stereochemical structure determined (DeLuca, 1988). This led to chemical synthesis of the naturally occurring isomer and its testing in various biological assays *in vitro* and *in vivo*. Currently, only the compounds representing the main pathway of vitamin D activation, namely vitamin D_3, 25-hydroxyvitamin D_3 (25-OH-D_3), and 1α,25-$(OH)_2D_3$, are synthesized and available for use as drugs.

Vitamin D Prodrugs

Table II lists some of the important prodrugs of vitamin D. All of these compounds require a step (or more) of activation *in vivo* before they are biologically active. Included here is vitamin D_2, which is derived from the fungal sterol, ergosterol, by irradiation. Because vitamin D_2 is found rarely in nature and is hard to detect in humans eating

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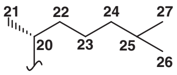
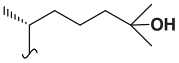
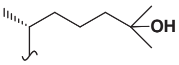
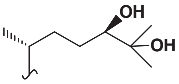
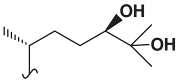
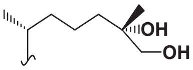
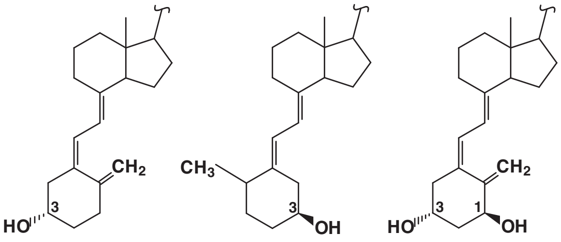
TABLE I Vitamin D and Its Natural Metabolites

Vitamin D metabolites [ring structure][*]	Side-chain structure	Site of synthesis	Relative VDR-binding affinity[†]	Relative DBP-binding affinity[‡]	Reference
Vitamin D_3 [1]		Skin	≤0.001	3,180	Mellanby, 1919 McCollum *et al.*, 1922
25-OH-D_3 [1]		Liver	0.1	66,800	Blunt *et al.*, 1968
1α,25-$(OH)_2D_3$ [3]		Kidney	100	100	Fraser & Kodicek, 1970 Holick *et al.*, 1971
24(R),25-$(OH)_2D_3$ [1]		Kidney	0.02	33,900	Holick *et al.*, 1972
1α,24(R),25-$(OH)_3D_3$ [3]		Target tissues[§]	10	21	Holick *et al.*, 1973
25(S),26-$(OH)_2D_3$ [1]		Liver ?	0.02	26,800	Suda *et al.*, 1970

[*] *Structure of the vitamin D nucleus (secosterol ring structure).*
[†] *Values reproduced from previously published data* (Stern, 1981).
[‡] *Values reproduced from previously published data* (Bishop *et al.*, 1994)
[§] *Known target tissues included intestine, bone, kidney, skin, and the parathyroid gland.*

Vitamin D nucleus



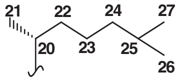
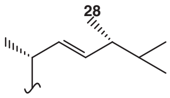
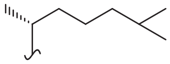
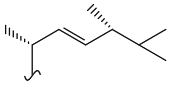
[1] [2] [3]

nonfortified foods, we can consider it to be an artificial form of vitamin D or prodrug. Vitamin D_2 is used as a substitute for the natural form, vitamin D_3, in pharmaceutical preparations or over-the-counter supplements in the United States. Vitamin D_2 possesses two specific modifications of the side chain (see Table II) but these differences do not preclude the same series of activation steps as vitamin D_3, these giving rise to 25-OH-D_2, 1α,25-$(OH)_2D_2$, and 24,25-$(OH)_2D_2$, respectively. Recently, there has been much debate in the vitamin D field, particularly in the United States where vitamin D_2 is the sole agent available, about the relative utility of vitamin D_2 and vitamin D_3 to raise the circulating 25-OH-D level (Vieth, 2005). Evidence from research studies suggests that oral doses of vitamin D_3 are significantly more effective than equivalent doses of vitamin D_2 for increasing the 25-OH-D level into the sufficient range (more than 40 ng/mL) (Trang *et al.*, 1998; Armas *et al.*, 2003).

25-OH-D_3 was developed and approved as the pharmaceutical preparation Calderol in the 1970s by Upjohn, later acquired by Organon, but was withdrawn recently and is currently unavailable. Two other prodrugs, 1α-OH-D_3 and 1α-OH-D_2, were synthesized in the early 1970s (Barton *et al.*, 1973; Paaren *et al.*, 1978) as alternative sources of 1a,25-$(OH)_2D_3$ and 1a,25-$(OH)_2D_2$, respectively, that in the process circumvent the renal 1α-hydroxylase enzyme, which was shown to be tightly regulated and prone to damage in renal disease.

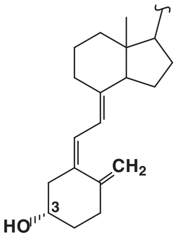
The final compound in the list, dihydrotachysterol (DHT) has lived a complex history as a prodrug. Originally it was believed to be "active" when converted to 25-OH-DHT by

TABLE II Vitamin D Prodrugs

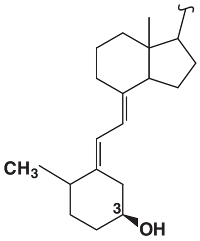
Vitamin D prodrug [ring structure][*]	Side-chain structure	Company	Possible target diseases	Mode of delivery	Reference
1α-OH-D_3 [3]		Leo	Osteoporosis	Systemic	Barton *et al.*, 1973
1α-OH-D_2 [3]		Genzyme	Secondary hyperparathyroidism	Systemic	Paaren *et al.*, 1978
Dihydrotachysterol [2]		Duphar	Renal failure	Systemic	Jones *et al.*, 1988
Vitamin D_2 [1]		Various	Rickets Osteomalacia	Systemic Systemic	Fraser *et al.*, 1973

[*] *Structure of the vitamin* D *nucleus* (*secosterol ring structure*).

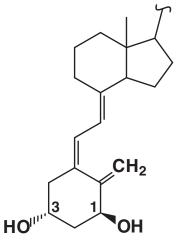
Vitamin D nucleus



[1]



[2]



[3]

virtue of an A ring rotated 180° such that the 3β-hydroxyl function assumes a pseudo-1α-hydroxyl position (Jones *et al.*, 1988). The mechanism of action of DHT has become less clear with the description of the extrarenal metabolism of 25-OH-DHT to 1α,25-$(OH)_2$-DHT and 1β,25-$(OH)_2$-DHT, two further metabolites that have greater biological activity than either 25-OH-DHT or DHT itself (Qaw *et al.*, 1993).

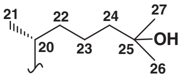
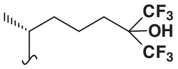
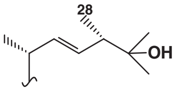
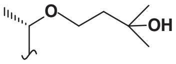
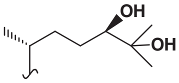
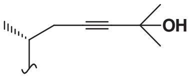
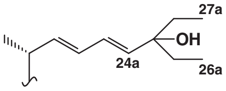
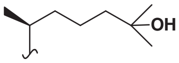
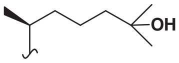
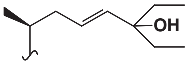
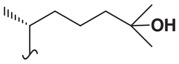
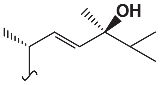
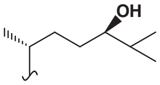
Vitamin D Analogues

Table III lists some of the most promising vitamin D analogues of 1α,25-$(OH)_2D_3$ already approved by governmental agencies or currently under development by various industrial or university research groups. Because the number of vitamin D analogues synthesized now lists in the thousands, the table is provided mainly to give a flavor of the structures experimented with thus far, the worldwide scope of the companies involved, and the broad spectrum of target diseases and uses.

The first generation of calcitriol analogues included molecules with fluorine atoms placed at metabolically vulnerable positions in the side chain and resulted in highly stable and potent "calcemic" agents such as 26,27-F_6-1α,25-$(OH)_2D_3$. A second generation of analogues focused on features that make the molecule more susceptible to clearance, such as in calcipotriol (MC903), where a C22–C23 double bond, a 24-hydroxyl function, and a cyclopropane ring have been introduced into the side chain or in 22-oxacalcitriol (OCT) where the 22-carbon has been replaced with an oxygen atom. Both modifications have given rise to highly promising analogues marketed initially in Europe and Japan, respectively (Kragballe, 1992; Abe-Hashimoto *et al.*, 1993).

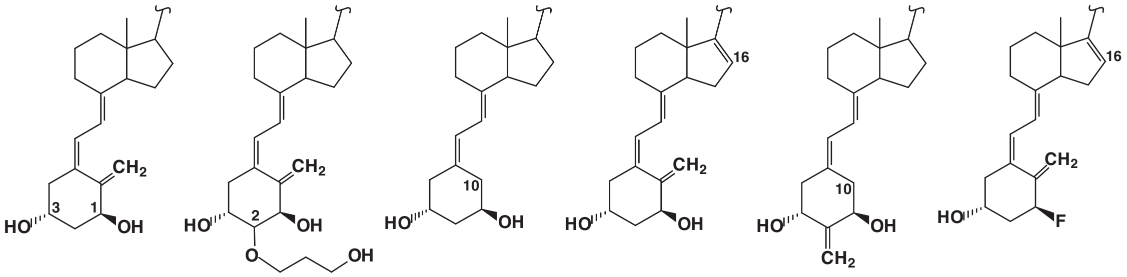
The C-24 position is a favorite site for modification and numerous analogues contain 24-hydroxyl groups, e.g., 1α,24(*S*)-$(OH)_2D_2$ and 1α,24(*R*)-$(OH)_2D_3$ (Strugnell *et al.*, 1995). Other analogues contain multiple changes in the side chain in combination, including unsaturation; 20-epimerization, 22-oxa replacement; and homologation in the side chain or terminal methyl groups. The resultant molecules such as EB1089 and KH1060 attracted strong attention of researchers because of their increased potency *in vitro* and were pursued as possible anticancer and immunomodulatory compounds, respectively.

TABLE III Analogues of 1α,25-(OH)$_2$D$_3$

Vitamin D analogue [ring structure][*]	Side-chain structure	Company	Possible target diseases	Mode of delivery	Reference
Calcitriol, 1α,25-(OH)$_2$D$_3$ [3]		Roche, Duphar	Hypocalcemia Psoriasis	Systemic Topical	Baggiolini *et al.* (1982)
26,27-F$_6$-1α,25-(OH)$_2$D$_3$ [3]		Sumitomo-Taisho	Osteoporosis hypoparathyroidism	Systemic Systemic	Kobayashi *et al.*, 1982
19-Nor-1α,25-(OH)$_2$D$_2$ [5]		Abbott	Secondary hyperparathyroidism	Systemic	Perlman *et al.*, 1990
22-Oxacalcitriol (OCT) [3]		Chugai	Secondary hyperparathyroidism Psoriasis	Systemic Topical	Murayama *et al.*, 1986
Calcipotriol (MC903) [3]		Leo	Psoriasis Cancer	Topical Topical	Calverley, 1987
1α,25-(OH)$_2$-16-ene-23- yne-D$_3$ (Ro 23-7553) [6]		Roche	Leukemia	Systemic	Baggiolini *et al.*, 1989
EB1089 [3]		Leo	Cancer	Systemic	Binderup *et al.*, 1991
20-epi-1α,25-(OH)$_2$D$_3$ [3]		Leo	Immune diseases	Systemic	Calverley *et al.*, 1991
2-methylene-19-nor-20-epi-1α,25-(OH)$_2$D$_3$ (2MD) [7]		Deltanoids	Osteoporosis	Systemic	Shevde *et al.*, 2002
BXL-628 (formerly Ro-269228) [8]		Bioxell	Prostate cancer	Systemic	Marchiani *et al.*, 2006
ED71 [4]		Chugai	Osteoporosis	Systemic	Nishii *et al.*, 1993
1α,24(S)-(OH)$_2$D$_2$ [3]		Genzyme	Psoriasis	Topical	Strugnell *et al.*, 1995a
1α,24(R)-(OH)$_2$D$_3$ (TV-02) [3]		Teijin	Psoriasis	Topical	Morisaki *et al.*, 1975

[*] Structure of the vitamin D nucleus (secosterol ring structure).

Vitamin D nucleus



[3] [4] [5] [6] [7] [8]

A few attempts have been made to modify the nucleus of calcitriol. The Roche compound 1α,25-$(OH)_2$-16-ene-23-yne-D_3, touted as an antitumor compound *in vivo,* possesses a D-ring double bond. Declercq and Bouillon have introduced a series of biologically active analogues without the C/D rings but with a rigid backbone to maintain the spatial arrangement of the A-ring hydroxy1 groups and the side chain (Verstuyf *et al.*, 2000). Relatively recently, the A-ring-substituted 2-hydroxypropoxy-derivative, ED71, has been tested as an antiosteoporosis drug. Other bulky modifications at the C2 position of the A ring are accommodated well by the vitamin D receptor, as indicated by retention of biological activity (Suhara *et al.*, 2001; Shevde *et al.*, 2002). The Abbott compound, 19-nor-1α,25-$(OH)_2D_2$, lacks a 19-methylene group and is styled upon the *in vivo* active metabolite, 1α,25-$(OH)_2$ DHT_2, formed from dihydrotachysterol, which retains biological activity though the C-19 methylene, is replaced by a C-19 methyl. Many other compounds have been developed with rigid or altered *cis*-triene structures (Okamura *et al.*, 1995) or modifications of the 1α,3β-, or 25-hydroxy1 functions, not for the purpose of developing active molecules for use as drugs, but to allow us to establish minimal requirements for biological activity in structure/activity studies (Calverley and Jones, 1992; Bouillon *et al.*, 1995).

Two recent compounds, Bioxell's BXL-628 and Deltanoids' 2-MD, combine modifications in the side chain with those in the nucleus. BXL-628 combines 1-fluorination, 16-ene and 23-ene unsaturations, 26,27-homologation, and 20-epimerization all found in earlier generations of analogues to make a antiproliferative agent currently in clinical trials for the treatment of prostate cancer and prostatitis (Crescioli *et al*., 2003; Adorini *et al*., 2007). Likewise, 2-MD, touted as being bone-specific, combines a novel 2-methylene substitution and the 19-nor feature with side chain 20-epimerization (Shevde *et al*., 2002).

One series of compounds depicted in Table IV are the substituted biphenyls originally developed by Ligand, representing nonsteroidal scaffolds selected by high-throughput screening, which show weak VDR-binding but good transactivation through VDRE-driven, vitamin D-dependent genes and produce hypercalcemia *in vivo* (Boehm *et al.*, 1999). This family has recently been extended by the synthesis of some highly potent, tissue-selective nonsecosteroidal VDR modulators with nanomolar affinity (e.g., LY2109866) by a research group at Eli Lilly (Ma *et al.*, 2006). This is the first class of vitamin D mimics that lack the conventional *cis*-triene secosteroid structure while maintaining the spatial separation of the A-ring and side-chain hydroxy1 functions needed to bind to certain key residues of the ligand-binding pocket of the VDR. Though these nonsecosteroidal compounds exhibit a 270-fold improvement of the therapeutic index over calcitriol in animal models, they are still to be tested clinically. On the contrary, Table IV also shows the structures of two different classes of VDR/cacitriol antagonists made by Teijin and Schering, respectively. The former compounds, most notably TEI-9647, are based on the natural metabolite 1α,25-$(OH)_2D_3$-26,23-lactone and have found clinical utility in the treatment of Paget's disease (Ishizuka *et al.*, 2005; Saito and Kittaka, 2006).

Another group of compounds which impact the vitamin D field that are under development are the CYP24 inhibitors. By blocking CYP24A1, the main catabolic pathway within the vitamin D-target cell, these agents extend the life of the natural agonist, calcitriol, giving rise to a longer-lasting biological effect (Prosser and Jones, 2004). Sandoz/Novartis developed a group of molecules that have greater specificity toward CYP24 and CYP27B1 from the general cytochrome P450 (CYP) inhibitor, ketoconazole, which showed utility to block cell proliferation *in vitro,* but these compounds were discontinued after early clinical trials (Schuster, 2001). Cytochroma has synthesized a group of inhibitors based on vitamin D templates and these have currently reached phase IIB human clinical trials for the treatment of psoriasis (Posner *et al*., 2004; Kahraman *et al.*, 2004). Some of these molecules show promise for use in secondary hyperparathyroidism, presumably because they counter the role of CYP24 in attenuating the effect of calcitriol on preproPTH gene suppression.

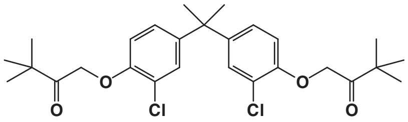
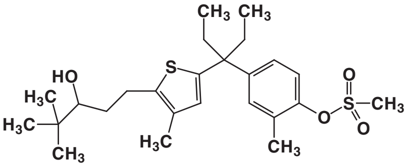
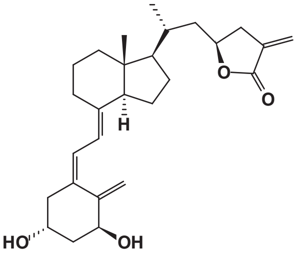
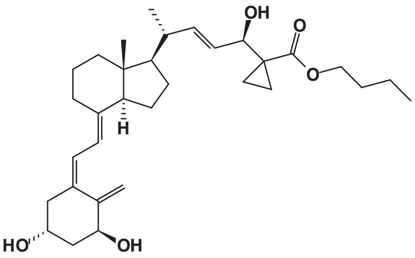
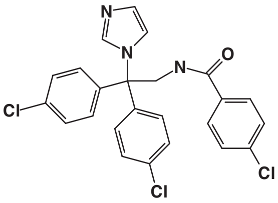
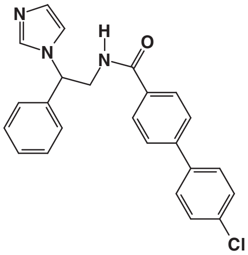
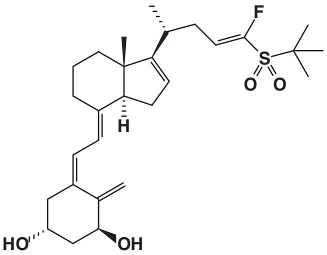
CLINICAL APPLICATIONS OF VITAMIN D COMPOUNDS

The clinical usefulness of vitamin D analogues has been reviewed comprehensively by both Bikle (1992) and Bouillon *et al.* (1995) in overviews and also within this book. This chapter summarizes some of the highlights in this area.

Rickets and Osteomalacia

When the nutritional basis of rickets and osteomalacia became apparent in the first half of the twentieth century, vitamin D (particularly vitamin D_2 because it was less expensive) became the treatment of choice for these diseases. Of course, low-dose prophylactic vitamin D (400 IU) in the form of supplements to milk, margarine, and bread replaced much of the need for therapeutic vitamin D to abolish overt rickets and osteomalacia. In fact, since then full-blown vitamin D deficiency rickets (defined as plasma 25-OH-D levels below 10 ng/mL or 25 nmol/L) has become very uncommon in North America because vitamin D supplementation is required by law, whereas it was quite common before the practice of food fortification and it is still more prevalent in the world where food fortification is not permitted. On the other hand, vitamin D insufficiency (defined as plasma 25-OH-D levels in the range 10 to 40 ng/mL or 25 to 100 nmol/L) remains common in the general

TABLE IV Miscellaneous Vitamin D Compounds

Name	Structure	Name	Structure
LG190090 Ligand Pharmaceuticals Nonsteroidal agonist Boehm *et al.*, 1999		LY2108491 Eli Lilly Nonsteroidal agonist Ma *et al.*, 2006	 CH3 CH3 HO H3C H3C CH3 S H3C CH3 O O S CH3 O
TEI-9647 Teijin Calcitriol antagonist Dehydration product of 1α,25(R)-$(OH)_2D_3$-26,23(S)-lactone Saito and Kittaka, 2006 Ochiai *et al.*, 2005 Toell *et al.*, 2001		ZK159222 Schering Calcitriol antagonist Toell *et al.*, 2001	
SDZ 89-443 Sandoz/ Novartis P450 inhibitor Schuster *et al.*, 2003		VID400 Sandoz/ Novartis P450 inhibitor Schuster *et al.*, 2003	
CTA016 Cytochroma CYP24A1 inhibitor Posner *et al.*, 2004			

population and is being increasingly correlated with poor outcomes in several health-related areas including optimal bone mineral density (Bischoff-Ferrari *et al.*, 2006).

Vitamin D deficiency and insufficiency are also quite prevalent in the elderly and are usually treated with modest doses of 800 to 1000 IU of vitamin D (Chapuy *et al.*, 1992). In recent years, several world, continent-wide, and national food agencies have put out new guidelines raising the recommendations for vitamin D intake for all age groups, but particularly for those in the elderly or post-menopausal category, to try to ensure adequate intakes irrespective of geographical, dietary, and sun exposure differences (National Academy of Sciences Reference Intakes, 1997; FAO/WHO Nutritional Guidelines, 2000). However, the need for the use of expensive pharmaceutical preparations containing calcitriol or its analogues to cure simple rickets and osteomalacia is usually not warranted.

Though many of the hallmarks of rickets and osteomalacia are successfully relieved by doses of vitamin D in the range of 400 to 800 IU/day (10 to 20 μg/day), there are epidemiological data to suggest that current recommended dietary allowances (also known as DRIs) do not result in plasma 25-OH-D levels greater than 40 ng/mL which correlate with maximal bone mineral density (Holick, 2007; Bischoff-Ferrari *et al.*, 2004) or the other health benefits of vitamin D (Bischoff-Ferrari *et al.*, 2006). Consequently, there has been much recent debate over the optimal level of vitamin D intakes and this has led to a general view that vitamin D intakes might need to be increased above 1500 IU/day (Heaney, 2004) and possibly higher (Dawson-Hughes *et al.*, 2005) in order to achieve target plasma 25-OH-D levels greater than 40 ng/mL. However, many quasi-governmental agencies have yet to translate the latest recommendations for increase into new public health guidelines.

Osteoporosis

Although the etiology of this disease is complex and likely to be multifactorial (Seeman *et al.*, 1995; Nordin, 1997), there have been consistent claims that levels of 1α,25-$(OH)_2D_3$ are low in osteoporosis (Riggs and Melton, 1992; Eastell and Riggs, 2005). In addition, the recent debate over VDR genotypes correlating with bone mineral density (Morrison *et al.*, 1994; Whitfield *et al.*, 2000; Uitterlinden *et al.*, 2005) suggests some genetically inherited basis involving vitamin D exists leading to increased susceptibility to osteoporosis. As a consequence it is not surprising that clinical trials of 1α-OH-D_3 (Orimo *et al.*, 1987), 1α-OH-D_2 (Gallagher *et al.*, 1994), and 1α,25-$(OH)_2D_3$ (Gallagher *et al.*, 1989; Ott and Chestnut, 1989; Tilyard *et al.*, 1992) have been undertaken. Modest gains in bone mineral density and reductions in fracture rates are reported in many of these studies, and this subject has been reviewed by Seeman *et al.* (1995).

With the demonstration that ovariectomy results in enhanced production of osteoclastogenic cytokines such as interleukin-6, tumor necrosis factor (TNF), and interleukin-1 as well as cytokine-mediated osteoclast recruitment and increased bone resorption has come a clearer understanding of the molecular processes underlying postmenopausal osteoporosis (Manolagas and Jilka, 1995; Teitelbaum and Ross, 2003). Theories focusing on osteoblast/osteoclast communication led to the discovery of receptor activator of nuclear factor κB (RANK), its ligand RANKL, and the decoy receptor, osteoprotegerin, and how agents such as 1α,25-$(OH)_2D_3$ can influence osteoclastogenesis and bone resorption (Aubin and Bonnelye, 2000). Although 1α,25-$(OH)_2D_3$ treatment might be expected to exacerbate the excessive bone-resorptive component of osteoporosis, the vitamin D hormone also raises plasma Ca^{2+} levels and stimulates synthesis of bone matrix formation in osteoblasts. In fact, Raisz and coworkers (Hock *et al.*, 1986) have shown that pharmacological doses of 1α,25-$(OH)_2D_3$ administered to rats, in great excess over the doses used in osteoporosis, result in hypercalcemia and nephrocalcinosis that is accompanied by a hyperosteoid or undermineralized condition in the long bones.

Although small doses of vitamin D (800 to 1000 IU) have proven effective in treating vitamin D deficiency accompanying osteoporosis and even reduce fracture rates (Chapuy *et al.*, 1992), the use and effectiveness of active vitamin D metabolites in the treatment of osteoporosis are controversial. Nevertheless, the experience seems to have been that beneficial effects can be observed and bone loss reduced, but at the expense of occasional hypercalcemia. In North America, where dietary Ca intakes and absorption rates are higher, this has led to intolerable side effects and the discontinuation of the use of 1α,25-$(OH)_2D_3$ and 1α-OH-D_3 for the treatment of osteoporosis. In the United Kingdom, Australia, Italy, Japan, New Zealand, and 16 other countries in rest of the world, these drugs are approved or side effects tolerated. Nevertheless, some pharmaceutical companies have sought to develop "milder" but "longer-lived" calcitriol analogues for use in osteoporosis. ED-71 represents such an analogue, which by virtue of an A-ring substituent at C-2 and tighter-binding affinity to DBP has a longer $t_{1/2}$ in the plasma (Nishii *et al.*, 1993). ED-71 has performed well at restoring bone mass without causing hypercalcemia in long-term studies involving ovariectomized rats (Okano *et al.*, 1991) and in phase I and II clinical trials (Matsumoto and Kubodera, 2007). Another bone-specific analogue with potential for treatment of osteoporosis, 2-MD (Shevde *et al.*, 2002) is at a relatively early stage of development.

Renal Osteodystrophy

Chronic renal disease (CKD) is accompanied by the gradual loss of renal 25-OH-D_3-1α-hydroxylase (CYP27B1) activity over the five-stage natural history of the disease which culminates in dialysis (stage 5). As early as stage 2 of CKD, the 1α-hydroxylase declines leading to reduced plasma levels of 1α,25-$(OH)_2D_3$, which results in hypocalcemia and secondary hyperparathyroidism. Unchecked, these biochemical events, together with the other sequelae of renal failure such as phosphate retention, can result in renal osteodystrophy. Active vitamin D analogues, such as 1α-OH-D_3 and 1α,25-$(OH)_2D_3$, raise plasma Ca^{2+} concentrations and, in addition, lower PTH levels by direct suppression of PTH gene transcription at the level of the PTH gene promoter. Slatopolsky and colleagues (Delmez *et al.*, 1989) showed that intravenous infusion of "active" vitamin D preparations results in a more effective suppression of plasma PTH levels without such a profound increase in plasma $[Ca^{2+}]$ in end-stage renal disease. Subsequent work has employed "low-calcemic" vitamin D analogues such as OCT or 19-nor-1α,25-$(OH)_2D_2$ as substitutes for the more calcemic natural hormone (Brown *et al.*, 1989; Slatopolsky *et al.*, 1995). More recently, the Food and Drug Administration (FDA) approved both oral and intravenous, 1α-OH-D_2 (trade name, Hectorol) for the treatment of secondary hyperparathyroidism at earlier stages 3 and 4 of the disease. In clinical trials, 1α-OH-D_2 effectively suppressed PTH in renal failure patients with very few incidences of hypercalcemia and hyperphosphatemia (Frazao *et al.*, 2000). Recently, oral formulations of 19-nor-1α,25-$(OH)_2D_2$ were also approved.

In 2003, a body of leading nephrologists released guidelines (KDOQI, 2003) recommending more aggressive use of vitamin D preparations and "active" vitamin D analogues in the treatment of secondary hyperparathyroidism in CKD. KDOQI guidelines suggested that treatment as early as stage 3 [glomerular filtration rate (GFR) less than 60] might benefit the patient by limiting the extreme rises in plasma PTH levels and preventing the parathyroid gland resistance to vitamin D treatment often observed in end-stage renal disease (ESRD). KDOQI guidelines also recognized the high frequency of vitamin D deficiency (25-OH-D less than 10 ng/mL) and vitamin D insufficiency (25-OH-D 10 to 30 ng/mL) in the CKD and ESRD population (Gonzalez *et al.*, 2004) and recommended an initial attempt at vitamin D repletion with escalating doses of vitamin D_2 prior to administration of "active" vitamin D analogue replacement therapy. Currently, both oral and intravenous formulations of various active vitamin D analogues are available for use in stage 3, 4, and 5 patients to take over, if and when vitamin D repletion fails to regulate PTH levels.

The emergence of the potential importance of the extrarenal 1α-hydroxylase in normal human physiology has led to a reevaluation of the vitamin D repletion and "active" hormone replacement arms of the CKD therapy (Jones, 2007). The value of the vitamin D repletion is now seen as providing the substrate 25-OH-D for both the renal 1α-hydroxylase, which is the main determinant of circulating $1\alpha,25\text{-(OH)}_2D_3$, and the extrarenal 1α-hydroxylase, which is postulated to augment $1\alpha,25\text{-(OH)}_2D_3$ synthesis for local or paracrine actions around the body. Although the decline of the renal enzyme during CKD is well established, the fate of the extrarenal 1α-hydroxylase in the face of uremia is largely a matter of conjecture. Evidence from anephric patients treated with large doses of 25-OH-D_3 (Dusso, *et al.,* 1988) suggests that the extrarenal enzyme survives in CKD patients, arguing that provision of a source of 25-OH-D to vitamin D-deficient and -insufficient patients throughout all stages of CKD is warranted. It also argues for the more judicious use of "active" vitamin D analogues as hormone replacement therapy layered on top of conventional vitamin D repletion therapy. Early attempts at this type of combined vitamin D/"active" vitamin D analogue approach in a pediatric population have resulted in a more efficient PTH control without many of the usual problems of soft-tissue calcification observed in patients treated only with active vitamin D analogues (Briese *et al.*, 2006; Fournier *et al.*, 2007).

Psoriasis and Cancer

The demonstration that $1\alpha,25\text{-(OH)}_2D_3$ is an antiproliferative, prodifferentiating agent for certain cell types *in vivo* and many cell lines *in vitro* suggested that vitamin D analogues might offer some relief in hyperproliferative disorders such as psoriasis and cancer. Early psoriasis trials with $1\alpha,25\text{-(OH)}_2D_3$ were moderately successful but plagued with hypercalcemic side effects. Modifications to the protocol included: (1) administration of calcitriol overnight when intestinal concentrations of $[Ca^{2+}]$ were low, (2) substitution of "low-calcemic" analogues for the calcitriol.

According to Holick (1995), oral calcitriol is an effective treatment for psoriasis when administered using an overnight protocol. However, by far the most popular treatment for psoriasis is the topical administration of the "low-calcemic" analogue calcipotriol, formulated as an ointment (Kragballe, 1992). When given orally, calcipotriol is ineffective because it is rapidly broken down (Binderup and Bramm, 1988). When given topically as an ointment, calcipotriol survives long enough to cause improvement in more than 75% of patients (Kragballe *et al.*, 1991). Both $1\alpha,25\text{-(OH)}_2D_3$ and calcipotriol are effective in psoriasis because they block hyperproliferation of keratinocytes, increase differentiation of keratinocytes, and help suppress local inflammatory factors through their immunomodulatory properties. Calcipotriol has now been marketed worldwide for use in psoriasis for more than 15 years. The success of calcipotriol has spawned the development of second-generation analogues.

Several hundreds of vitamin D analogues have been tested *in vitro* and *in vivo* with some degree of success in controlling the growth of tumor cells offering potential for use as anticancer drug therapies (reviewed extensively in Masuda and Jones, 2006). Many vitamin D compounds are extremely effective antiproliferative or pro-differentiation agents *in vitro* using a variety of mechanisms involving gene expression of cell division and pro-apototic genes to produce their effects. Preclinical studies in laboratory animals have also resulted in promising data (Masuda and Jones, 2006). For example, in mice inoculated with fulminant leukemia, moderate leukemia, or slowly progressive leukemia, the Roche compound $1\alpha,25\text{-(OH)}_2\text{-16-ene-23-yne-}D_3$ administered at 1.6 μg q.o.d. was significantly more effective than 0.1 μg q.o.d. $1\alpha,25\text{-(OH)}_2D_3$ at increasing survival time even though the $1\alpha,25\text{-(OH)}_2D_3$-treated group developed mild hypercalcemia and the analogue-treated animals remained normocalcemic (Zhou *et al.*, 1990). With the analogue EB1089, the promising antiproliferative effects observed *in vitro* and in the NMU-induced mammary tumor and in LNCaP prostate cancer xenograft models (Colston *et al.*, 2003; Blutt *et al.,* 2000) were also extended into the clinic. Early trials in limited numbers of breast cancer patients have been followed up with more extensive ongoing phase II and phase III clinical trials in a number of different cancers (Gulliford *et al.*, 1998; Evans *et al.*, 2002; Dalhoff *et al.,* 2003). Several other analogues have entered clinical trials for the treatment of a variety of hyperproliferative diseases, usually involving VDR-positive tumors (see Masuda and Jones, 2006). Many trials are still ongoing including the testing of BXL-628 (see Table III) in prostate-related diseases (Crescioli *et al.*, 2003)

Despite the enormous promise of vitamin D analogues as anticancer agents, this has yet to result in an approved vitamin D analogue for use in any type of cancer (Masuda and Jones, 2006). The principal problem in anticancer studies involving orally administered vitamin D compounds is hypercalcemia. Though the newer analogues appear to be less calcemic than calcitriol itself, they still retain some ability to raise serum calcium; they are not "noncalcemic" as is sometimes claimed. Another problem emerging from experience with clinical trials is that effective doses needed to retard cell growth (~1 n*M* or higher) cannot be attained *in vivo* because of low bioavailability (Beer *et al.*, 2004; Trump *et al.*, 2004). One of the principal determinants of tumor cell vitamin D analogue levels is the catabolic enzyme CYP24A1, which is upregulated in vitamin D-target cells, and limits the effective drug concentration reached. Another approach to effective vitamin D therapy in cancer patients is the potential use of CYP24-inhibitors (see Table IV). Nevertheless, it remains uncertain whether we will ever develop a vitamin D compound sufficiently devoid of calcemic activity while retaining sufficient antiproliferative activity to be valuable in cancer and also deliver it to target cells in appropriate concentrations

Immunosuppression

The immunosuppressive properties of 1α,25-$(OH)_2D_3$ and its analogues have been the subject of several excellent reviews (Bouillon *et al.*, 1995; Van Etten, 2000; Mathieu and Adorini, 2002). 1α,25-$(OH)_2D_3$ is believed to work by regulation of the expression of various cytokines, particularly those involved in suppressing inflammation and which raise the Th2/Th1 ratio. The hormone also stimulates the transcription of a natural bacterial peptide, cathelicidin, which kills *Mycobacterium tuberculosis* resulting in increased resistance to tuberculosis (Wang *et al.*, 2004; Holick, 2007). The spectrum of effects exhibited by 1α,25-$(OH)_2D_3$ and its analogues on the immune system results in beneficial effects on a wide variety of autoimmune diseases. Researchers have demonstrated the ability of calcitriol to suppress the onset of experimental encephalitis (Lemire and Clay, 1991) and type I diabetes in NOD mice (Mathieu *et al.*, 1995), and to work synergistically with cyclosporine to provide immunosuppression in transplantation medicine (Mathieu *et al.*, 1994a). This latter development has led to some optimism that coadministration of a vitamin D analogue with cyclosporin can reduce the dosage of the latter drug and minimize the serious side effects associated with its use. Several studies (Mathieu *et al.*, 1995, 1994b; Veyron *et al.*, 1993) have focused on the immunosuppressive effects of Leo drugs KH1060 and 20-epi-1α,25-$(OH)_2D_3$, both of which contain the 20-*S* side-chain configuration. Recent generations of compounds such as BXL-628, that contain multiple modifications found in the Leo Pharma drugs, are being tested in prostatitis, an inflammation of the prostate (Adorini, 2007). Again, it remains unclear whether analogues that show promise in immunological studies will prove to be effective immunomodulators in the clinic.

CRITERIA THAT INFLUENCE PHARMACOLOGICAL EFFECTS OF VITAMIN D COMPOUNDS

Activating Enzymes

It has been shown by using *in vitro* models that some vitamin D compounds lacking 1α-hydroxylation (e.g., 24(*R*),25-$(OH)_2D_3$) are capable of interacting with the vitamin D receptors (VDRs) and transactivating reporter genes but this occurs only at high concentrations of ligand (Uchida *et al.*, 1994). It seems unlikely that these concentrations will be reached *in vivo* except in hypervitaminosis D. Consequently, most of the compounds described in Tables I and II lack lack vitamin D biological activity *unless* they are activated *in vivo.* This is particularly the case for the parent vitamin D_3 itself, for its main circulating form 25-OH-D_3 or for any of the prodrugs listed in Table II. Vitamins D_2 and D_3 depend on both the liver 25-hydroxylase and kidney 1α-hydroxylase enzyme systems in order to be activated, whereas most prodrugs require only a single step of activation. Indeed, the 1α-OH-D drugs were designed to overcome the tightly regulated 1α-hydroxylase step that is easily damaged in chronic renal failure. In essence, prodrugs depend on the weakly regulated 25-hydroxylase step in the liver for activation. In recent years, the cytochrome P450 originally thought to be responsible for 25-hydroxylation of vitamin D_3, CYP27A1, has been cloned and shown to be a bifunctional polypeptide that executes both activation of vitamin D_3 and the 27-hydroxylation of cholesterol during bile acid biosynthesis (Okuda *et al.*, 1995). However, the CYP27A1 enzyme has a relatively low affinity for vitamin D, does not 25-hydroxylate vitamin D_2, and when mutated results in cerebrotendinous xanthomatosis not rickets. Consequently, another "physiologically relevant" 25-hydroxylase may exist and there are now several candidate P450s (Prosser and Jones, 2004), the main one being CYP2R1 (Cheng and Russell, 2003), a high-affinity microsomal enzyme with known human mutations that cause rickets, that has been recently shown to 25-hydroxylate the prodrug 1α-OH-D_2 (Jones *et al.*, 2006). However, it is clear that the mitochondrial CYP27A1 efficiently 25-hydroxylates 1α-OH-D_3 to give 1*a*,25-$(OH)_2D_3$ (Guo *et al.*, 1993); and is present in a variety of tissues as well as the liver (e.g., kidney and bone). In fact, studies using cultured bone cells and even keratinocytes *in vitro* are able to demonstrate synthesis of 1α,25-$(OH)_2D_3$ from 1α-OH-D_3 (Ichikawa *et al.*, 1995; Jones *et al.*, 1999a) or 1α,24-$(OH)_2D_2$ from 1α-OH-D_2 (Masuda *et al.*, 2006). If these findings can be extrapolated to the *in vivo* situation, the implications of this work are that in CYP27A1, vitamin D target cells may have some ability to synthesize the active form from a prodrug *without* the need for the hormone to enter the bloodstream.

The ability of extrarenal tissues to 1α-hydroxylate various 25-hydroxylated metabolites and analogues has always been a controversial story. However, it was widely accepted that extrarenal 1α-hydroxylase activity is associated with certain granulomatous conditions (e.g., sarcoidosis) (Adams and Gacad, 1985). Currently, there is little information for why the enzyme is overexpressed in sarcoidosis. In these patients, 25-OH-D can be converted to 1α,25-$(OH)_2D$, a step that, unlike the renal case, is not subject to tight regulation and thus potentially more likely to result in hypercalcemia. Exposure of such patients to sunlight or administration of 25-OH-D can result in excessive plasma levels of 1α,25-$(OH)_2D$. The cloning of the cytochrome P450 representing the 1α-hydroxylase (officially named CYP27B1) (St. Arnaud *et al.*, 1997; Takeyama *et al.*, 1997) has been followed by confirmation that the cytochrome can be expressed extrarenally in skin and lung cancer cells (Fu *et al.*, 1997; Jones *et al.*, 1999b). This has extended over the past decade with further studies of CYP27B1 mRNA levels using real-time PCR and specific anti-CYP27B1 antibodies (Hewison and Adams, 2005) to show the widespread distribution of this enzyme in many normal tissues as well as pathological situations.

As alluded to earlier, the concept of the extrarenal 1α-hydroxylase suggests that this enzyme plays an important physiological as well as pathological role (Jones, 2007) and this has in turn raised the level of importance given to ensuring maintenance of adequate 25-OH-D levels by vitamin D or direct 25-OH-D_3 supplementation rather than just by calcitriol hormone replacement.

Most of the calcitriol analogues listed in Table III are thought to be active as such, not requiring any step of activation prior to their action on the transcriptional machinery or in nongenomic pathways. It remains a theoretical possibility, though, that the biological activity of one of these parent analogues could be altered by enzyme systems *in vivo*, either by the generation of a more potent metabolite or by giving rise to a less active but more long-lived catabolite.

Vitamin D-Binding Protein

The vitamin D-binding protein (DBP) serves several functions including providing transport for a lipid-soluble vitamin D analogue. Most of the analogues of calcitriol, designed to date, contain modifications to the side chain and this is usually detrimental to binding to DBP. Several analogues, for example, calcipotriol or OCT, have very weak affinities for DBP, reduced by two to three orders of magnitude relative to 1α,25-$(OH)_2D_3$. This property has important implications for metabolic clearance rates, delivery to target cells, and tissue distribution (Bouillon *et al.*, 1991; Kissmeyer *et al.*, 1995). Detailed studies with one analogue, OCT, have shown it to bind primarily to β-lipoprotein and exhibit an abnormal tissue distribution *in vivo*, with abnormally high concentrations (ng/g tissue) in the parathyroid gland (Tsugawa *et al.*, 1991). It was thus proposed that this unusual distribution may make OCT a useful systemically administered drug with a selective advantage in the treatment of hyperparathyroidism.

Another vitamin D analogue with a modified side chain is 20-epi-1α,25-$(OH)_2D_3$, where the 20-*S* configuration of the side chain is opposite to the normal 20-*R* configuration. The DBP binding affinity of this analogue is virtually unmeasurable because it does not displace $[^3H]$25-OH-D_3 from the plasma-binding protein (Dilworth *et al.*, 1994). Confirmation that this is indeed the case comes from GH-reporter gene transactivation assays where 20-epi-1α,25-$(OH)_2D_3$ transactivates equally well in COS cells incubated in the presence and absence of fetal calf serum (as a source of DBP). On the other hand, 1α,25-$(OH)_2D_3$-induced GH reporter gene expression is sensitive to DBP in the external growth medium, requiring 2-fold less hormone in the absence of DBP as in its presence (Dilworth *et al.*, 1994). It therefore appears that analogues that bind DBP less well than 1α,25-$(OH)_2D_3$ derive a target cell advantage over the natural hormone, *if they are able to find alternative plasma carrier proteins to transport them to their target cells.* However, these same alternative plasma carriers presumably result in changes in the tissue distribution and hepatic clearance of analogues over the natural metabolites of vitamin D. The recent development of a DBP-knockout mouse (Safadi *et al.*, 1999) suggests that 25-OH-D_3 clearance is more rapid in the absence of DBP. The availability of the model permits the study of alternate vitamin D analogue transport mechanisms in an *in vivo* setting.

Vitamin D Receptor/RXR/VDRE Interactions

Previous chapters in this book have established that 1α,25-$(OH)_2D_3$ is able to work through a VDR-mediated genomic mechanism to stimulate transcriptional activity at vitamin D-dependent genes. Considerable progress has been made recently toward delineating the precise conformational changes that take place when the natural ligand binds to the VDR (Wurtz *et al.*, 1997; Rochel *et al.*, 2001); and the nature of the postligand binding transcriptional events that occur, particularly the nature of the coactivator proteins involved (Rachez and Freedman *et al.*, 2000; Kato *et al.*, 2000). These developments have improved our thinking about how and where analogues might act differently from 1α,25-$(OH)_2D_3$ in the transcriptional cascade. Whether 1α,25-$(OH)_2D_3$ works through other non-VDR-mediated mechanisms to produce physiologically relevant effects is a question that currently remains unproven, but this question is also important to our understanding of the pharmacological effects of vitamin D analogues.

Much evidence exists to support the viewpoint that vitamin D analogues mimic 1α,25-$(OH)_2D_3$ and use a genomic mechanism. The first clue that vitamin D analogues can work through a VDR-mediated transcriptional mechanism came 25 years ago from the bone resorption studies reported by Stern in her classic review "Monolog on Analogs"(1981). Stern showed that there exists a strong correlation between chick intestinal VDR binding of an analogue and its potency in a [^{45}Ca] rat bone resorption assay. This suggests that a vitamin D analogue is only as good as its affinity for the VDR. Over the past 25 years since Stern's article, there have been many claims that VDR-binding affinity is not the only factor in determining biological activity of a given analogue but also that VDR-binding affinity is not even the major factor, transactivation activity stemming from a series of parameters such as conformation of the ligand/VDR complex, binding of the RXR partner, stability of the VDR/RXR/ ligand complex, or even the nature of the coactivator proteins recruited to the complex. Examples of these apparent discrepancies between VDR affinity and biological activity will be provided later but it should be pointed out that some of these discrepancies are almost certainly explained by other considerations such as DBP binding or pharmacokinetics. Preliminary results with the analogues KH1060, EB1089, and 20-epi-1α,25-$(OH)_2D_3$ (Binderup *et al.*, 1991) suggested that they might be active in immunoregulatory roles at concentrations orders of magnitude below their affinities for the VDR (e.g., at as low as 10^{-15} *M* for KH1060, whereas it binds VDR at 10^{-11}*M*). More recent results (Yang and Freedman, 1999; Dilworth *et al.*, 1994, 1997) suggest that 20-epi compounds including KH1060 are consistently only one to two orders of magnitude more potent than 1α,25-$(OH)_2D_3$ in gene transactivation assays and in differentiation assays, a difference that could be explained by fine-tuning the transcriptional model of analogue action (e.g., by including pharmacokinetic considerations) rather than discarding the genomic hypothesis altogether. The majority of researchers are keeping an open mind on this subject and are searching for differences in the newly delineated transcriptional machinery that might explain qualitative and quantitative differences between 1α,25-$(OH)_2D_3$ and its analogues.

Over the past decade it has been clearly established that the liganded VDR functions transcriptionally as a vitamin D-VDR-RXR heterodimer (Macdonald *et al.*, 1993; reviewed in Haussler *et al.*, 1998) and not as a VDR-VDR homodimer (Carlberg, 1995). The role of the RXR ligand is still controversial, many studies suggesting that pan RAR and RXR ligands such as 9-*cis*-retinoic acid inhibit VDR-RXR heterodimer formation, whereas other studies demonstrate the synergistic effects of pure RXR ligands (so-called rexinoids) and 1α,25-$(OH)_2D_3$ on VDR-RXR-driven transcription at a CYP24-VDRE (Zou *et al.*, 1997). Whether vitamin D analogues might differ from 1α,25-$(OH)_2D_3$ and act transcriptionally through VDR-VDR homodimers or other VDR-nuclear transcription factor heterodimers are ideas that have been considered as theoretical possibilities, and in some cases even shown to occur weakly *in vitro*, but largely dismissed as occurring *in vivo*.

Adding to the complexity of the target cell action of 1α,25-$(OH)_2D_3$, and thus that of vitamin D analogues, is the type and context of the VDRE involved (Haussler *et al.*, 1998). One possibility is that vitamin D analogues could show selectivity for certain genes based on the type of VDRE within their promoter. Morrison and Eisman (1991) showed that a noncalcemic analogue such as calcipotriol is easily capable of transactivating a calcemic VDRE such as the human osteocalcin promoter-VDRE placed in front of the CAT reporter gene and stably transfected into ROS 17/2 cells provided that it can get into the target cell. One interpretation of this experiment is that a noncalcemic analogue with good VDR affinity is just as calcemic as 1α,25-$(OH)_2D_3$ if it can be delivered to the target cell. Another idea put forward by Morrison and Eisman (1991) is that noncalcemic analogues may be capable of stimulating both cell-differentiating and calcemic genes but that the former genes require only a short pulse of analogue to effect a switch in the cell cycle, whereas the latter genes require a sustained concentration of the vitamin D ligand. The concentration of 1α,25-$(OH)_2D_3$ may be sustained *in vivo* by renal synthesis and some protection by DBP, whereas systemically administered noncalcemic analogues reach a high initial concentration but do not bind DBP and are rapidly metabolized and cleared. This hypothesis remains to be adequately tested.

Carlberg *et al.* (1994) has also tested the idea that other vitamin D analogues (EB1089 and KH1060) might favor one specific VDRE using the mouse osteopontin gene VDRE (DR3-type) and the same human osteocalcin VDRE that Morrison and Eisman used. Carlberg *et al.* (1994) found that 1α,25-$(OH)_2D_3$ and the two analogues are unable to differentiate between the two different types of VDRE. Though many VDREs have been postulated in the literature, the direct repeat-3 spacer type (DR3) of VDRE seems to be the sequence that is gaining widespread acceptance as the most physiologically relevant (Haussler *et al.*, 1998; Jones *et al.*, 1998). Whether other more exotic DR4, DR6, or inverted palindrome (IP9) nucleotide sequences are recognized by the analogue-VDR/RXR complex *in vivo* still remains unclear (Carlberg, 1995). Even with the DR-3 type of VDRE, the gene and cell context seems to be important in determining the transactivation produced by the vitamin D analogue. The work of Williams' laboratory (Brown *et al.*, 1994; Williams *et al.*, 1995; Kane *et al.*, 1996) suggests that 1α,25-$(OH)_2D_3$, KH1060, and EB1089 show different patterns of gene activation in bone marrow, osteoblastic cells (ROS17/2, ROS25/1, and UMR106), and intestine (HT29 and CaCo-2) that appear to be gene and cell-specific. Part of the explanation for gene- and tissue-specific effects probably lies in the influence of neighboring response elements to the VDRE and the binding of tissue-specific transcription factors at these sites. More recent work (Lin *et al.*, 2002) using expression profiling (gene array) to investigate the differences in gene expression exhibited by squamous carcinoma cells (SCC25) in response to EB1089 and 1α,25-$(OH)_2D_3$ concluded that the two agents did not have qualitatively different effects. In fact, the differences in gene expression between EB1089 and 1α,25-$(OH)_2D_3$ were the result of potency differences and nullified by coadministration of a general P450 inhibitor such as ketoconazole, implying that they were the result of excessive metabolism of 1α,25-$(OH)_2D_3$. On the other hand, the laboratories of Lian and Stein have elegantly demonstrated (Guo *et al.*, 1997) that the response to 1α,25-$(OH)_2D_3$-VDR-RXR complex to the gene promoter of the osteocalcin gene in osteoblasts depends on occupancy of an adjoining YY1-binding site that allows for temporal changes in responsiveness to 1α,25-$(OH)_2D_3$. Though this work explains why 1α,25-$(OH)_2D_3$ might have gene- and tissue-specific effects, it does not explain the analogue-specific differences in Williams' work, whereas the work of Lin *et al.* suggests that there are differences of potency not selectivity.

As alluded to earlier, the emergence of important new information about (1) the structure of the ligand-binding domain of the VDR and (2) coactivator characterization and involvement in the 1α,25-$(OH)_2D_3$-VDR-RXR transcriptional machinery have opened up additional possibilities about where vitamin D analogues might differ in their action from the natural ligand. Evidence suggests that the "Trap Door Hypothesis" for retinoid binding to RAR/RXR (Renaud *et al.*, 1995) also applies to 1α,25-$(OH)_2D_3$ binding to VDR. In this model, 1α,25-$(OH)_2D_3$ binding to a central binding pocket triggers a dramatic conformational change of helix 12, a domain close to the C terminus of the VDR, such that it moves from a position on the exterior of the VDR to one within the interior of the receptor, thereby closing the access channel to the ligand-binding pocket. In the process, amino acid residues of the AF-2 domain that are hidden in the unliganded VDR become exposed in the liganded VDR and are now available to interact with coactivator proteins. The recruitment of coactivators to the 1α,25-$(OH)_2D_3$-VDR-RXR subsequently leads to the recruitment of other transcription factors which result in chromatin remodeling and gene transcription (Whitfield *et al.*, 2005).

In the execution of this work, members of Moras' laboratory have modeled the ligand-binding pocket of VDR and shown it to be able to accommodate with ease several other analogues depicted in Table III (Rochel *et al.*, 2001). Though many of the active vitamin D analogues, especially the 20-epi analogues, have bulky side-chain substituents or radically different side-chain orientations (Yamamoto *et al.*, 1999), the pocket appears to have a great reserve capacity for binding (Rochel *et al.*, 2001). As a result, from modeling alone it is difficult to forecast radical changes in VDR conformations as a result of binding to these different analogues. Nevertheless, there is some indirect evidence, most notably from experiments measuring susceptibility to protease digestion, that subtle differences do occur in VDR-RXR-containing transcription complexes when different ligands are used (Peleg *et al.*, 1995; Carlberg *et al.*, 1995; Van den Bemd *et al.*, 1996). Binding of 20-epi-analogues (e.g., MC1288 and KH1060) to the VDR results in increased resistance to protease digestion compared with 1α,25-$(OH)_2D_3$, which has been interpreted as evidence for differences in accessibility of protease to cleavage sites (Peleg *et al.*, 1995). Interestingly, there appears to be a direct correlation between transactivation activity of an analogue and the resistance of the VDR-transactivation complex to protease, a relationship that applies to different analogues and even to metabolites from a single analogue (Peleg *et al.*, 1995; Liu *et al.*, 1997; Van den Bemd *et al.*, 2000). Because the rearrangement in helix 12 of the VDR brings about exposure of the AF-2 domain and this is critical to coactivator binding, it might be expected that subtle conformational differences in VDR observed for different vitamin D analogues might also be reflected in differences in coactivator recruitment.

Consequently, several groups have looked for qualitative differences in the pattern of coactivators recruited or quantitative differences in the strength of RXR heterodimerization or coactivator binding following ligand binding. Liu *et al.*, (2001) used a series of AF-2 domain mutants to reach the conclusion that the conformational changes occurring in the VDR upon hormone or 20-epi-1α,25-$(OH)_2D_3$ binding have a bigger impact on RXR-heterodimerization than on coactivator recruitment. This is in complete contrast to the work of Freedman and coworkers, who have shown repeatedly (Cheskis *et al.*, 1995; Yang and Freedman, 1999) that analogue binding [20-epi-1α,25-$(OH)_2D_3$ or 1α,25-$(OH)_2$-16-ene, 23-yneD_3] to VDR results in no difference in RXR-heterodimerization compared with binding of 1α,25-$(OH)_2D_3$. Instead, Freedman's group reports that the ability of a various analogues to transactivate vitamin D-dependent genes or to stimulate differentiation of cells is best correlated with their ability to recruit the coactivator, DRIP-205, one of the many components of the DRIP complex isolated by Freedman's group (Rachez *et al.*, 1999; Freedman and Reszka, 2005). Among the other coactivators/transcription factors implicated in vitamin D analogue action is GRIP-1 (TIF-2), which has been purported to have a particular propensity to interact with the analogue OCT (Takeyama *et al.*, 1999). In another study by Issa *et al.* (2002), a broad panel of vitamin D analogues showed that GRIP-1 was more consistently recruited at levels closer to that of 1α,25-$(OH)_2D_3$ than was another coactivator AIB-1. Work by Peleg *et al.* (2003) offers an insight into the purported bone tissue selectivity of the Roche analogue Ro 26-9228 (see Table III, renamed BXL-628) which recruits GRIP-1 in osteoblasts but not CaCo-2 colon cancer cells; though these authors may now need to explain why BXL-628 is now being pursued clinically in prostatic diseases rather than osteoporosis as was originally attempted. Thus, it appears that there is a fairly strong basis for the hypothesis that differences in the biopotency advantage of certain vitamin D analogues over 1α,25-$(OH)_2D_3$ are caused in part by changes in the recruitment of dimerization partner and/or coactivators, but there is no clear consensus on which of these coactivator proteins is the important one or if these different coactivators can explain tissue/cell selectivity.

Target Cell Catabolic Enzymes

In recent years, much evidence has accumulated to support the hypothesis that 1α,25-$(OH)_2D_3$ is subject to target cell catabolism and side-chain cleavage to calcitroic acid via a 24-oxidation pathway (Makin *et al.*, 1989). The cloning of CYP24, the cytochrome P450 involved, has confirmed that it is vitamin D-inducible because its gene promoter contains a VDRE, carries out multiple steps in the side-chain modification process, and is present in many (if not all) vitamin D-target cells (Akiyoshi-Shibata *et al.*, 1994; Prosser and Jones, 2004). We have postulated that the purpose of this catabolic pathway is to desensitize the target cell to continuing hormonal stimulation by 1α,25-$(OH)_2D_3$ (Lohnes and Jones, 1992). Recently, support for this hypothesis has emerged when St. Arnaud's group engineered a CYP24-knockout mouse that exhibits 50% lethality at weaning, death resulting from hypercalcemia and nephrocalcinosis (St. Arnaud, 1999). Surviving mice show an inability to rapidly clear a bolus dose of 1α,25-$(OH)_2D_3$ from the bloodstream and tissues (Masuda *et al.*, 2005) and a metabolic bone disease reminiscent of the excessive osteoid bone pathology observed in rodents given excessive amounts of 1α,25-$(OH)_2D_3$ (Hock *et al.*, 1986). Recent work with this model has shown that the bone defect is probably caused by excessive 1α,25-$(OH)_2D_3$ levels because crossing the CYP24-knockout mouse with the VDR-knockout mouse results in a phenotype without the bone defect (St. Arnaud *et al.*, 2000). Given the demonstrated importance of CYP24 to 1α,25-$(OH)_2D_3$ clearance, one must ask the question of whether vitamin D analogues might be subject to the same catabolic processes that determine their pharmacokinetics? If not, what other drug-catabolizing systems are present within vitamin D-target cells to inactivate the vitamin D analogue?

Certainly there are vitamin D analogues such as calcipotriol, OCT, EB1089, and KH1060 that are metabolized by vitamin D-target cells to clearly defined and unique metabolites (Masuda *et al.*, 1994, 1996; Shankar *et al.*, 1997; Dilworth *et al.*, 1997), which resemble products of the 24-oxidation pathway for 1α,25-$(OH)_2D_3$ or which are unique to the particular analogue. Furthermore, some of these metabolites are products only of vitamin D target cells and are D-inducible, implying that CYP24 is involved in their formation, and this has been confirmed with some analogues such as calcipotriol (Jones *et al.*, 2006). Moreover, in the case of several analogues blocked at C-24 and subject to metabolism elsewhere on the side chain, the direct involvement of CYP24 is strongly implicated or proven. Examples where CYP 24 involvement is strongly suspected include 23-hydroxylation of 26,27-hexafluro-1α,25-$(OH)_2D_3$ (Sasaki *et al.*, 1995); 26-hydroxylation of 24-difluro-1α, 25-$(OH)_2D_3$ (Miyamoto *et al.*, 1997); 26-hydroxylation of 1α,25-$(OH)_2$-16ene-23yne-D_3 (Satchell and Norman, 1995); and 26- and 28-hydroxylation of 1α, 25-$(OH)_2D_2$ (Rao *et al.*, 1999; Shankar *et al.*, 2001). Because many of these same products are observed *in vitro* and *in vivo* and because pharmacokinetic parameters often parallel target cell metabolic parameters (Kissmeyer *et al.*, 1995; Jones, 1997), one concludes that target cell metabolism of vitamin D analogues must contribute to the pharmacokinetics and biological activity observed *in vitro* and *in vivo*. In fact, there is little doubt that the poor performance of some promising vitamin D analogues during *in vivo* testing is because of their poor metabolic stability. Accordingly, greater attention to the metabolic potential of *in vitro* testing systems and/or greater use of defined target cell (and hepatic) metabolic systems is warranted.

One factor regarding target cell metabolism considered in recent years is the possibility that vitamin D analogues might be *activated* rather than *catabolized* by the same enzymes (Siu-Caldera *et al.*, 1999; Swami *et al.*, 2003). Although this is potentially more important for prodrugs (see Table II), the generation of large numbers of metabolites from such analogues as KH1060 (Dilworth *et al.*, 1997) or the formation of long-lived metabolites such as 26,27-hexafluro-1α,23,25-$(OH)_3D_3$ from 26,27-hexafluro-1α,25-$(OH)_2D_3$ (Sasaki *et al.*, 1995) complicates the picture. In most cases, however, this issue can be resolved on pharmacokinetic grounds.

Other Factors

Hepatic Clearance of Vitamin D Analogues

The poor DBP-binding properties of many side-chain modified calcitriol analogues open up the possibility of alternative plasma carriers and accelerated degradation. The liver plays a major role in such metabolic clearance and a small number of detailed studies performed to date have included *in vitro* incubation with liver preparations. Calcipotriol (Sorensen *et al.*, 1990), OCT (Masuda *et al.*, 1996), EB1089 (Kissmeyer *et al.*, 1997), and KH1060 (Rastrup-Andersen *et al.*, 1992) are all subject to metabolism by liver enzymes. One such liver enzyme capable of 23- and 24-hydroxylation of 1α,25-$(OH)_2D_3$, and possibly some of its analogues, is the abundant general cytochrome P450, CYP3A4 (Xu *et al.*, 2006). Indeed, this enzyme is upregulated by 1α,25-$(OH)_2D_3$ in duodenum suggesting that a physiologically relevant loop exists (Thummel *et al.*, 2001). Because, over the years, there have been frequent reports of drug-induced osteomalacia associated with coincidental use of anticonvulsants (e.g., diphenylhydantoin) or barbiturates and vitamin D preparations (e.g., Onodera *et al.*, 2002), the direct association between CYP3A4 and 1α,25-$(OH)_2D_3$ is potentially important to explain the putative accelerated clearance of vitamin D metabolites (Gascon-Barre *et al.*, 1984).

These cytochrome P450 enzymes give rise to intermediate polarity molecules or truncated metabolites, which can be further glucuronidated and excreted in bile (e.g., OCT; Kobayashi *et al.*, 1991). A recent study has defined UGT1A3 as the isoform of UDP-gluronosyltransferase involved in glucuronidation of the 23-hydroxylated metabolite of the analogue 26,27-F_6-1α,25-$(OH)_2D_3$ (Kasai *et al.*, 2005), whereas UG1A4 appears to be the isoform involved in conjugation of 1α,25-$(OH)_2D_3$ (Hashizume *et al.*, 2008).

Few, if any, studies have separately considered the *rate* of catabolism or glucuronidation relative to 1α,25-$(OH)_2D_3$. However, data are available comparing the *in vivo* rate of metabolic clearance of vitamin D analogues with 1α,25-$(OH)_2D_3$, though inevitably this probably measures a few *in vitro* parameters, such as the rate of both hepatic and target cell metabolism, in addition to the affinity of DBP binding within a single *in vivo* parameter. Thus, in lieu of detailed *in vitro* metabolic analyses, the $t_{1/2}$ of the vitamin D analogue is a useful term for indicating the general survival of the vitamin D drug *in vivo*. Data for this parameter have been published for some of the most interesting analogues (Kissmeyer *et al.*, 1995).

Nongenomic Actions of Vitamin D Analogues

The nongenomic actions of 1α,25-$(OH)_2D_3$ have been reviewed elsewhere (Norman *et al.*, 1992; Bouillon *et al.*, 1995) and were described in detail in Chapter 35 and will not be repeated here. One analogue purported to discriminate between genomic and nongenomic actions is 1β,25-$(OH)_2D_3$, the epimeric form of 1α,25-$(OH)_2D_3$, which is an antagonist of nongenomic but not genomic actions (Bouillon *et al.*, 1995). The membrane VDR initially described by Nemere *et al.* (1994) and identified as annexin II (Baran *et al.*, 2000) may be involved in mediating putative nongenomic effects. It will be interesting to see whether an annexin-II knockout mouse will possess a distinct phenotype that will aid in delineating the nongenomic actions of vitamin D in the same way that the VDR-knockout mouse has aided our understanding of the genomic actions. In recent years, further attempts to purify and identify the putative membrane receptor have resulted in a newly-named membrane-associated response system (MARRS) in chick intestinal cells (Rohe *et al.*, 2005) that may explain rapid nongenomic actions (Norman, 2005).

But at this point in time, little work has been performed on the specificity of the vitamin D-binding site of membrane VDR/annexin II or MARRS complex and thus the possibility that the nongenomic actions/membrane VDR might explain vitamin D analogue actions seems premature.

PROPOSED MOLECULAR MECHANISMS OF ACTION OF VITAMIN D COMPOUNDS

Building on the data acquired from a variety of *in vitro* tests performed over the past 20 years and described briefly in the previous section of this chapter, one is able to identify those criteria that are important to vitamin D analogue action. This in turn allows us to put forward a model for how vitamin D analogues may work *in vivo*. This is depicted in Figure 1. As a general model, it allows for consideration of both prodrugs (those requiring 25-hydroxylation by CYP27A1 or CYP2R1; those requiring 1α-hydroxylation by the kidney or extrarenal 1α-hydroxylase) and 1α,25-$(OH)_2D_3$ analogues. This model therefore makes a distinction between those target cells that express an extrarenal 1α-hydroxylase (CYP27B1) and therefore have the ability to make and respond to their own "local" 1α,25-$(OH)_2D_3$ and those that simply respond to circulating hormonal 1α,25-$(OH)_2D_3$ through their VDR with altered transcription. This model features a conventional VDR-RXR heterodimer working through a DR-3 type VDRE in most genes. Crucial characteristics for each new analogue (all measurable *in vitro*) are in our opinion:

1. affinity for DBP,
2. affinity for VDR,
3. ability to recruit RXR and coactivators followed by transactivation of genes,
4. rate of target cell metabolism (reflected partly in pharmacokinetic measurements), and
5. rate of hepatic clearance (reflected partly in pharmacokinetic measurements).

All parameters contribute significantly to the overall biological activity. Target cell distribution differences might be expected for those analogues that do not bind DBP and this model does not explain the differences observed at different genes within the same or different cells.

However, it might be useful to outline broad expectations for a new compound. Based on this model one would predict that those analogues that have good VDR binding affinity but slow rates of target cell metabolism owing to side-chain blocks, such as strategically placed fluorine atoms or double bonds, might be more active than 1α,25-$(OH)_2D_3$ *in vitro* and perhaps also *in vivo*. Hexa-fluoro-1α,25-$(OH)_2D_3$ and EB1089 are such compounds; looking alike *in vitro* and differing *in vivo* owing to differences in DBP binding and metabolism. On the other hand, a compound such as calcipotriol binds VDR moderately well but is rapidly metabolized in both the liver and the vitamin D target cell; is very active *in vitro* or when applied to the skin topically; but is inactive when administered orally and is forced to enter the bloodstream and pass the liver to get to its target site. It remains to be seen if this general model can be applied to all vitamin D analogues (e.g., the 20-epi- superanalogues such as KH1060, which has a very complex metabolic picture but very high biological activity *in vitro*) or must be adapted.

FUTURE PROSPECTS

A number of researchers remain optimistic that the unraveling of the genomic (or nongenomic) mechanism of action

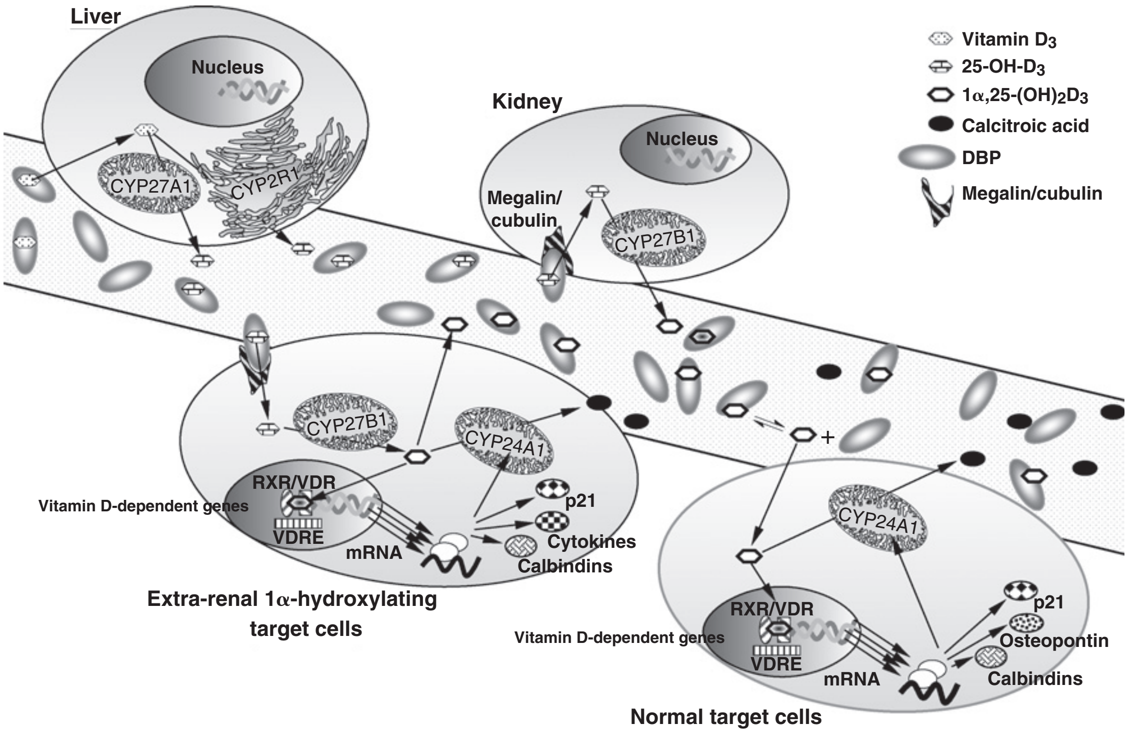


FIGURE 1 Current concepts of the activation, mechanism of action, and catabolism of vitamin D. The model incorporates a plasma-binding protein (DBP) that acts as a carrier of vitamin D metabolites and analogues; activating enzymes (CYPs) involved in activation of vitamin D or prodrug; target cell transcriptional machinery (VDR, RXR, coactivators) involved in biological actions of 1α,25-$(OH)_2D_3$ or its analogues; and target cell catabolic enzyme system (CYP24A1) involved in degradation of 1α,25-$(OH)_2D_3$ or its analogue. The figure shows the metabolism of vitamin D in the context of the cells involved. Clockwise: (*Top Left*) *Hepatocyte* showing some of the candidate cytochrome P450s shown to 25-hydroxylate vitamin D and its prodrugs; note that VDR is believed to be absent from liver cells. (*Top Middle*) *Proximal Tubular Cell* showing the key elements in the uptake of 25-OH-D_3 and its conversion to 1α,25-$(OH)_2D_3$. Megalin/cubulin are cell surface receptors that execute endocytosis of the DBP/25-OH-D_3 complex, whereas CYP27B1 is the main component of the 1α-hydroxylase, responsible for synthesis of circulating 1α,25-$(OH)_2D_3$. (*Lower Right*) *Conventional Target Cell* that lacks megalin/cubulin and takes up only the free ligand, 1α,25-$(OH)_2D_3$, but not the DBP originally involved in transporting the ligand to the target cell. The key elements of the transcriptional machinery are shown, including VDR/RXR as well as representative gene products such as cell division protein p21, the bone matrix protein osteopontin, the calcium transport protein calbindin, and the autoregulatory protein CYP24A1. The role of the highly inducible CYP24A1 is to convert the hormone (or analogue) into inactive degradation products, such as calcitroic acid, which enter plasma and are excreted in bile. (*Lower Left*) *Target Cell that expresses extrarenal 1α-hydroxylase* (*CYP27B1*) and the megalin/cubulin machinery to take up 25-OH-D_3, and thus is capable of making 1α,25-$(OH)_2D_3$ locally. The cell can also respond in a likewise manner to the conventional target cell because it also possesses the VDR and other transcriptional machinery. The expectation is that cells involved in cell differentiation or controlling cell division require higher concentrations of 1α,25-$(OH)_2D_3$ in order to modulate a different set of genes, and the CYP27B1 boosts local production to augment that *circulating* 1α,25-$(OH)_2D_3$ arriving from the kidney in the bloodstream. Under normal physiological processes, locally produced 1α,25-$(OH)_2D_3$ would not enter the general circulation, though in pathological conditions (e.g., sarcoidosis) this could occur. At this time, it is not clear how many cell types can be considered simple target cells and how many possess the CYP27B1 and megalin/cubulin to allow for local production of hormone.

of 1α,25 $(OH)_2D_3$ will reveal new approaches by which the vitamin D signaling cascade can be exploited. Certainly, the significant progress made in characterizing the coactivator proteins and the rest of the transcriptional apparatus will continue. One is able to predict fairly confidently from success in related steroid hormone fields that a fully functional vitamin D-dependent *in vitro* reconstituted VDR-RXR transcriptional system, devoid of the complications of metabolic enzymes, will be the perfect model to test the transactivation activity of future vitamin D analogues. It seems likely that this approach will allow us to dissect out the exact features that give certain analogues a transcriptional advantage to provide increased potency and/or selectivity over 1α,25-$(OH)_2D_3$.

Studies of the vitamin D-binding pockets of VDR, DBP, and the three (or more) vitamin D-related cytochrome P450s will continue to be a major goal now that all these specific proteins have been cloned, overexpressed, and crystallized. Although the ligand-binding domains of the nuclear receptors have been studied, the full-length proteins are beyond the current limits of NMR or x-ray crystallography. It is also likely that technical problems with these procedures will be overcome shortly and the full-length proteins can be tackled. The initial work of the Moras group (Rochel *et al.*, 2001) on the ligand-binding domain of the VDR will be extended to new analogues and there will also be a growing focus on the other major proteins in the vitamin D signal transduction pathway.

The wide availability of recombinant proteins for hundreds of cytochromes P450 from species across the phylogenetic tree, including 58 CYPs in the human genome, has allowed for the elucidation of some crystal structures and also modeling studies of the enzymes involved in vitamin D metabolism (Prosser *et al.*, 2006; Hamamoto *et al.*, 2006; Masuda *et al.,* 2007). Current models are starting to reveal key substrate side-chain contact residues (e.g., Ala326 within CYP24A1) associated with hydroxylation (Prosser *et al.*, 2007). The membrane-associated region of cytochromes P450 has posed problems for expression and crystallization but enormous strides have been made based on models built with x-ray data from soluble prokaryotic isoforms and with truncated mammalian isoforms such as CYP2C5 (Williams *et al.*, 2000) and the first crystal structures of vitamin D-related CYPs e.g., CYP2R1 are now emerging (Strushkevich *et al.*, 2008).

Access to full-length CYP241 and CYP27B1 has also permitted a more efficient search for potential inhibitors. Such specific inhibitors of CYP241 and CYP27B1 (Schuster *et al.*, 2001, 2003; Muralidharan *et al.*, 1997; Posner *et al.*, 2004) may be of value in blocking $1\alpha,25\text{-}(OH)_2D_3$ catabolism or synthesis in certain clinical conditions where excessive breakdown is suspected. In general, modeling of VDR and cytochromes P450 is expected to lead to more rational vitamin D analogue design to take advantage of structural idiosyncrasies of all of these key proteins. Meanwhile, the not-so-rational synthesis of new analogues is likely to continue.

The list of applications for vitamin D compounds continues to increase (reviewed in Jones *et al.*, 1998; Holick, 2007; Jones, 2007). These applications have been further rationalized with the availability of VDR knockout mice to demonstrate vitamin D-dependent processes (Yoshizawa *et al.*, 1997; Li *et al.*, 1997). Elucidation of the mechanism by which $1\alpha,25\text{-}(OH)_2D_3$ and its analogues regulate the cell cycle and proliferation remains an important priority of the field. Current applications of vitamin D analogues still fall mainly into calcium-related and cell-proliferative/differentiating arenas but the "rediscovery" of the wide effects of vitamin D deficiency and insufficiency has reinvigorated the whole field. The goal of developing analogues that can completely separate the "calcemic" and "nonclassical" properties of $1\alpha,25\text{-}(OH)_2D_3$ has not yet been fully realized. However, some promising compounds have been synthesized and interesting idiosyncrasies of their biological actions have surfaced (e.g., tissue, cell, gene, and VDRE differences). It remains to be seen whether these differences can be exploited. On the other hand, it must be stated that if vitamin D analogues work *only* through a VDR-mediated genomic mechanism, it is difficult to appreciate how the "calcemic" properties of $1\alpha,25\text{-}(OH)_2D_3$ can ever be fully resolved from the "cell-differentiating" properties given that pharmacokinetic differences have provided only a partial separation. On a more optimistic front, it can be stated that since the first/second editions of this book were published, several new vitamin D analogues (OCT, $1\alpha,24\text{-}(OH)_2D_3$, $1\alpha\text{-}OH\text{-}D_2$, and $19\text{-nor-}1\alpha,25\text{-}(OH)_2D_2$) have received governmental approval to be used in the treatment of various clinical conditions worldwide. We must remain upbeat that more vitamin D analogues will be developed and important new applications of vitamin D, particularly in the area of bone biology, remain to be uncovered.

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Chapter 84

Estrogen Effects on Bone in the Male Skeleton

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Over the past decade, we have seen fundamental changes in the landscape that used to describe osteoporosis as a rather exclusive disease of postmenopausal women. We now appreciate that the prevalence of osteoporosis in older men is substantial (Orwoll, 1998; Bilezikian, 1999; Gennari and Bilezikian, 2007), and that the consequences of a hip fracture in terms of morbidity and mortality are more severe in men than in women (Center *et al*., 1999a). Importantly, partly because the world population is aging, it is estimated that the total number of hip fractures in men in 2025 will be similar to current estimates for women (Genant *et al*., 1999; Cooper *et al*., 1992). To highlight this point, the 2004 Invest in Your Bones Campaign of the International Osteoporosis Foundation was fully dedicated to the problem of osteoporosis in men. Moreover, these observations have led, in turn, to efforts to understand better the physiology of the male skeleton, in terms of both protective factors and factors that place the male skeleton at risk. The purpose of this chapter is to review this information in the context of the developing and adult male skeleton and the role of estrogens.

Cartoons that describe the change in bone mass with time have tended to emphasize the role of aging per se because it is associated with a rather constant rate of decline in bone density from the middle years onward (Fig. 1). The downslope of this curve is more telling in women because they are exposed to an additional insult, besides age, namely the abrupt cessation of ovarian estrogen production at the time of the menopause. The accelerated bone loss characteristic of the early postmenopausal period is believed to place women at greater risk for osteoporosis than men, because men, under normal circumstances, do not experience a menopause equivalent during their middle years.

Such observations help to explain differences between men and women in their differential susceptibility to osteoporosis. However, equally important is the upswing in the curve describing acquisition of bone mass during the youthful years of skeletal accrual. Factors responsible for the establishment of peak bone mass are also key aspects to any discussion of the osteoporotic process. For example, the attainment of peak bone mass helps to define the reserve that is called upon during the period of age-related bone loss. Someone who has achieved optimal peak bone mass is likely to be relatively more protected from losing so much bone mass that places him/her at risk for osteoporotic fracture. In contrast, someone whose peak bone mass is suboptimal may show evidence for fracture risk earlier in the aging process. Although a number of factors help to account for the degree to which an individual achieves optimal peak bone mass (e.g., genetics, nutrition, environmental, exercise, calcium intake), this discussion will deal only with the role of the sex steroids, with particular emphasis on estrogens.

Estrogen deficiency states such as delayed puberty, amenorrhea of any cause, and, perhaps, the use of progestational agents for contraception, can lead to suboptimal acquisition of peak bone mass. In the male, similarly, androgens are thought to be critical to the establishment of peak bone mass. Finkelstein has shown, for example, that delayed puberty in otherwise normal boys is associated with reduced bone density for as long as 10 years thereafter compared with boys who entered puberty “on time” (Finkelstein *et al*., 1992, 1996). Although these results have been disputed by Bertelloni *et al*. (1998), who argue that volumetric bone density is not reduced among boys with delayed puberty, this point has been clarified by a recent study employing peripheral quantitative computer tomography (pQCT) in which both cortical and trabecular volumetric bone mineral density (BMD) were shown to be compromised in growing boys who are not exposed to androgens at a critical

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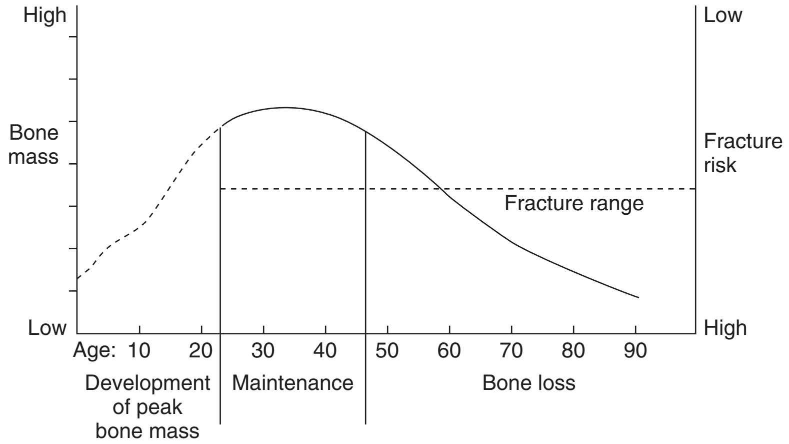

FIGURE 1 The change in bone mass as a function of the aging process. This schema illustrates the importance of establishing optimal peak bone mass in the chronology of events that take place thereafter. Adapted from Wasnich *et al.* (1991).

time in their skeletal development (Kindblom *et al.*, 2006). This study also demonstrates that late puberty is a risk factor for previous fractures in young male adults.

In sex steroid-sufficient, growing boys and girls, a 7% to 10% difference in the achievement of peak bone mass is believed to be a key protective factor in the male. Thus, not only do men not normally experience an abrupt cessation of androgen production in their middle years, but they also achieve a greater reservoir of bone owing to the attainment of greater peak bone mass in youth. Different observations have helped to clarify these differences in the achievement of peak bone mass as a function of androgens and estrogens. When true bone density is measured, that is as a volumentric quantity (g/cm^3), there is relatively little difference between the growing male and female skeleton throughout childhood (Gilsanz *et al.*, 1994; Schoenau *et al.*, 2001). The major difference in bone density between the growing female and male skeleton at puberty relates to areal density (g/cm^2) as determined by dual-energy x-ray absorptiometry (DXA). Thus, the difference appears to be bone size, a parameter that has been attributed to androgens (Beck *et al.*, 1992).

Recent pQCT studies have demonstrated that reductions in trabecular volumetric BMD begin before midlife in both sexes, and that they are greater in women than in men at central sites, but similar at peripheral sites (Riggs *et al.*, 2004, 2007). Cortical bone loss begins in the perimenopause in women and later in life in men, and is higher in women than in men, consistent with the menopausal-induced increases in bone turnover and skeletal porosity. Seeman and other investigators emphasize the point that androgens have region-specific actions on bone growth and remodeling so as to lead to larger bones (Orwoll, 1995b; Duan *et al.*, 1999; Seeman, 2001, 2002, 2004; Kim *et al.*, 2003). Bones that are exposed to androgen display greater periosteal apposition, which, in turn, leads to larger cross-sectional diameter. Under the influence of androgens, therefore, bones are bigger, but not necessarily more "dense." Although young men clearyly have larger bones than young women, age-related increases in bone size (at least as assessed by QCT) appear to be similar at most sites in men and women (Riggs, 2004); these findings are in contrast to measurements with DXA, which do suggest greater periosteal apposition over life in men compared with women (Duan *et al.,* 2003). Structural and biomechanical basis of sexual dimorphism in femoral neck fragility has its origins in growth. Nonetheless, at any age, for a given reduction in cortical bone mass, an expected concomitant of aging, the male achieves a relative mechanical advantage by increased cross-sectional diameter. This effect may well be a specific androgen action, although estrogen and IGFI may play a role as well (Vanderschueren *et al.*, 2006).

Of interest, consistent gender differences in the age-related changes of bone microstructure at the ultradistal radius have also been described in recent population-based cross-sectional studies using high-resolution pQCT (HRpQCT; Khosla *et al.*, 2006a). This is a new technology that can image the skeleton noninvasively with a resolution that is as high as 100 μm. Using HRpqCT, women show a progressive loss of trabeculae with an increase in trabecular separation with age, whereas men show trabecular thinning but no substantial loss of trabecular number or any major increase in trabecular separation. Because decreases in trabecular number have been shown to have a much greater impact on bone strength compared with decreases in trabecular thickness, these findings may help to explain how men maintain their skelelal strength with aging better than women.

Although it may have seemed clear that differences between the growing female and male skeleton as well as the skeleton in decline could be attributable satisfactorily, if not completely, to the sex-specific steroids (estrogens in women; androgens in men), recent observations have shattered this simple concept. It is no longer straightforward. The new observations underscore the normal biosynthetic pathway by which estrogens are made. C-18 estrogens are derived from androgens via the activity of aromatase, a cytochrome P450 product of the CYP 19 gene (Bulun *et al.*, 1993). The obligate precursors are the C-19 androgenic steroids. The human P450 aromatase enzyme is found in many tissues such as placenta, ovary, testis, brain, and adipocyte. In human bone, aromatase activity is reported to be expressed in osteoblast or osteoblast-like cells from fetal and normal tissue, in articular cartilage chondrocytes, in adipocytes adjacent to bone trabeculae, and in osteocytes, but not in osteoclasts (Sasano *et al.*, 1997). Apart from contributing to the circulating estrogen pool, extragonadal sources of estrogen may be locally active in a paracrine or intracrine fashion (Simpson *et al.*, 2001; Labrie *et al.*, 1997). Thus, although the total amount of estrogen synthesized at any site could be small, local tissue concentrations could be substantial. Regulation of aromatase activity occurs through tissue-specific promoters and alternative splicing mechanisms (Simpson *et al.*, 1997, 2001). The translated gene product, however, is the same among all tissues that harbor an active aromatase gene.

With this background, one can quite reasonably revisit the classical observation assigning differences among male and female skeletal growth to androgens or estrogens, respectively. It is possible that the growing male skeleton is also dependent upon estrogens and that some skeletal events previously noted to be androgenic may actually be caused, at least in part, by estrogens. Such thinking has received seminal support by two fundamental experiments of nature. In two rare syndromes, the estrogen receptor or the aromatase gene has been "knocked out" by a point mutation, rendering subjects either estrogen resistant (receptor defect; Smith *et al.*, 1994) or estrogen deficient (aromatase defect; Morishima *et al.*, 1995; Carani *et al.*, 1997). A key role of estrogens in male skeletal development has emerged from insights gleaned from the study of these unusual subjects.

ALPHA-ESTROGEN RECEPTOR DEFICIENCY IN THE MALE

The pivotal importance of estrogen in the growing male skeleton was first demonstrated by the single case report of complete estrogen resistance in a young 28-year-old male owing to a disruptive homozygous mutation in exon 2 of the alpha-estrogen receptor gene (ERα) (Smith *et al.*, 1994). The gene defect was inherited as an autosomal recessive from his consanguineous parents who were heterozygotes for the gene defect. A cytosine to thymidine transition at codon 157 resulted in a stop codon and an alpha estrogen receptor that is severely truncated and cannot bind estrogen. He had no detectable response to administration of large doses of exogenous estrogen (transdermal ethinyl estradiol), achieving serum concentrations of estradiol 10-fold higher than the typical male, 270 pg/mL (nl, 10 to 50). His own baseline estradiol (119 pg/mL) and estrone (145 pg/mL) levels were markedly above normal. Although bound and free testosterone as well as dihydrotestosterone concentrations were normal, luteinizing hormone (LH; 37 mIU/mL) and follicle-stimulating hormone (FSH; 33 mIU/mL) levels were in the mildy castrate range. He was extremely tall (204 cm) without acromegalic features and still growing with open epiphyses (bone age, 15 years) and genu valgum. The growth curve did not show a pubertal growth spurt, although his puberty in all other respects was normal. His body proportions were eunuchoid. Bone density of the lumbar spine as measured by dual energy x-ray densitometry was 0.745 g/cm^2, corresponding to 2 standard deviations below average for a 15-year-old boy.

AROMATASE DEFICIENCY IN THE MALE

So far, there are eight known cases of aromatase deficiency in men, owing to inactivating mutations in the CYP19A1 gene (Morishima *et al.*, 1995; Carani *et al.*, 1997; Deladoey *et al.*, 1999, Hermann *et al.*, 2002; Herve *et al.*, 2004; Boullion *et al.*, 2004; Maffei *et al.* 2004, 2007). The disorder occurs in an autosomal recessive pattern, and in at least 6 of the 8 described subjects parental consanguinity was evident. A summary of described mutations is reported in Table I. All known mutations exhibit no or minimal enzyme activity in transient expression systems. Accordingly, circulating estrogen levels are markedly low or undetectable but androgens are normal or elevated. Serum FSH was elevated or in the upper range, whereas serum LH was normal except in one patient.

Interestingly, the skeletal phenotypes of aromatase-deficient men are virtually identical to the man with a loss-of-function mutation at the ERα gene (Table II and Table IV). Common characectistics include tall stature, continued longitudinal growth, unfused epiphyses, delayed bone age, lack of pubertal growth spurt, eunuchoid skeletal proportions (upper segment/lower segment ratio from 0.84 to 0.92), genu valgum, elevated bone resorption markers, and reduced bone mass. Lipid abnormalities, increased body mass index, hyperinsulinemia, and various degrees of glucose impairment (including diabetes and achantosis nigricans in one and two cases, respectively) have also been reported. When some of these subjects were treated with intramuscular testosterone, there was no benefit, an understandable observation because aromatase deficiency does not lead to low testosterone levels. Indeed, in two cases (Morishima *et al.*, 1995; Hermann *et al.*, 2002), testosterone levels were markedly elevated. On the other hand, estrogen treatment in all cases of aromatase deficiency is associated with marked changes in the skeletal phenotype (Fig. 2 and Fig. 3). Epiphyses closed quickly, longitudinal growth ceased, and BMD increased dramatically (Carani *et al.*, 1997; Deladoey *et al.*, 1999; Bilezikian *et al.*, 1998; Maffei *et al.*, 2004; Rochira *et al.*, 2000). A comparison between the syndromes of estrogen resistance and estrogen deficiency in the male is shown in Table III.

TABLE I Human Aromatase Gene (CYP19A1) Mutations

Reference	CYP1A1 Mutation	AA change	Aromatase activity
Morishima *et al.*, 1995	C1123T, ex. 9, homoz.	Cys375Arg	0.20%
Carani *et al.*, 1997	G1094A, ex. 9, homoz.	Glu365Arg	0.40%
Deladoey *et al.*, 1999	156delC, ex.5, homoz.	Frameshift and stop codon	(0%)*
Hermann *et al.*, 2002	IVS6-3 C > A, int. 5, homoz.	Frameshift and stop codon	(0%)*
Herve *et al.*, 2004	Insertion, ex. 9 (c353), homoz.	Frameshift	–
Maffei *et al.*, 2004	G > A last nt. of ex. 5, homoz.	Glu210Lys	(0%)*
Boullion *et al.*, 2004	C-deletion in ex.5, homoz.	Frameshift and stop codon	(0%)*
Maffei *et al.*, 2007	T380G ex. 4, heteroz. G1124A ex. 9, heteroz.	Arg127Met His375Arg	0% (ex. 4) 7% (ex. 9)

*Presumed due to truncated protein (lacking functional domains).

TABLE II Skeletal Phenotypes of Aromatase-Deficient Men

Reference	Age (yr)	Bone Age (yr)	Height (cm)	Unfused epiphyses	BMD T score
Morishima *et al.*, 1995	24	14.5	204	Yes	−4.46 (forearm) −1.96 (lumbar spine) −0.36 (femoral neck)
Carani *et al.*, 1997	31	14.8	187	Yes	−2.07 (lumbar spine)
Deladoey *et al.*, 1999	infant	–	–	–	–
Hermann *et al.*, 2002	27	16.5	197	Yes	−2.24 (lumbar spine)
Herve *et al.*, 2004	26	–	190	–	–
Maffei *et al.*, 2004	29	15.0	177	Yes	−3.30 (lumbar spine) −2.30 (femoral neck)
Boullion *et al.*, 2004	17	12.5	176	Yes	−2.31 (lumbar spine) −0.46 (femoral neck) −3.40 (ultradistal radius) −3.63 (mid radius)
Maffei *et al.*, 2007	25	15.3	192	Yes	−1.80 (ultradistal radius) −3.70 (mid radius)

Importantly, a dose-dependent effect of estrogen replacement therapy on bone mass in aromatase-deficient men exists, because a very low dose of estradiol (below 25 μg twice weekly) was not sufficient for maintaining a normal bone mineral density (Rochira *et al.*, 2000). Long-term data are available with a 5-year follow-up in the patient described by Morishima, Bilezikian, and their colleagues. Starting on low-dose conjugated estrogen, 0.3 mg, he was quickly raised to 0.75 mg daily, the dosage that he has consistently taken for most of the period of observation. Estradiol levels rose from undetectable into the normal range for men. Androgen levels fell from markedly elevated levels to normal. The concomitant return of the gonadotropins, LH and FSH, to normal illustrates well the role of estrogens in controlling gonadotropin production in the male (Fig. 4). He immediately stopped growing with closure of all open epiphyses within 6 months of starting estrogen therapy (see Fig. 2). Bone markers that were elevated at baseline returned slowly to normal (Fig. 5). Most impressively, this man has experienced a dramatic increase in bone density with estrogen therapy. During the first 3 years, increases in the lumbar spine, femoral neck, and forearm were 20.7%, 15.7%, and 12.9%, respectively. Over the next 2 years of estrogen therapy, the gains in the lumbar spine and femoral neck were maintained with further marked increases in the forearm bone density, now totaling 26% (see Fig. 3). The improvement in bone mass is seen

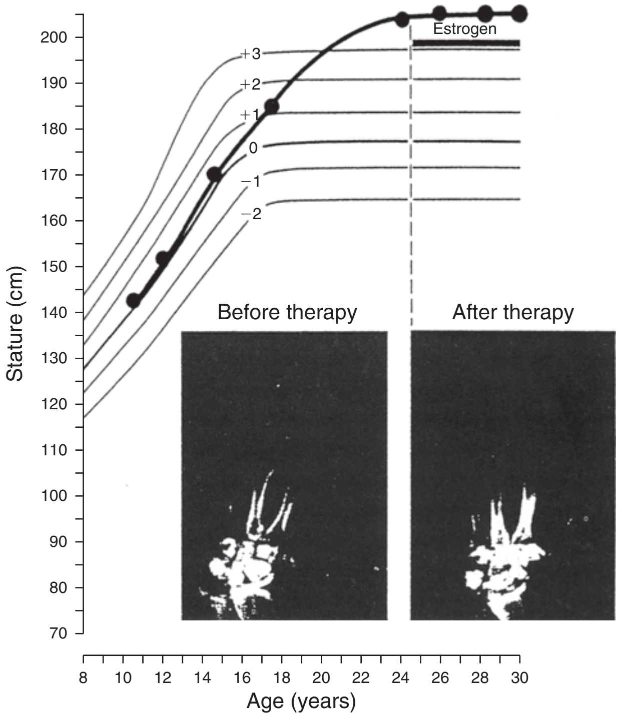

FIGURE 2 Growth curve and bone age before and after 5 years of estrogen therapy in a man with aromatase deficiency. After conjugated estrogen therapy was begun (bar), linear growth ceased immediately. Height has remained at 204 cm since therapy, whereas previously it was showing continued increases. All epiphyses were closed within 6 months (*insets*). The curves with + and – numbers represent the mean and standard deviations for normal young men.

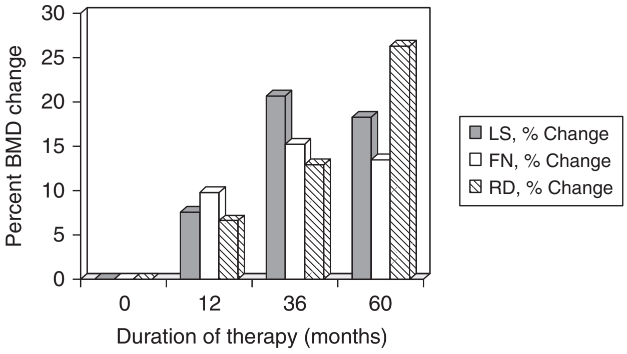

FIGURE 3 Change in lumbar spine (LS), femoral neck (FN), and radial (RD) BMD with estrogen therapy in a man with aromatase deficiency.

also by the change in *T* score with normal values at the lumbar spine and femoral neck and from markedly osteoporotic levels in the forearm to mildy osteopenic levels ($T = -4.65$ to -1.84). The effect of estrogen to improve bone mass is best described as anabolic because of the magnitude of the change and also because further bone growth did not occur. This anabolic action of estrogen is quite different from effects in the postmenopausal woman, in whom the estrogen effect is more accurately described as antiresorptive.

A recent report of a 16-year-old boy with aromatase deficiency has sharpened our views of how bone density is abnormal in this syndrome (Boullion *et al.*, 2004). All reports up to this recent one assessed BMD by dual-energy x-ray absorptiometry (DXA), an approach that gives only areal density, not true volumetric bone density. In this case, the skeleton of the 16-year-old boy with congenital aromatase deficiency was assessed by pQCT of the radius as well as by DXA. The use of pQCT allows measurements not easily made or even possible by DXA, such as cross-sectional area (CSA), cortical thickness, trabecular volumetric BMD, and cortical volumetric BMD. As expected, estrogen replacement in this boy was associated with normalization of sex hormone levels, reduced bone turnover rate, and increased areal BMD of the lumbar spine (23%) and femoral neck (14%). However, the gain in volumetric BMD, either estimated by the calculation of the bone mineral apparent density from DXA or assessed directly by pQCT was limited at the lumbar spine and even absent at the femoral neck and the radius. Interestingly, longitudinal bone growth, CSA, and cortical thickness, as measured by pQCT, increased significantly by 8.5%, 46%, and 12%, respectively. Thus, the observed increase in areal BMD at peripheral sites was mainly driven by an increase in bone size, rather than bone density. These changes are similar to those associated with normal pubertal growth and support the notion that in growing bones, except for the spine, true density does not increase (Seeman *et al.*, 2001; Sundberg *et al.*, 2003). Moreover, these observations not only account more completely for the densitometric observations but they also lead to a reconsideration of functional sex steroid assignments on bone size. It is well known that during normal male puberty, periosteal diameter continues to expand and cortical thickness increases because of reduced endocortical expansion and accelerated periosteal apposition (Seeman, 2002). These effects, leading to increased bone size, have classically been attributed to androgens and in fact help to account for greater areal density in the male skeleton. Conversely, when females enter puberty, periosteal apposition is inhibited, an action classically believed to be an estrogen effect. According to the results of the parameters altered by estrogen administration to the young boy with aromatase deficiency (Boullion *et al.*, 2004), some actions on bone size, previously attributed to androgens, must at least in part be an estrogen effect. It is likely that estrogen, rather than androgens, at least in this single example of a growing boy, is helping to drive periosteal bone apposition. A biphasic, dose-dependent effect of estrogen could exist. At low levels (as observed in males and in early pubertal females) estrogen may stimulate periosteal apposition and increase bone size, whereas at higher concentrations (as observed in late pubertal and adult females) estrogen may inhibit cross-sectional bone growth. This latter effect could be caused by stimulation of the estrogen receptor β.

Recently, the concomitant presence of mild hypogonadism in a man with aromatase deficiency has offered a useful

TABLE III Extraskeletal Characteristics of Aromatase-Deficient Men

Reference	Obesity (BMI)	Diabetes	Insulin resistance	Achantosis nigricans	Dyslipidation	Steatohepatitis
Morishima *et al.*, 1995	Yes (32.5)	No	Yes	No	Yes	–
Carani *et al.*, 1997	No (27.6)	No	No	No	Yes	–
Hermann *et al.*, 2002	Yes (30.9)	No	Yes	No	No	–
Maffei *et al.*, 2004	No (25.4)	Yes*	Yes	Yes*	Yes	Yes
Maffei *et al.*, 2007	Yes (35.8)	No	Yes	Yes	Yes	Yes

**Developed during high-dose testosterone replacement.*

TABLE IV Comparison of α-Estrogen Receptor and Aromatase Deficiency Syndromes

	ERα deficiency	Aromatase deficiency
Testosterone	Normal	High/normal
Estrogen	High	Low
Gonadotropins	High	High/normal
Bone turnover	High	High
Bone age	Reduced	Reduced
Bone mass	Reduced	Reduced
Response to estrogen	No	Yes
Testicular size	Normal	Large/normal
Tallness	Yes	Yes
Eunuchoid proportions	Yes	Yes
Masculinized	Yes	Yes

model to study the effects of testosterone and estradiol replacement, separately or in combination (Rochira *et al.*, 2007). In this man, estradiol treatment alone increased BMD with a greater gain than the one obtained with testosterone alone. Interestingly, the combination of testosterone (6 mg/day) and estrogen (25 μg twice weekly) replacement led to a further increase in cortical thickness at the radius and the tibia as measured by pQCT, further supporting the concomitant importance of both sex steroids for periosteal apposition. In this case, however, an increase in volumetric BMD at the tibia and the radius as well as an increase of areal BMD of the lumbar spine and the femoral neck was also described after 2 years of combined therapy.

The converse of the inactivating mutation of the aromatase gene, namely a mutation leading to overly active aromatase activity, has also been instructive with regard to assigning a role for estrogens in the male skeleton (Hemsell *et al.*, 1977; Bulun *et al.*, 1999; Stratakis *et al.*, 1998). The observations are opposite to those made in the aromatase-deficient male. Characteristic findings are accelerated bone growth, advanced bone age, and short stature, features that are all consistent with the precocious presence of estrogen in an immature skeleton. Other common characteristics of this syndrome include prepubertal gynecomastia and mild hypogonadotropic hypogonadism. Despite increased estrogen levels and testosterone levels that are frankly low or in the lower range of normal, males with aromatase excess appear to be fertile. In some cases, short-term treatment with an aromatase inhibitor (testolactone or anastrozole) was successful in reducing serum estrogen levels, restoring gonadotropin

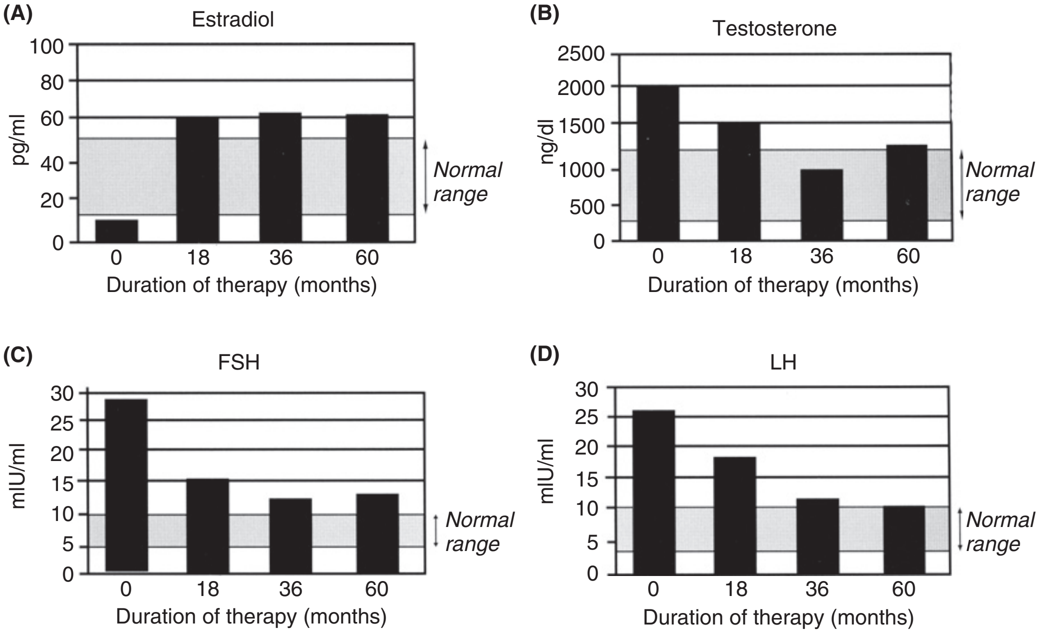

FIGURE 4 Changes in sex steroids and in gonadotropins with estrogen therapy in a man with aromatase deficiency. The data are shown for estradiol (**A**), testosterone (**B**), FSH (**C**), and LH (**D**). Over 5 years, estradiol levels initially rose and then were maintained at levels in the normal range for males when administered at 0.75 mg/day. Testosterone levels fell into the normal range, as did FSH and LH levels.

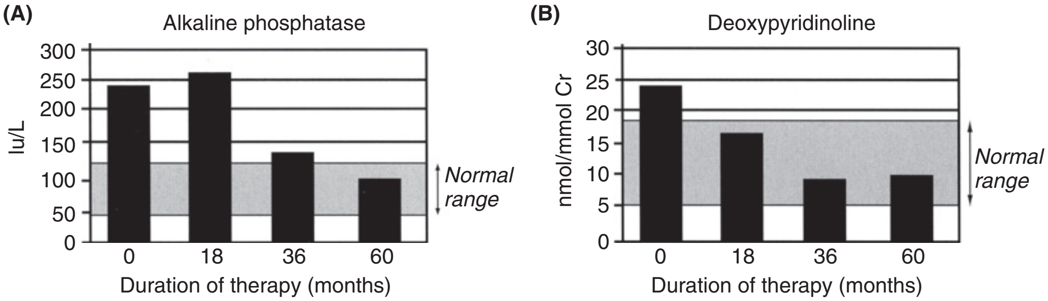

FIGURE 5 Changes in bone markers with estrogen therapy in a man with aromatase deficiency. The data are shown for alkaline phosphatase (**A**) and for deoxypyridinoline (**B**). The resorption markers, deoxypyridinoline, appear to fall faster over time than did the formation marker, alkaline phosphatase.

and testosterone levels to normal, and arresting skeletal aging (Stratakis *et al.*, 1998; Shozu *et al.*, 2003; Martin *et al.*, 2003). Even though at least 5 families with 13 affected male subjects and 2 sporadic cases of aromatase excess syndrome have been described, the effects of estrogen excess on bone turnover and bone density in men remain as yet unknown. The only available data in this respect relate to bone density that has been described to be in the upper limits of the normal range in two siblings with familial hyperestrogenism. It is a remarkable observation considering the additional point that the man had hypogonadism and the woman had irregular menses (Martin *et al.*, 2003).

Of special note is the relationship between the pubertal growth spurt and estrogen. In the syndromes of estrogen deficiency or resistance, the tallness and continued growth well into adulthood was not associated with a pubertal growth spurt. Conversely, in the example of aromatase excess, owing to an activating mutation of the aromatase gene, with elevated estrogen levels, puberty occurs early and is accompanied by skeletal maturation. Additional evidence for an important role of estrogens in the pubertal growth spurt is the testicular feminization syndrome in which XY males do not respond to androgens because of a mutation in the androgen receptor. Responsiveness to estrogens is normal. In this syndrome, the pubertal growth spurt is seen (Grumbach and Auchus, 1999; Zachmann *et al.*, 1986). Finally, in estrogen-secreting tumors, similar observations of premature skeletal maturation have been made (Coen *et al.*, 1991; Simpson *et al.*, 1994; Grumbach and Styne, 1998). These observations, therefore, argue rather persuasively that in the male, as in the female, the pubertal growth spurt is a function of estrogens, not androgens. It is interesting to speculate that the difference in height among women and men may reflect the difference in the time of onset of puberty. Boys gain a few more years of linear growth because their puberty occurs later. The notion, namely, that the additional stature in the male is owing to estrogen, not androgens, is particularly intriguing.

From these human experiments of nature, it seems reasonable to conclude that in the male, estrogens are important in the control of linear growth, in the pubertal growth spurt, in epiphyseal maturation, and in the achievement of optimal peak bone mass and size. Such conclusions, obviously, must be tempered by an appreciation that androgens are also important in the acquisition of peak bone mass. In the testicular feminization syndrome, for example, the peak bone mass achieved is consistent with expectations for an estrogenic influence only because these XY boys had bone density most consistent with expectations for an XX girl (Kelch *et al*., 1972). In either event, the failure to achieve peak bone mass may be a risk factor for the development of osteoporosis later in life. Although one does not expect to "find" many examples of complete estrogen deficiency or resistant syndromes, more subtle abnormalities are possible in those genes (i.e., polymorphisms) that would render men at risk for osteoporosis on this basis (Gennari *et al*., 2004a).

ANIMAL "KNOCKOUTS" OF THE ESTROGEN RECEPTOR AND AROMATASE GENES

Male and female mice in which either of the two ERs or the aromatase genes have been knocked out (KO), generated by several different investigative groups, include the following phenotypes: ERα KO, ERβ KO, or deletion of both ERα and ERβ (DERKO) mice. Overall, these models show a less dramatic phenotype than corresponding alterations in human subjects born with inactivating mutations of either the ERα or the aromatase gene. Particularly, deletions of both the ERα and the ERβ in these mice do not seem to completely account for loss of estrogen action in bone, because the impact of DERKO is seemingly not as great as the impact of gonadectomy on the skeleton (Gentile *et al*., 2001; Bilezikian, 2002). Initial studies reported a reduction in BMD and a tendency toward an increase in bone turnover in male ERα KO mice (Couse *et al*., 1999; McCouley *et al*., 2003). Conversely, male ERβ KO mice have a skeletal phenotype that is identical to that of the wild type, whereas ERβ KO females showed an increase in cortical bone that was associated with enhanced periosteal apposition, and diminished age-related bone loss (Windahl *et al*., 1999, 2001; McDougall *et al*., 2002; Chagin *et al*., 2004). Some male DERKO models showed reductions in bone density that are similar to single ERα KO animals suggesting that the ERα is more protective than the ERβ in bone (Vidal *et al*., 2000; McCouley *et al*., 2003; Tozum *et al*., 2004). However, more recent and extensive investigations of KO models by histomorphometry, DXA, and pQCT have been puzzling, showing different and contrasting skeletal phenotypes (Sims *et al*., 2002, 2003; Lindberg *et al*., 2002; Parikka *et al*., 2005). In this respect, it should be emphasized that ERα and DERKO, but not ERβ, KO mouse models have clearly increased serum levels of testosterone and estradiol. These elevated, supraphysiological sex hormone levels could have accounted for the lack of some of the expected skeletal changes. Importantly, variations in IGF-I levels have also been observed in ERα and DERKO animals (Vidal *et al*., 2000). Moreover, some of these KO models do not seem to be completely functional KOs, because it has been shown that they express truncated ER transcripts that are able to bind estradiol and, in the case of ERα, capable of activating transcription in an estrogen-dependent manner (Couse *et al*., 1995; Krege *et al*., 1998). Finally, the recent generation of mice carrying a mutation that eliminates classical (estrogen-responsive element mediated) but not nonclassical (non-estrogen-responsive element mediated) ERα signaling (nonclassical ERα knockin mice, NERKI) suggest that there is a critical balance between classical and nonclassical ERα pathways, which, when altered, can result in markedly aberrant responses to estrogen (Syed *et al*., 2005). It is possible that the expressed truncated ER transcripts in some of these ER KO models, together with supraphysiological estrogen levels, may alter this balance and account for these apparent but unexpected estrogen effects. To address this issue, Nakamura *et al*. (2007) recently generated mice with deletion of ERα specifically in osteoclasts and demonstrated osteopenia in female, but not male mice, suggesting that in this model androgen action via the AR was sufficient to compensate for loss of ERα in male, but not female mice. Overall, however, the data from the ER KO models indicates that ERα mainly regulates bone remodeling in male mice, whereas in female mice both ERα and ERβ influence this process and could partially compensate for each other.

Recent findings from ERs KO mice also suggest that the activity of ERα may explain the positive interaction between estrogen and exercise at endocortical and trabecular bone surfaces (Jessop *et al*., 2004). In contrast estrogen may inhibit the anabolic exercise response at the periosteal surface by the activation of ERβ. Signaling through this receptor retards periosteal bone formation and suppresses gains in bone size and bone strength, thus acting as an antimechanostat (Saxon *et al*., 2005).

The skeletal consequences of estrogen deficiency are illustrated further by studies of the aromatase knockout mouse (ArKO) model (Fisher *et al*., 1998; Honda *et al*., 1998; Toda *et al*., 2001). The newborn skeleton of ArKO animals demonstrates no consistent differences in bone and mineralized cartilage from wild-type littermates

(Oz *et al*., 2001). In contrast, growing and adult male and female ArKO mice show reduced BMD and micro-CT parameters, particularly at trabecular sites (Oz *et al*., 2000, 2001; Miyaura *et al*., 2001). The reduction in BMD in these mice was completely prevented by estrogen replacement (Miyaura *et al*., 2001). Histologically, knockout animals of both sexes have significant reductions in trabecular bone volume, trabecular thickness, and cortical thickness, compared with wild-type littermates (Oz *et al*., 2000). Analysis of the effects of aromatase inactivation on bone-remodeling parameters in different ArKO mice models has shown conflicting results. In one study on static and histomorphometric parameters of the spine, adult ArKO males showed a low-turnover pattern, with significant reductions in osteoblastic, osteoid, and eroded surfaces and with reduced mineralizing surface as observed from tetracycline uptake (Oz *et al*., 2000). In contrast, adult ArKO females showed a high-turnover pattern, suggesting a sexual dimorphism in bone remodeling of 5- to 7-month-old ArKO animals (Oz *et al*., 2000). These observations were confirmed in a subsequent study utilizing fluorine-18 PET imaging (Oz *et al*., 2001). By this technique, ArKO males clearly demonstrated reduced vertebral remodeling compared with wild-type littermates, and estradiol treatment in these mice increased fluorine-18 uptake to normal levels. The sexual dimorphism in bone remodeling in ArKO animals was not appreciated in another ArKO mice model, in which increased bone resorption was observed in both male and female animals (Miyaura *et al*., 2001). Treatment with estrogen restored the increased parameters of bone remodeling to the wild-type level in both sexes (Miyaura *et al*., 2001). Although it is not clear how to account for the differences in these observations, what is clear is that aromatase deficiency profoundly influences processes associated with bone turnover in both male and female animals. To further clarify the relationship between estrogen and androgen in bone, male ArKO mice were orchidectomized to induce a double deficiency of estrogen and androgen (Matsumoto *et al*., 2006). In this model, ORX induced a further decrease in BMD and in endosteal bone resorption compared with sham-operated ArKO mice, without any major difference in periosteal bone formation.

In different experiments, aromatase inhibition in aged male rats by vorozole has been associated with increases in bone resorption and bone loss to an extent similar to that of orchidectomy (ORX) (Vanderschueren *et al*., 1996, 1997, 2000). Importantly, vorozole treatment does not significantly affect cortical thickness in contrast to ORX, suggesting a direct role of androgens on this geometric property at cortical sites (Vanderschueren *et al*., 1997). Consistently, androgen receptor KO mice and ORX mice show a similar degree of reduction in cortical area and thickness, as a result of a reduced bone formation at the periosteum (Venken *et al*., 2006). Moreover, testosterone replacement stimulated periosteal bone formation in ORX but not ARKO mice, again supporting the concept that androgen receptor activation plays a major role at the periosteum (Venken *et al*., 2006). Interestingly, administration of an aromatase inhibitor in these mice partly reduced testosterone action on periosteal bone formation in ORX but not ARKO mice.

Taken together, these studies on ERs and CYP19 KO mice, as well as other rodent models of altered sex steroid action in the male, provide general confirmation of the human gene KO disorders and suggest that estrogen may be more protective in the skeleton than androgens in males. A minimum circulating estrogen level is also required for maximal androgen-induced stimulation of periosteal apposition. In contrast, higher circulating estrogen levels may have negative effects on the periosteum. This concept has been further emphasized by the observation that transgenic male mice overexpressing aromatase activity have increased trabecular BMD, but decreased bone formation rate at the periosteum (Peng *et al*., 2004). Moreover, ERα appears to be the main effector of estradiol's protective function in bone in both male and female, as also indicated by *in vivo* animal studies on the use of ERα-selective ligands (Harris *et al*., 2002).

STUDIES IN HUMANS-OBSERVATIONAL DATA

The data noted earlier from the human "experiments of nature" clearly indicate that estrogen plays a significant role in the male skeleton. In particular, they demonstrate that estrogen is required for epiphyseal closure, that it has significant effects on skeletal modeling during growth, and that it is necessary for optimal acquisition of bone mass and size during puberty in boys. These observations, however, leave several major questions unresolved. In particular, they leave open the issues of what, if any, are the roles of estrogen in regulating bone remodeling in adult men with mature skeletons, whether estrogen is necessary for the maintenance of bone mass in adult men, and what, if any, is the role of estrogen (or estrogen deficiency) in mediating age-related bone loss in men.

Before addressing these issues, it is useful to review the available data on age-related changes in serum sex steroid levels in normal men. As shown in Figure 6A, serum total testosterone levels do not change significantly in otherwise healthy men until the ninth decade. Similarly, there is little or no change in serum total estradiol levels in men over life (see Fig. 6B). By contrast, serum bioavailable (or non-sex hormone-binding globulin (SHBG) bound) testosterone levels decrease by approximately 70% and serum bioavailable estradiol levels decrease by approximately 50% over life. To the extent that the non-SHBG bound sex steroid fraction is the one that has access to target tissues (Manni *et al*., 1985; Giorgi and Moses, 1975), available data from

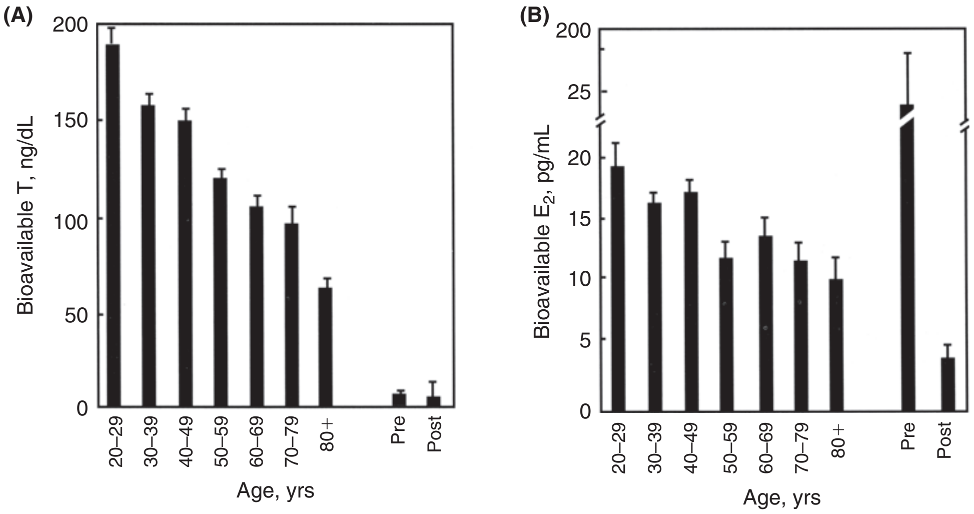

FIGURE 6 Serum total testosterone (**A**) and estradiol (**B**) levels as a function of age in a population-based sample of men. Data are adapted from Khosla *et al.* (1998), with permission.

either cross-sectional or longitudinal studies in different populations indicate that while men do not have the equivalent of the menopause, they clearly have significant (but gradual) age-related decreases in bioavailable testosterone and estradiol levels over life (Harman *et al.*, 2001; Khosla *et al.*, 2001; Gennari *et al.*, 2003; Orwoll *et al.*, 2006; Liu, 2007). These changes are primarily owing to a large increase (of approximately 125%) in serum SHBG levels over life in men (Khosla *et al.*, 1998). Although the reason(s) for this increase in SHBG levels are unclear, it is apparent that the age-related increase in SHBG serves to limit the availability of testosterone and estradiol to target tissues in elderly men.

A first indication about the relative roles of estrogen versus testosterone in the male skeleton came from cross-sectional observations in middle-aged and elderly men relating sex steroid levels to BMD at various sites. It was shown that BMD is more directly related to declining estrogen levels than declining androgen levels, particularly when circulating bioavailable fractions of these steroids were considered (Slemenda *et al.*, 1997; Greendale *et al.*, 1997; Khosla *et al.*, 1998; Center *et al.*, 1999b; Ongphiphadhanakul *et al.*, 1998; Amin *et al.*, 2000; Barrett-Connor *et al.*, 2000; Szulc *et al.*, 2001). Indeed, serum estradiol levels were more robust predictors of BMD than serum testosterone levels even in a sample of androgen-deficient men from the Framingham Study (Amin *et al.*, 2000). Conversely, more conflicting data have been presented regarding the association between testosterone and BMD. Although these findings add to the evidence from the ER-alpha and the aromatase-deficient males in building the case for an important role for estrogen in the male skeleton, there are clear limitations to cross-sectional observational data. Thus, because BMD in adult men is a function both of peak bone mass and bone loss with aging, these studies cannot dissociate the effects of estrogen on the acquisition of peak bone mass in early adulthood from its effects on bone loss later in life. Moreover, existing differences on the relative contribution of estrogen versus androgen among cross-sectional studies may be also related to site-specific effects of sex steroids on BMD as well as to additive effects on bone geometric properties that cannot be completely addressed by the measurements of areal BMD. A recent study in a large cohort of elderly men suggests that testosterone is an independent positive predictor of BMD particularly at cortical (hip and forearm) rather than at trabecular (spine) sites (Mellström *et al.*, 2006). In the same cohort, estradiol was an independent predictor of BMD at all bone sites, with the highest predictive value seen for lumbar spine. Moreover, the predictive effect of testosterone for prevalent fractures was not affected by adjustement for BMD. In a different pQCT-based study on young Swedish men at the age of peak bone mass, free estradiol was a positive predictor of volumetric BMD, whereas it was a negative independent predictor of cortical cross-sectional area, periosteal circumference, and endosteal circumference. Conversely, free testosterone did not significantly affect volumetric BMD, but positively regulated cortical cross-sectional area, periosteal circumference, and endosteal circumference (Lorentzon *et al.*, 2005). Age- and sex hormone-related differences on bone microstructure have also been described in a recent HRpQCT cross-sectional study (Khosla *et al.*, 2006b).

Importantly, cross-sectional data have been confirmed in general in longitudinal studies. Over a 2-year period, Slemenda *et al.* (1997) described lower estradiol levels in men losing BMD at more than 1% per year compared with men with higher estradiol levels and lower rates of bone loss. In a more recent 4-year study by Khosla *et al.* (2001), elderly men with bioavailable estradiol levels below the median value of 40 pmol/L showed higher rates of bone loss at the midradius and ulna than men with bioavailable estradiol levels above the median. A similar longitudinal 4-year study on 200 elderly Italian men (Gennari *et al.*, 2003) confirmed and extended these observations by showing a negative correlation between estrogen levels and bone turnover markers or rates of bone loss at the lumbar spine and the distal femur (see Fig. 8). In the same study, the ratio between estradiol and testosterone, presumed to be an indirect index of aromatase activity, increased significantly with age and was higher in normal than in osteoporotic subjects. In a larger cohort of older subjects, rapid bone loss at the hip was more likely in men with low testosterone or estradiol levels (Fink *et al.*, 2006; Ensrud *et al.*, 2006), while in male participants from the Tromso study (mean age, 25 80 years), the correlation between sex steroids and forearm bone loss was only modest (Bjornerem *et al.*, 2007). Taken together, these observations illustrate an important role for estrogen in the maintenance of bone mass in aging men. In a longitudinal observation within the Framingham Study cohort, men with low estradiol levels were at greatest risk for hip fracture (Amin *et al.,* 2006). Fracture risk further increased when both low estradiol and low testosterone levels were considered.

Considerable indirect evidence also suggests that a threshold value for estrogen in the male skeleton is needed to control bone remodeling and to maintain bone mass (Khosla *et al*., 2002). In a cross-sectional analysis from the MINOS Study, men in the lowest quartile for bioavailable estradiol level showed significantly lower BMD at multiple sites than men in the upper three estradiol quartiles (Szulc *et al.*, 2001). Moreover, in two longitudinal studies in older men, rates of bone loss at different skeletal sites were unrelated to serum estradiol levels if they were above the median value, but clearly associated with estradiol levels if they were below the median value (Khosla *et al.*, 2001; Gennari *et al.*, 2003) (see Figs 7 and 8). The threshold concentration of bioavailable estradiol appears to be remarkably similar across all studies, ranging from 40 to 55 pmol/L. This apparent threshold value is higher than typical estradiol concentrations for postmenopausal women who are not receiving exogenous estrogens. On the other hand, premenopausal women and young men are typically above this apparent threshold level. Because about 50% of middle-aged men fall below this estradiol threshold, it could be a determinant in age-related bone loss in a large number of men. A recent cross-sectional study in which volumetric BMD and bone geometry at different sites were assessed by QCT further extended these observations, and indicated that in men the relationship between bioavailable estradiol and volumetric BMD at cortical versus trabecular sites appears to be different (Khosla *et al.*, 2005a). The supposed threshold level for estrogen deficiency appeared to be lower for cortical than for trabecular bone. In other words, trabecular bone seems to be more sensitive than cortical bone to small age-related declines in estrogen levels. The same associations were confirmed in a similar QCT study in women (Khosla *et al.*, 2005b).

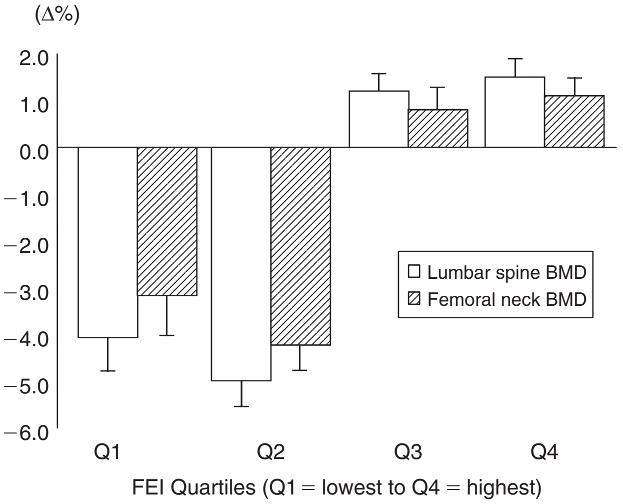

FIGURE 7 Rates of BMD loss (percentage per 4 year) and in elderly men grouped according to free estrogen index quartiles. The free estrogen index, as indirect measure for bioavailable estradiol, has been calculated by the ratio of total estradiol to SHBG. Data are adapted from Gennari *et al.* (2003), with permission.

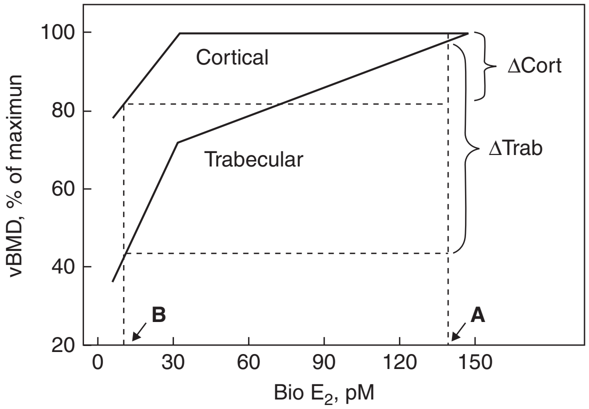

FIGURE 8 Femur neck cortical vBMD (**A**) and vertebral trabecular vBMD (**B**) as a function of bio E2 levels below and above the median (30 pM, 8 pg/mL) from an age-stratified, random sample of Rochester male residents. Data are adapted from Khosla *et al.* (2005), with permission.

Because only a small fraction of circulating estradiol is derived directly from the testes, it is likely that peripheral aromatization of testicular and adrenal androgen precursors to estrogen exerts a key role in maintaining estradiol levels above the threshold with aging (Gennari *et al.*, 2004a). It is also likely that individual differences in aromatase activity with aging may help to distinguish among men and their rates of bone loss. Recent studies on aromatase CYP19 gene suggested that polymorphic variation at this gene may account, at least in part, for these differences in aromatase activity (Gennari *et al.*, 2004a, 2004b). A major unsolved issue is which tissue site of aromatase activity is the most important in terms of bone metabolism in men. The consistent association between circulating estradiol levels, BMD, and fracture risk, reported in a large number of cross-sectional and longitudinal studies, suggests that although local aromatization in bone may contribute significantly to skeletal homeostasis (Simpson *et al.*, 1999, 2000), a minimum circulating level of estradiol (derived from nonskeletal peripheral aromatization) is necessary to prevent bone loss in elderly men. However, it is possible that circulating estrogen levels simply reflect local estrogen status within the bone, and that locally produced estradiol exerts an even greater impact on bone physiology than circulating plasma estradiol levels.

STUDIES IN HUMANS—INTERVENTIONAL DATA

In order to definitively dissect out estrogen versus testosterone effects on the adult male skeleton, more dynamic short-term interventional observations have been performed. Falahati-Nini *et al.* (2000) studied 59 elderly men (mean age, 68 years) in whom they eliminated endogenous testosterone and estrogen production using a GnRH agonist (leuprolide acetate) and an aromatase inhibitor (letrozole). Physiological testosterone and estrogen levels were maintained by concurrently placing the men on testosterone and estradiol patches. Following baseline studies in which markers of bone resorption (urinary deoxypyridinoline, Dpd, and N-telopeptide of type I collagen, NTx) and formation (serum osteocalcin and amino-terminal propeptide of type I procollagen, PINP) were measured, the men were randomized into one of four groups: group A had both patches withdrawn, group B continued the estradiol patch but had the testosterone patch withdrawn, group C continued the testosterone patch but had the estradiol patch withdrawn, and group D continued both patches. After 3 weeks, the baseline studies were repeated. As shown in Figure 9, estrogen had the dominant effect in preventing the increase in both urinary Dpd (Fig. 9A) and NTx (Fig. 9B) excretion that was observed in group A. In the same study (Fig. 10A), serum osteocalcin levels decreased following acute sex steroid withdrawal in these men, and either estrogen or testosterone was able to prevent the decrease in serum osteocalcin levels. By contrast, estrogen, but not testosterone, was effective in preventing the decrease in serum PINP levels (Fig. 10B). Because osteocalcin is produced primarily by mature osteoblastic cells and osteocytes (Lian *et al.*, 1999), these findings are consistent with an important role for both estrogen and testosterone in maintaining the functional integrity of these cells, perhaps by preventing apoptosis (Manolagas, 2000). Type I collagen, by contrast, is produced by cells of the entire osteoblastic lineage (Orwoll, 1995b), and these data would suggest that it is primarily estrogen that regulates this process. In a similar study in younger individuals, Leder *et al.* (2003) confirmed an increase in bone resorption markers following induction of the hypogonadal state by the GnRH agonist, goserelin acetate. In this model, evidence was provided for independent effects of testosterone and estrogen on bone resorption. Moreover, in that study, bone formation markers also appeared to be dependent both on androgens and estrogens. These observations are in keeping with the increase in bone formation markers described in aromatase-deficient or gonadectomized men (Stepan *et al.*, 1989). A study by Taxel *et al.* (2001) with a longer observation period (9 weeks) gave similar results, further indicating that treatment of elderly men with an aromatase inhibitor produces significant increases in bone resorption and decreases in bone formation. Conversely, a similar approach to boys with constitutional delay of puberty has given different results. Suppression of estrogen production by aromatase inhibitor, letrozole, for 1 year during testosterone treatment did not negatively affect bone mineral content, BMD, or apparent BMD, an estimate of true volumetric BMD, with respect to testosterone treatment alone (Wickman *et al.*, 2003). In testosterone alone and testosterone

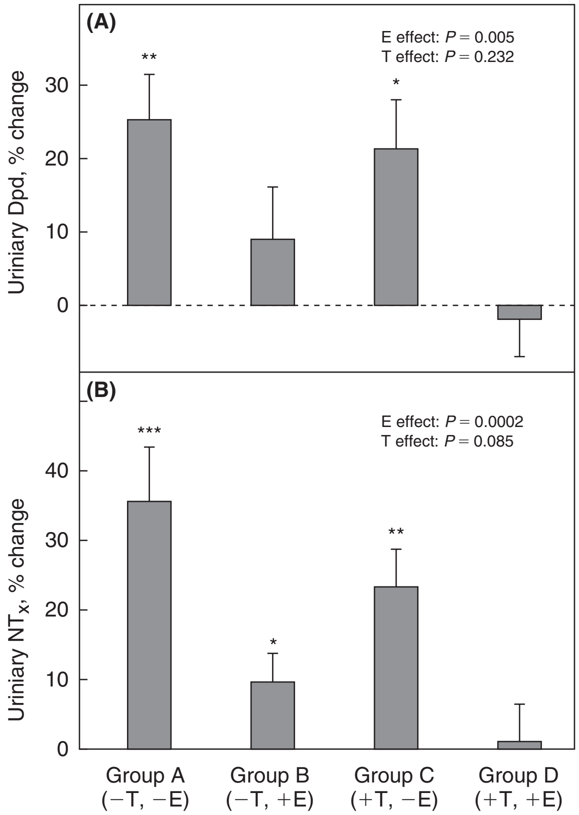

FIGURE 9 Changes in urinary Dpd (**A**) and NTx (**B**) excretion between the baseline and final visits in the four groups as described in the text. $***P < 0.001$, $**P < 0.005$, and $*P < 0.05$ for change from baseline. The overall estrogen (E) and testosterone (T) effect on the bone markers was analyzed using a two-factor ANOVA model. Adapted from Falahati-Nini *et al.* (2000), with permission.

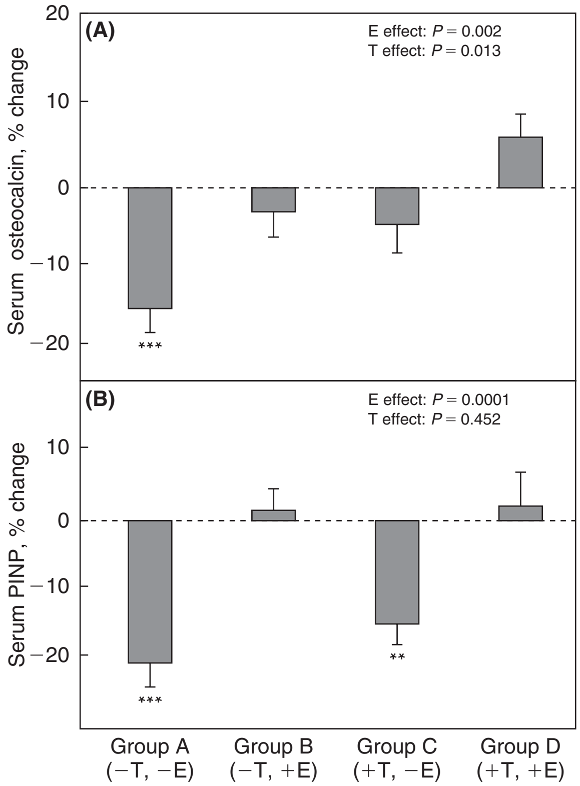

FIGURE 10 Changes in serum osteocalcin (**A**) and PINP (**B**) levels between the baseline and final visits in the four groups as described in the text. $^{***}P < 0.001$ and $^{**}P < 0.005$ for change from baseline. The overall estrogen (E) and testosterone (T) effect on the bone markers was analyzed using a two-factor ANOVA model. Adapted from Falahati-Nini *et al.* (2000), with permission.

plus letrozole groups, bone mass parameters increased with respect to baseline values and those in the untreated group. However, the increase in apparent BMD in the testosterone plus letrozole group was statistically significant only at 18 months, 6 months after discontinuation of letrozole treatment. Moreover, a decrease in BMD was observed in four letrozole-treated boys, but in only one of the boys treated with testosterone alone. These findings suggest that 1-year treatment with an aromatase inhibitor in pubertal boys is unlikely to have any major harmful effect on BMD and the attainment of peak bone mass, but such short-term studies do not diminish the clear effects of long-term estrogen deficiency (i.e., aromatase deficiency) on the developing male skeleton.

Consistent with these findings, several other interventional studies have found significant effects of estrogen on the adult male skeleton. Thus, in a preliminary study, Taxel and Raisz *et al.* (1997) treated nine elderly men with either 0.5 mg or 2.0 mg daily of micronized 17β-estradiol and found significant reductions in bone resorption markers. In addition, Anderson *et al.* (1997) treated 21 eugonadal men with osteoporosis with intramuscular testosterone and found a significant increase in lumbar spine BMD, which was correlated with changes in serum estradiol, but not testosterone levels.

Collectively, then, the observational and direct interventional data now provide conclusive evidence for an important and, indeed, dominant role for estrogen in the male skeleton. Moreover, given the data noted earlier on age-related decreases in serum bioavailable estradiol levels over life in men, a plausible hypothesis is that this decline is primarily responsible for age-related bone loss in men. Clearly, further studies are needed to address this issue.

SKELETAL EFFECTS OF ESTROGENIC COMPOUNDS IN MEN

Results from the short-term interventional observations also opened the way to studies on the use of estrogenic compounds in men. Indeed, short-term low doses of estradiol have been successfully employed to increase growth velocity in prepubertal boys (Caruso-Nicoletti *et al.*, 1985). Major concerns of either androgen and estrogen treatment in men relate to their possible collateral negative implications in other estrogen targets, such as the gonads, the prostate, and the cardiovascular system. Of interest, in the study on androgen supplementation in eugonadal men with osteoporosis, the increase in BMD and the reduction in bone turnover positively correlated with change in estradiol, but not in testosterone levels (Anderson *et al.*, 1997). Thus, it is likely that the therapeutic skeletal effects of androgens in men are owing, at least in part, to their conversion to estrogens.

Evaluating the potential of estrogen replacement therapy in healthy men is ethically and socially problematical because of the potential risk of estrogen-induced feminizing effects. To date, available data about estrogen replacement and bone in men pertain almost exclusively to the short-term and long-term treatment of aromatase-deficient subjects. Under estradiol (but not testosterone) treatment, these men experience the desired effects, namely epiphyseal closure, bone maturation, and restoration of bone mass and size. Importantly, a threshold estradiol treatment dosage was evident in these studies, with doses of transdermal estradiol below 25 μg twice weekly (corresponding to achieved serum estradiol levels below 88 pmol/L) being ineffective in preventing bone loss (Rochira *et al.*, 2000). In these cases, treatment with estradiol did not induce gynecomastia, hyperprolactinemia, behavioral or sexual dysfunction.

Given their preliminary *in vitro* inhibitory effects in human prostate cancer cell lines (Kim *et al.*, 2002) and the lack of feminizing effects, selective estrogen receptor modulators (SERMs) such as raloxifene, have been recently regarded for potential application in men, including the prevention of bone loss. Indeed, another first-generation SERM, tamoxifene, has been administered to men as a fertility-promoting drug, without relevant adverse side events (Vandekerckhove *et al.*, 2000). Its effects on male bone are unknown, but increases in serum LH, FSH, and testosterone were observed. In a single study of men receiving a GnRH agonist for prostate cancer, raloxifene treatment (60 mg/day) for 12 months increased BMD at the hip and the spine and decreased bone turnover markers (Smith *et al*., 2004). The changes in BMD were comparable to those observed in postmenopusal women treated with raloxifene at the same doses (Delmas *et al*., 1997). Apart from these observations in men with GnRH-induced severe hypogonadism other studies recently investigated the skeletal effects of raloxifene in healthy men. In a first study of 50 elderly men, raloxifene treatment (60 mg/day) for 6 months was no different from placebo in terms of its effects on urinary cross-linked N-telopeptide of type I collagen (NTX) (Doran *et al*., 2001). However, consistent with the threshold estradiol hypothesis, changes in urinary NTX were related directly to baseline serum estradiol levels. Subjects with serum estradiol levels below 96 pmol/L (26 pg/mL, corresponding approximately to serum bioavailable levels of 9 pg/mL) responded to raloxifene with a decrease in bone resorption marker. In this group, raloxifene was serving as an agonist. Above this estrogen value, raloxifene caused an increase in bone resorption. In this group with higher estrogen levels, raloxifene was acting as an estrogen antagonist. This seems to be remarkably comparable to the findings of a similar study in which raloxifene was given at a greater dose (120 mg/day) for 6 weeks in middle-aged eugonadal men (Uebelhart *et al*., 2004). In fact no major relevant effect on bone resorption and formation markers was observed in the overall raloxifene-treated group, whereas in the group of subjects with estradiol levels below a threshold value of 101.8 pmol/L, raloxifene treatment was associated with a significant decrease in bone turnover markers. In this study, as well as in a somewhat longer study (Duschek *et al*., 2004), 120 mg/day doses of raloxifene significantly increased serum concentrations of LH, FSH, estradiol, and total or bioavailable testosterone. The increase in estradiol after raloxifene treatment was more pronounced in those subjects with a low baseline serum estradiol level. No major treatment-related adverse events or feminizing effects were observed in these studies.

Thus, in men, raloxifene treatment might exert beneficial effects on bone metabolism only if serum concentrations of estradiol are low. Additional studies on raloxifene and other SERMs under development are needed to determine whether these compounds can be useful in the treatment of osteoporosis in hypogonadal men.

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Chapter 85

Mechanisms of Exercise Effects on Bone Quantity and Quality

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INTRODUCTION

By increasing peak bone mass and subsequently maintaining or decelerating bone loss, exercise is a critical deterrent to osteoporosis, a goal achieved through bone tissue's sensitivity to its mechanical loading environment. There is mounting evidence from clinical and animal studies that these physical stimuli are strong influences on both the quantity and quality of the skeleton, yet identifying those specific components of the loading milieu that can be both anabolic as well as antiresorptive has proven difficult. Perhaps improving our understanding of the biological mechanisms by which these physical signals regulate skeletal tissues will help to translate this information to the clinic, and enhance the potential of a non-drug-based deterrent and/or treatment for osteoporosis.

A host of human-based studies have worked toward quantifying the impact of general and specific exercise regimens on bone mass and morphology, taking into account interdependent variables such as gender, age, and nutritional status of the individuals. Although some studies have provided encouraging results, the large majority of data have been equivocal, perhaps as much a reflection of our limited understanding of which specific components of the mechanical signal are perceived as anabolic and anticatabolic by the resident bone cell populations (e.g., osteocytes, osteoblasts, lining cells, and osteoclasts), and which are irrelevant byproducts of loading. The design of an "optimal" exercise intervention (e.g., least amount of physical exertion for the greatest gain in skeletal strength) will require many critical questions to be addressed, such as: should the exercise protocol incorporate large loads or could they be small if they are applied rapidly? How long does an individual have to exercise to maximize benefits? Are the attributes of mechanical loading accumulated, or is there a threshold past which additional challenges no longer are perceived as regulatory influences to the skeleton? Can exercises be designed to stimulate bone formation at skeletal sites most prone to fracture or is the response systemic to a given skeletal segment?

Here, we define the functional mechanical environment of bone to demonstrate that the ability of physical signals to influence bone morphology strongly depends on the character of the signal, and we provide an overview of the molecular and cellular events that play a role in transducing the mechanical signal into a biochemical message. Understanding the precise physical and molecular factors of the mechanically derived signal is critical toward developing biomechanical prophylaxes that are clinically feasible, enjoy high compliance, and can prevent the bone loss leading to osteoporosis. If components of exercise can also suppress muscle loss and preserve postural stability, then these physical signals may represent a more comprehensive strategy than drugs to prevent the degradation of the musculoskeletal system and thus reduce chance of injury. As an example that mechanical signals need not be large to be effective and that the application of mechanical signals is not synonymous with strenuous exercise, the potential of extremely low-level, high-frequency mechanical signals is described.

BONE'S SENSITIVITY TO MECHANICAL SIGNALS

The skeleton's ability to adapt to altered levels and patterns of mechanical loading is well established but the difficulty lies in defining the specific osteoregulatory component(s)

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that is embedded within the bone's complex loading environment. Numerous clinical exercise studies have failed to relate adaptive changes in bone to specific aspects of a particular exercise regimen including exercise mode (e.g., running, swimming, weightlifting), intensity (e.g., percent of maximal heart rate), duration, and frequency (number of times per month, week, day), reflecting the need to characterize bone's mechanical environment directly by the prescribed exercise parameters. Considering that bone adaptation is site-specific and focal in nature at both the organ and the tissue level, it should not be surprising that altered levels of bone formation or resorption are only weakly related to parameters that are systemic to the entire skeleton such as exercise mode or intensity.

At the organ level, the site specificity of exercise-induced bone adaptation is particularly obvious in tennis and squash players who display significant bone hypertrophy in the playing extremity but not in the contralateral arm that is not subjected to significant changes in loading. During physical activity, changes in the loading environment are not only very heterogeneous across the entire skeleton, but also within a bone. Reflecting this nonuniform environment, adaptation within a bone is also site-specific and some bone surfaces will change their osteoblastic or osteoclastic activity but others will not. For example, subjecting an adult rooster to high-speed running may activate as little as 23% of previously quiescent periosteal surfaces in the middiaphysis of the tarsometatarsus (Judex *et al*., 1997).

To define the mechanical osteogenic components, experimental, analytic, and empiric models have been developed. Through the past three decades, specific components of the mechanical milieu have been proposed as the dominant stimulus for bone adaptation, including strain magnitude (Rubin and Lanyon, 1985), strain rate (O'Connor *et al*., 1982), electrokinetic streaming currents (Pollack *et al.*, 1984), piezoelectric currents (Bassett, 1968), fluid shear flow (Weinbaum *et al*., 1994), and strain energy density (Fyhrie and Carter, 1986). Although several of these parameters correlate with specific skeletal morphologies, few have validated their regulatory role with an accurate, prospective prediction of adaptation as stimulated by changes in loading conditions (Gross *et al.*, 1997). The difficulty in identifying a unifying principle that can anticipate the mechanical control of bone adaptation may be aggravated by an underlying "structural" assumption that tissue efficiency (minimal skeletal strain/minimal skeletal mass) is itself the driving stimulus that regulates the remodeling process.

Alternatively, bone cells may be responding to "biologically relevant" parameters of the functional milieu that are not necessarily linked to minimizing deformation of the structure under load. In support of "other than peak" signals regulating bone adaptation, examples of biological systems that perceive and respond to exogenous stimuli, such as vision, hearing, and touch are regulated in this fashion. It is also well accepted that too much loading will damage the bone, leading to failure (just as too much light, noise, or pressure will overwhelm sight, hearing, and touch). To identify the criteria by which the skeleton responds to its loading environment, it is important to look beyond the material consequences of a structure subject to load and consider the biological benefit of a viable tissue subject to functional levels of strain.

BONE'S MECHANICAL MILIEU ENGENDERED BY PHYSICAL ACTIVITY

Strains in Bone

To gain insight into the structural demands that are placed onto the skeleton during daily activities, the mechanical environment that bone is subject to can be characterized. In contrast to the notion that bone is purely loaded under compression, these experiments have definitively demonstrated that the long bones of the appendicular skeleton are also subject to a complex array of forces and moments produced by torsion and bending even during very simple activities such as steady-state locomotion. The mechanical state in the bone matrix is commonly quantified by spatial and temporal measures that are based on mechanical strain (ε), the dimensionless expression of deformation as explained elsewhere in this book (ε = change in length, ΔL, normalized to the original length, L, of any given specimen). Because of the very small deformations that bone experiences during physiological loading, strain is typically expressed as microstrain (1% deformation $= 0.01$ strain $= 10{,}000 \times 10^{-6}$ strain $= 10{,}000\,\mu\varepsilon$).

Strain is a ubiquitous product of a functionally loaded skeleton, regardless of its specific design or function. Mechanical strain and its derivatives are therefore considered an efficient means of translating exercise intensity, duration, and manner into a site-specific regulatory signal that can be recognized by the cells that control bone adaptation. One obvious goal of this strain-mediated form/function adaptation is to avoid catastrophic failure, or bone fracture. This requires that bone loading and architecture must be coordinated to avoid tissue's yield strain over 0.7% (7,000 microstrain). Through strain gauges surgically implanted onto bone's surface, strain magnitudes have been determined during functional activities in a great variety of species including humans, dogs, primates, roosters, horses, sheep, and rats (Burr *et al.*, 1996; Gross *et al.*, 1992; Lanyon, 1971). Although these gauges only record deformation from the specific sites that they are attached to, mechanical models can be used to extrapolate measured deformations to other sites within the bone.

Peak strain magnitudes measured in diverse vertebrates range from 2000 to 3000 microstrain (Rubin and Lanyon, 1984a). Whether measured in the third metacarpal of a galloping horse, the tibia of a running human, the humerus of a flying goose, the femur of a trotting sheep, or the mandible of a chewing macaque, this "Dynamic Strain Similarity" suggests that skeletal morphology is adjusted such that functional activity elicits a very specific (and perhaps beneficial) level of strain to the bone tissue (Rubin *et al*., 1990). That strains of this magnitude are at least a factor of two below the yield point of bone material emphasizes that bone has a safety factor of about 2 to 3 to accommodate abnormal loading events. However, the realization of a mechanism by which the skeleton anticipates to be loaded to about half its yield strength seems overly complex and unlikely. Instead, *in vivo* and *in vitro* data suggest that the functional criteria that regulate adaptation, and the means by which bone cells perceive and respond to their functional milieu are more sophisticated than a mechanism that simply focuses on the magnitude of the deformation generated in the tissue. In fact, accumulating data suggest that morphological adaptation to biophysical stimuli occurs to encourage specific components of the strain milieu, as a means of promoting site-specific adaptation.

Locomotion Induces a Nonuniform Strain Environment

During locomotion, bending is the dominant form of loading in the middiaphysis of limb bones; more than 85% of the strain measured in diaphyseal long bones is accounted for by bending moments (Rubin and Lanyon, 1982). These middidphyseal bending moments are caused by bone curvature as well as applied bending moments and generate compressive strains on one side of the cortex and tensile strains on the opposite side (Fig. 1). The dominance of bending in the functional loading environment is somewhat counterintuitive because far less bone mass would be required to support the same loads if the bone were loaded axially. Nevertheless, bending may be beneficial to bone because it creates a nonuniform strain environment that is more diverse than uniform compression, thereby enabling signaling pathways that could not be generated by uniform loading. Perhaps more importantly, bending causes consistent, predictable loading conditions in which specific regions within the cortex are loaded in a similar fashion independent of the functional activity. To demonstrate this stability of the functional strain environment, roosters were subjected to three distinct loading environments engendered by treadmill walking, treadmill running, or drop jumps for which roosters were released from a 50- to 60-cm height. Tarsometatarsal middiaphyseal strain magnitudes and distribution were determined via strain gauges and linear beam theory (Judex and Zernicke, 2000b). We found that peak compressive strains produced by these three activities increased from −1570 με for walking to −1870 με for running and −2070 με for jumping. Despite this increase in peak strains, the relative distribution of strain across the middiaphysis was qualitatively identical across the three activities.

With bending causing tension on one surface and compression on another, the transition between these two areas creates a region of the cortex that experiences very low peak strain magnitudes. Even though this neutral axis is far removed from the area of the cortex subject to the peak strains, somehow tissue is retained in this low-magnitude strain state. A conceivable mechanism to save bone from resorbing in this region could be differential coupling of bone cells to the matrix with cells in low-strain regions being tightly coupled and cells from peak strain regions being more loosely coupled to the matrix. In this way, the cells have "tuned" themselves to the mechanical strain environment, a means of functional adaptation at the level of the cell.

Although a high degree of bending in loaded bone may provoke nonuniform strain distributions, it does not necessarily preclude the possibility of an adaptive mechanism mediated by some aspect of strain. It might be suggested that bone cells in different regions of the cortex are differentially sensitive to strain (e.g., some cells strive to 3000 με in compression, some to 1500 με in tension; others—near

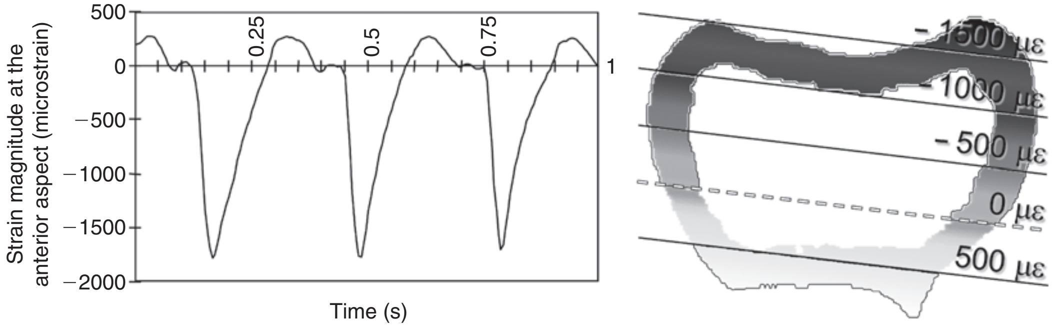

FIGURE 1 Recording from a strain gauge attached to the anterior aspect of the middiaphyseal tarsometatarsus while the animal is running on a treadmill at a high speed (*Left*). The distribution of longitudinal normal strain magnitude about the middiaphysis of the rooster tarsometatarsus. The distribution above was recorded at that point in the stride in which peak strain is achieved. Although the distribution of peak strain is very spatially very nonuniform, the manner in which the bone is loaded remains constant (i.e., the site of peak strain varies very little during the stance phase of a gait cycle. Adapted from Judex *et al*. (1997).

the neutral axis—are content with strains of 50 or 100 με). Although this is appealing in its simplicity, the apparent limitations of a spatially specific strain sensitivity are apparent with respect to the required genetic logistics. Alternatively, it is possible that strain information is spatially integrated in three dimensions via a cell network facilitated by gap junction intercellular communication (Donahue *et al.*, 1995), such that the area of the cortex subject to 100 με resists resorption owing to sufficient homeostatic signals received from neighboring areas subject to much higher strains. This "information integration" perspective is supported by the observation that the bone loss that parallels disuse occurs uniformly about the cortex and through the diaphysis, even though the net change in bone strain caused by the absence of function varies widely (Gross and Rubin, 1995).

The Influence of Muscle on Bone's Strain Environment

Although the symbiotic relationship between muscle and bone is inherently obvious, only seldom is it explicitly considered in the context of one defining the other. In the absence of ground reaction forces, the spectral components of muscle contraction typically impose smaller strains on the skeleton than those caused by functional impact loading. Even though muscle-induced strains may be small, they are sustained for extended periods of time (e.g., in postural muscle activity), and thus, over time, may dominate a bone's characteristic "strain history." Examining this hypothesis, strain data from a variety of animals reveal the existence of a broad frequency range of strains in the appendicular skeleton, even during activity such as quiet standing (Fritton *et al.*, 2000) (Fig. 2).

From a stimulus standpoint, these persistent, low-amplitude, high-frequency signals may, when summed, be at least as important as the seldom occurring, and somewhat unpredictable, peak strain events. If there is an age- or pathologically-induced change in the dynamics of these muscle oscillations, it could be argued that bone mass may deteriorate because these muscle-based signals also attenuate. To determine the role of muscle dynamics in the etiology of osteopenia, the spectral characteristics of muscle activity as a function of age were obtained through measurements of muscle surface vibration (Huang *et al.*, 1999).

During the contraction of a muscle, radial expansion of the individual fibers results in fiber collisions and the production of muscle sound or acoustic vibrations of the muscle body. The frequency of these vibrations reflects the firing rate of the motor units, and correspondingly, the force output of the motor unit. When recorded in 40 volunteers (20 to 83 years of age) using a low-mass accelerometer, the spectra of the acoustic vibrations normal to the surface of the soleus muscle showed that muscle activity in the frequency range above 20 Hz decreased by a factor of three in the elderly compared with that seen in young adults, a sarcopenia consistent with loss of fast oxidative-type fibers. Simultaneous with the decay of high-frequency signals, there is a marked decrease in the amount of type IIa and IIb fibers (Lee *et al.*, 2006), compounding the suppression of the source of the signal.

As the high-frequency components seen in bone during load bearing almost certainly arise through muscle activity, the deterioration of the muscle contraction spectra with age would consequently decrease the spectral content of bone strain above 20 Hz. From this perspective, it can be argued that the sarcopenia of aging may be a principal etiological factor in osteoporosis, as this portion of the strain spectra is demonstrably osteogenic. If aging leads to the loss of specific muscle fibers critical to the maintenance of bone mass, osteoporosis could presumably be inhibited by providing a "surrogate" for the lost spectral strain history.

REGULATION OF BONE MORPHOLOGY BY MECHANICAL STIMULI

Toward Identifying the Osteogenic Parameters of the Strain Milieu

It is clear that the skeleton is subject to a wide range of mechanical signals, including low- to high-frequency strains, normal and shear strains, and compressive and tensile strains. It is also clear that the cells on and within the mineralized matrix are subject not only to mechanical parameters such as strain, but derivatives of tissue deformation such as fluid flow and electrokinetic currents, parameters that may represent an important physiological pathway in mediating an adaptive response. But which physical parameters are most important to regulating the adaptive response?

Models that have been used to investigate bone's adaptive response to its mechanical environment include overloads by osteotomies (Lanyon *et al.*, 1982), vigorous exercise (Judex and Zernicke, 2000a), or exogenous loading models in which external forces are applied to the bone. Physical exercise represents a physiological means of enhanced mechanical loading but the limited exercise repertoire of most laboratory animals makes it difficult to generate and control distinct mechanical milieus. Exogenous loading models such as the functionally isolated avian ulna (Rubin and Lanyon, 1984b), the axially loaded rat ulna (Torrance *et al.*, 1994), or the rat tibia placed in a four-point bending apparatus (Turner *et al.*, 1991) allow the generation of controllable mechanical environments, but a disadvantage of some these models is that the morphological response may be confounded by injury caused by the means of load application.

Perhaps guided by putative form–function relations between bone's strain environment and modeling and remodeling processes in bone, researchers' attention has focused primarily on strain magnitude as the dominant determinant of

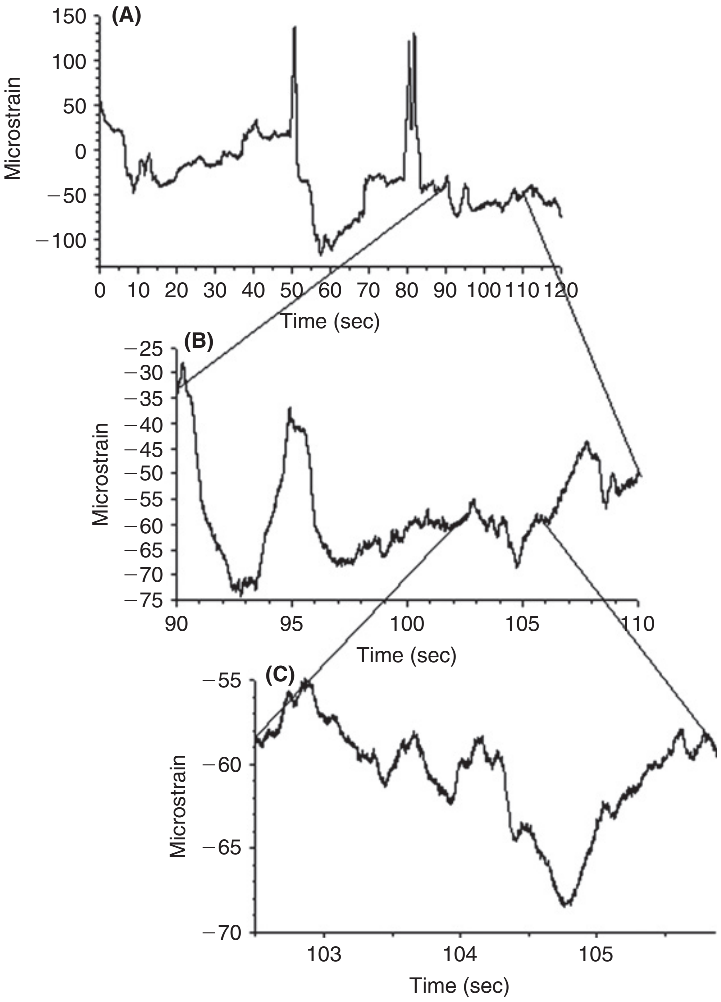

FIGURE 2 (**A**) A 2-minute strain recording from the caudal longitudinal gauge of the sheep tibia while the animal took a few steps with peak strains on the order of 200 με. (**B**) A 20-second portion of that strain record shows peak strain events as large as 40 με. (**C**) Further scaling down to a 3-second stretch of the strain recording illustrates events on the order of 5 με. Adapted from Fritton *et al*. (2000).

bone mass and morphology. Other parameters have received only limited attention. Isolating the effect of a single mechanical parameter is not trivial owing to the interdependence of many of the parameters. For a commonly used sinusoidal loading pattern, changing strain magnitude while maintaining a constant loading frequency, for example, results in a concomitant change in strain rate. Despite these difficulties, several mechanical parameters have emerged from controlled experimental studies that related the mechanical environment to induced morphological changes.

Strain Magnitude

When holding strain frequency and number of loading events constant, longitudinal normal strain magnitude (strain in the direction of bone's longitudinal axis) is highly related to the osteogenic response. In other words, the larger the maximal deformation that is generated in the bone, the larger the overall response of the bone. Strains, when applied at 1 Hz and to not reach a certain magnitude are permissive to bone loss. This relationship was first demonstrated in the functionally isolated turkey ulna preparation to which strains in the range of 500 to 4000 με were applied for 100 cycles per day. In this model, the ulna of adult male turkeys is functionally isolated by proximal and distal epiphyseal osteotomies, leaving the entire diaphyseal shaft undisturbed. The only stimuli applied to the diaphysis are the mechanical regimen prescribed by the investigators, with no aberrant biophysical signals entering the preparation. In this model, strains smaller than 1000 με caused bone loss with strains larger than 1000 με leading to new bone formation in a dose-dependent relationship.

The question as to how much strain in bone has to be generated to obtain an osteogenic effect depends on the interrelationship between strain magnitude, strain rate, and strain frequency. Although in the previously described isolated turkey ulna preparation, 100 loading cycles per day at 1 Hz inducing 1000 $\mu\varepsilon$ prevented bone loss from occurring, this threshold can be reduced to 700 $\mu\varepsilon$ when 600 loading cycles are applied at 1 Hz, to 270 $\mu\varepsilon$ when 36,000 loading cycles are applied at 60 Hz, or to 100 $\mu\varepsilon$ when 108,000 loading cycles are applied at 30 Hz (Qin *et al.*, 1998). This demonstrates that the search for a particular strain (loading) threshold has to take other mechanical parameters into account as well and that this relationship can be exploited to design safer exercise regimens using smaller loads.

Differential Modeling/Remodeling to Distinct Components of the Strain Tensor

This goal of investigating the osteogenic effects of different aspects of the strain tensor has been approached using the turkey ulna model of disuse osteopenia, in which the modeling and remodeling response was quantified following 4 weeks of either axial or torsional loading or disuse (Rubin *et al.*, 1996). Each of the two load groups were subject to peak principal strains of 1000 $\mu\varepsilon$ (predominately normal strain in the axial case, and shear strain when subject to torsion). Of the three distinct groups, only disuse caused a significant change in gross areal properties compared with controls (13% loss of bone). This suggests that both axial and torsional loading conditions are substitutes for the functional signals normally responsible for retention of bone mass, leaving the periosteal and endosteal envelopes unphased by disparate components of the strain tensor.

The intracortical response, however, was found to depend strongly on the manner in which the bone was loaded. Disuse failed to increase the number of sites within the cortex actively involved in bone turnover (intracortical events), yet significant area was lost within the cortex owing to a threefold increase in the mean size of each porotic site. Axial loading increased the degree of intracortical turnover compared with intact controls, yet the average size of each porotic event remained identical to that of control. Conversely, compared with control, torsion elevated neither the number of porotic events, the area of bone lost from within the cortex, nor the size of the porotic event. It appears that bone tissue can readily differentiate between distinct components of the strain tensor, with strain per se necessary to retain coupled formation and resorption, shear strain achieving this goal by maintaining the status quo, whereas normal strain elevates intracortical turnover, but retains coupling. Thus, not the aggregate of strain per se may define bone remodeling, but independent components of the strain tensor may have differential responsibilities in achieving and maintaining bone mass.

Strain Rate

Although strain magnitude appears to be an important determinant of bone mass, it is critical to realize that dynamic but not static strains have osteogenic potential. At the extreme, static loading (strain rate = 0) at strain magnitudes capable of stimulating formation when applied dynamically produces a remodeling response similar to disuse resulting in bone resorption (Lanyon and Rubin, 1984). Several studies support the notion that bone is sensitive to the applied strain rate, with higher strain rates being more osteogenic (Lamothe *et al.*, 2005).

For instance, high-speed running (1.7 ms^{-1}) increases peak strain magnitudes by approximately 20% in the middiaphyseal tarsometatarsus of roosters compared with walking (0.5 ms^{-1}). This increase in strain magnitude was not accompanied by additional bone formation in the tarsometatarsal middiaphysis when growing roosters were exercised for 15 minutes per day (~2600 loading cycles) for 8 weeks. An exercise intervention was then designed, that employed high-impact drop jumps for which growing roosters were lifted off the ground and released. Middiaphyseal tarsometatarsal peak strain magnitudes induced by this exercise were similar to those induced by high-speed running but strain rates were increased by 260% (0.32 ε/s vs. 0.09 ε/s). In contrast to the running protocol, 200 drop jumps per day for 3 weeks significantly increased bone formation rates at periosteal (+40%) and endocortical surfaces (+370%). As the other mechanical parameters considered between running and drop-jumping were similar, the differential osteogenic effect associated with these two exercise protocols could be attributed directly to the large difference in generated strain rates. Site-specific analyses within the middiaphyseal cortex revealed that drop-jumping deposited additional bone preferentially in those regions that were subjected to the largest strain rates, further emphasizing bone's sensitivity to high strain rates.

Extrapolated for the design of exercise interventions, these results imply that loads should be applied rapidly. Although exercise studies have been unable to identify a specific exercise intervention that is most effective in producing beneficial skeletal effects, a trend has emerged with high-impact exercise being more efficient than low-impact exercises in terms of stimulating new bone formation. This trend may support the notion that high strain rates have a critical impact on bone morphology as high-impact exercises ostensibly induce higher strain rates.

Cycle Number

A threshold behavior exists for the number of loading cycles. The full response can be triggered after only a limited number of loading cycles (Umemura *et al.*, 1997). In the functionally isolated turkey ulna preparation, a loading regime inducing peak strains of approximately 2000 $\mu\varepsilon$ maintained bone mass with only four cycles a day. When the cycle number was increased, this particular loading regime stimulated new bone formation. Thirty-six load cycles saturated the osteogenic response, with as many as 1800 cycles being not more effective than 36 cycles. The notion that a finite number of loading cycles employing large loads may increase BMD or inhibit bone loss is supported by exercise studies involving weight lifters. It is critical to realize, though, that the saturation threshold to cycle number is influenced by other mechanical parameters including strain magnitude.

Further, recent studies have indicated that the manner in which loading cycles are distributed plays an important role in defining the magnitude of the anabolic response. Partitioning a given bout of loading cycles into several loading sessions can increase bone's response to the mechanical intervention (Robling *et al*., 2002). Going one step further, recent studies have shown that this concept can be exploited to produce a mechanical intervention that is more efficacious despite providing fewer loading cycles (Gross *et al*., 2004). This loading paradigm that has been labeled rest inserted loading adds as little as ten seconds of rest after each loading cycle within a bout and has been shown to transform an otherwise ineffective loading regime into a highly osteogenic stimulus. The mechanisms by which the sensitivity of cells to mechanical signals is increased by rest inserted loading may be associated with high cell refractory periods that, upon the application of a single loading event, impose a "time out" to the cells during which it cannot respond to another cycle. Alternatively or in addition, including rest into the loading regime may enhance bone fluid flow, synchronize osteocytic activity, and enhance cell communication.

Strain Distribution

Although a relation between peak strain magnitude generated in a bone and the resulting adaptive response has been proposed, bone also appears to be sensitive to how the strains are distributed across a bone section. Simply imposing a strain distribution that produces similar peak strain magnitudes as habitual loading conditions—but at different locations within the section (i.e., rotating the strain distribution)—may initiate new bone formation (Rubin and Lanyon, 1987). Thus, unusual strain events (strain errors) have been suggested to drive bone adaptation. Running, for instance, may not be the osteogenically optimal exercise partly because it may generate strain distributions that are very similar to strain distributions induced by normal walking. Interestingly, sports that involve a great variety of changes in loading directions such as soccer or badminton have been suggested to possess a higher osteogenic capacity, although it has not been confirmed that these changes in loading directions actually cause altered bone strain distributions.

Strain Gradients

Parameters such as peak strain magnitude or strain rate were primarily tested at the organ level. In other words, the region of the bone that was studied in response to a given mechanical stimulus was large (e.g., the middiaphysis of a long bone) and encompassed a large range of strain magnitudes. Rather than simply considering the peak magnitude of the stimulus and averaging the morphological response across a section, one could investigate whether new bone is actually deposited in those regions where the applied stimulus is the largest (i.e., the distribution of a mechanical parameter can be correlated with the distribution of bone's response). If such a site-specific relationship exists, then the knowledge of this specific osteogenic component may provide information about a mechanism by which bone cells perceive their mechanical environment. Further, if a particular mechanical parameter is capable of consistently predicting the specific sites of bone formation in different models, then exercise interventions could be designed that deposit bone at sites where additional structural strength is required.

This issue was addressed in an exercise study in which young adult roosters were ran on treadmill for 9 minutes per day (~1500 gait cycles) for 3 weeks. Strain gauges were attached to the tarsometatarsus to determine the distribution of candidate mechanical parameters across a middiaphyseal section. Periosteal activation (as measured by histomorphometry) as well as mechanical parameters, such as strain magnitude, strain rate, and strain gradients, were spatially quantified in transverse section thus, enabling a site-specific correlation with each other. The brief daily running regime activated periosteal surfaces but the amount of periosteal mineralizing surfaces per sector was only weakly associated with strain magnitude ($R^2 = 0.24$, negative correlation). In contrast, circumferential strain gradients (changes in strain magnitude across a volume of tissue) correlated strongly ($R^2 = 0.63$) with the sites of periosteal activation, consistent with earlier results from an external loading model. In general, circumferential strain gradients are largest where strains (deformations) are the smallest. Although it is counterintuitive from a structural (engineering) perspective that new bone formation is activated at sites subjected to low strains rather than large strains, strain gradients drive fluid flow in bone in the matrix, a process that has been implicated to play an important role in mechanotransduction in bone. These data further emphasize that bone adaptation is not necessarily guided by form–function relations, rather that bone cells respond to a biological signal resulting from the application of mechanical loads.

Fluid Flow

It is clear that the cells on and within the mineralized matrix are subject not only to mechanical parameters such as strain, but derivatives of tissue deformation such as fluid flow and electrokinetic currents, parameters that may represent an important physiological pathway in mediating an adaptive response. Bone cells, in particular osteocytes, are surrounded by extracellular fluid. The driving force for fluid to flow is a pressure differential between two ends of a lucanar–canalicular system that experience different amounts of mechanical strain, both temporally and spatially. Fluid flow is thus a direct by-product of mechanical strain and a large body of *in vitro* studies has shown the sensitivity of bone cells to flow (Rubin *et al.*, 2006). Because of the tight coupling between mechanical strain and fluid flow under physiological conditions *in vivo*, only few studies have successfully increased fluid flow in controlled animal models (Qin *et al.*, 2003). Similar to *in vitro* data, raising intramedullary pressure, and thus enhancing interstitial bone fluid flow, can increase bone formation (Stevens *et al.*, 2006). The *in vivo* mechanism(s) by which bone cells sense and respond to fluid flow have not been elucidated but may be related to one of the following processes: (1) fluid pressurization directly stimulates cells, (2) interstitial fluid flow fluid excites osteocytes through fluid shear stresses, (3) drag forces perturb the osteocytic processes in the pericellular matrix, (4) fluid flow aids in the transport of nutrients to bone cells, or (5) electrokinetic effects such as streaming potentials. Together, *in vitro* and *in vivo* studies clearly demonstrate that bone fluid flow is capable of transducing mechanical signals from the matrix to the cell but it should be noted that no definite study has been performed to demonstrate that biophysical events produced by fluid flow are involved in mechanotransduction of physiological loading events *in vivo*.

LOW-MAGNITUDE, HIGH-FREQUENCY MECHANICAL SIGNALS

A common theme of the mechanical parameters described earlier is that only peak events are considered (e.g., peak strain magnitude, peak strain rate, peak strain gradients). From this, one could conclude that mechanical modulation of bone physiology depends on large signals to have any morphological impact. However, the weak correlation of new bone formation with exercise intensity or with the specific sites of peak strain magnitudes suggests that other factors may also be relevant for defining bone mass and morphology. As discussed earlier, *in vivo* data suggest a nonlinear interdependence between cycle number, strain frequency, and strain magnitude. When the turkey ulna was loaded at 1 Hz, peak strains larger than $700\,\mu\varepsilon$ were necessary to maintain bone mass. This loading threshold can be reduced to $400\,\mu\varepsilon$ at 30 Hz and to $70\,\mu\varepsilon$ at 30 Hz. Although the reduction in strain threshold could be associated with an increase in cycle number, it is most likely that the increase in frequency at which loading occurred played a large role. Indeed, over the past decade, we and others have demonstrated that bone can sense and respond to even extremely small mechanical signals if they are applied at high frequencies. Below are examples of studies suggesting that the skeleton can benefit from these small mechanical signals under normal as well as disturbed physiological conditions.

Low-Level Mechanical Signals Increase Bone Quantity and Strength

To examine the long-term influence of applying short bouts of extremely low-magnitude, high-frequency signals, adult sheep stood in a chute such that only the hind limbs were subject to a vertical ground-based vibration, oscillating at 30 Hz, to create peak–peak accelerations of $0.3\,g$ (Rubin *et al.*, 2001a). When the animals were not being treated, they pastured with controls. After 1 year of stimulation, pQCT at the lesser trochanter demonstrated a 34.2% increase in trabecular density in mechanically stimulated sheep ($P < 0.01$). Microcomputed tomography indicated that not only the quantity of trabecular bone had benefited but also its microarchitecture (Rubin *et al.*, 2002b). Trabecular Bone Pattern factor, an index of connectivity, decreased 24.2% in animals subject to the noninvasive stimulus ($P < 0.03$), reflecting an increase in connectivity of the trabecular struts. Histomorphometry emphasized that the increase in volume fraction was achieved through an anabolic stimulus (Rubin *et al.*, 2002a). The elastic modulus and stiffness of the bone subject to the low-level mechanical stimulus also increased significantly (Fig. 3) and analyses of the tissue-level stresses and strains indicated that, micromechanically, the bone had to adapt to become a mechanically more efficient structure (Judex *et al.*, 2003).

Low-Level Mechanical Signals Normalize Bone Formation

The one-year sheep experiments indicated the anabolic potential of low-level mechanical signals. The rat tail-suspension

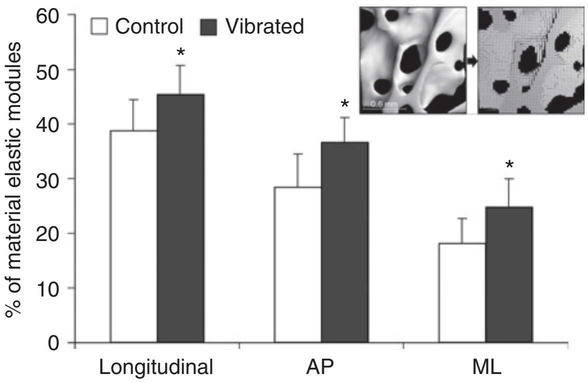

FIGURE 3 Finite-element modeling was used to convert the trabecular structure into a mechanical model (see *Inset*). Simulated mechanical testing in all three orthogonal loading directions indicated that the application of short bouts of high-frequency mechanical signals increased trabecular stiffness. Shown is the apparent elastic tissue modulus expressed as percentage of the tissue elastic modulus for control and experimental sheep in the three directions (mean + SD). AP, anterior-posterior direction; ML, medial-lateral direction. *: $P < 0.05$. Adapted from Judex *et al.* (2003).

model of disuse osteopenia was to determine whether resorptive remodeling, as stimulated by disuse, could be suppressed with brief exposure to the vibrating plate (Rubin *et al.*, 2001b). A single-element strain gauge, attached to the tibia of calibration rats showed that the oscillation, at 0.3 *g*, generated strains less than 5 $\mu\varepsilon$ at 45 Hz. Compared with long-term controls, 28 days of tail suspension caused mineralizing surface (MS/BS) of the tibiae to drop 52% ($P < 0.004$), and bone formation rate per bone volume (BFR/BV) to drop 72% ($P < 0.0002$). The suppression of formation was not significantly different from the animals subject to disuse for most of the day (23 hours, 50 minutes) and then allowed to freely bear weight for 10 minutes per day. However, 10 min/day of weight bearing on an active vibration platform normalized mineralizing surfaces and bone formation rates to normal weight-bearing levels. Based on these observations, high-frequency, low-magnitude, mechanical strains effectively served as a "surrogate" for musculoskeletal forces and, thus, may represent a countermeasure to the osteopenia that parallels disuse.

Vibrations can Decrease Resorptive Activity

To test the effects of these mechanical signals in the growing skeleton in which resorptive activity is relatively high, 8-week-old mice were subjected to daily 15-min bouts of whole-body vibrations at 45 Hz and 0.3 *g*. After three weeks, osteoclastic activity in the trabecular metaphysis and epiphysis of the tibia was 30% lower ($P < 0.05$) in vibrated mice than in age-matched controls. Bone formation rates (BFR/BS) on the endocortical surface of the metaphysis were 30% greater ($P < 0.05$) in WBV than in age-matched control mice, but trabecular and middiaphyseal BFR were not significantly altered. Importantly, the intervention did not negatively influence body mass, bone length, or chemical bone matrix properties of the tibia. These data indicate that, in the growing skeleton, short daily periods of extremely small, high-frequency mechanical signals can inhibit trabecular bone resorption, site-specifically attenuate the declining levels of bone formation, and maintain a high level of matrix quality. If WBV prove to be efficacious in the growing human skeleton, they may be able to provide the basis for a nonpharmacological and safe means to increase peak bone mass and, ultimately, reduce the incidence of osteoporosis or stress fractures later in life.

Genetic Variations Modulate Bone's Response to Mechanical Signals

It is well accepted that the genetic makeup of any given individual is a strong predictor of their risk of osteoporosis (Deng *et al.*, 2001). What is not as clear is if the genetic template can account for variability in the response to bone's ability to adapt to mechanical signals. To address this question, inbred mouse strains with distinct bone phenotypes (Judex *et al.*, 2004) were used to examine whether they were differentially sensitive to changes in their habitual mechanical environment (Judex *et al.*, 2002). Adult female mice, with relative thin (C57BL/6J), medium (BALB/cByJ), and robust (C3H/HeJ) skeletons were subjected to 10 min/day of low-level, high-frequency mechanical signals (0.25 *g* at 45 Hz). After three weeks, bone formation rates, with tissue volume as referent of C57BL/6J mice subject to the low-level mechanical signal, was 69% greater ($P < 0.04$) than in intrastrain control mice. Increased trabecular bone formation rates coincided with an 85% ($P < 0.01$) larger bone volume (BV/TV) and 50% larger trabecular thickness ($P < 0.009$) in the vibrated mice. In BALB/cByJ mice, the low-level mechanical signals increased BFR/BV by 34% ($P < 0.02$), but bone structural indices including BV/TV remained unaffected. In contrast to the responsiveness of the C57BL/6J and BALB/cByJ strains, no significant effects of mechanical stimulation were measured in tibial trabecular bone of C3H mice. Extrapolating these results to the human skeleton may provide insight into the preferential efficacy of mechanical interventions, such as exercise or low-level vibrations. The results of this study also suggest that some people who benefit from a genetically predetermined higher bone mass may ultimately be less sensitive to any form of physical intervention (Torvinen *et al.*, 2003).

Inhibition of Postmenopausal Bone Loss by Low-Level Vibrations

With the demonstration that mechanical factors can be anabolic and anticatabolic to the skeleton, a challenge becomes whether these "regulatory factors" can be safely and effectively administered in the clinic to inhibit or reverse osteopenia. The ability of a low-magnitude (0.2 *g*), high-frequency (30 Hz) mechanical stimulation to inhibit postmenopausal osteopenia was evaluated in a prospective, randomized, double-blind, placebo-controlled clinical trial (Rubin *et al.*, 2004). Sixty-two postmenopausal women were enrolled in the study. Thirty-one women underwent mechanical loading of the lower appendicular and axial skeleton for two ten-minute periods per day, induced via floor-mounted devices that produced the mechanical stimulus, whereas 31 women received placebo devices and underwent daily treatment for the same period of time.

An intention to treat analysis of DXA data of the spine (L1–4), proximal femur, and nondominant radius showed little effect of the intervention. In a *post hoc* analysis of those subjects that used the device at least 60% of the prescribed time, a linear regression of the means was used to show that lumbar spine bone mineral density (BMD) declined by −3.3% (±0.83) in the placebo group compared with only −0.8% (±0.82) in the treated group ($P < 0.03$), reflecting a 2.5% benefit of the biomechanical intervention. A 3.3% treatment benefit was observed in the trochanter region of the hip, with a −2.9% (±1.2) loss observed in the placebo group, yet with a 0.4% (±1.2) gain in the treated group ($P < 0.03$). At the distal radius, no significant differences were observed as a function of time or between groups, emphasizing the mechanical influence to be locally delivered.

Stratifying the results based on patient body mass index (BMI), endpoint analysis confirms the relationship between svelte stature and a greater degree of osteoporosis (Aloia *et al.*, 1995; Martin *et al.*, 1993); subjects with a BMI of less than 24 lost 2.5% (±0.6) BMD over the course of the year, whereas those with a BMI greater than 24 did not show any change over the 12-month period. This stratification also demonstrates the ability of mechanical stimulation to inhibit this bone loss in the group at greatest risk; in subjects with a BMI of less than 24 who received the mechanical stimulus, the bone loss in the spine was not significantly different than zero (+0.2%±0.7). The 2.7% difference between placebo and treatment groups was significant at $P < 0.01$. Treated subjects with BMI greater than 25 showed no apparent affect of treatment, perhaps because there was no bone loss to inhibit. Overall, these results indicated the potential of a noninvasive biomechanical therapy for osteoporosis, representing a nondrug means of inhibiting the decline of bone mineral density that typically follows the menopause.

Low-Level Mechanical Signals Are Anabolic to the Musculoskeletal System

Susceptibility to osteopenia is present early in life, the amount of bone gained during adolescence is a main contributor to peak bone mass in the young adult, and peak bone mass in the young adult is a determinant of osteoporosis in the elderly (Loro *et al.*, 2000). A study was designed to establish if brief, daily exposure to extremely low-level mechanical stimuli were anabolic to musculoskeletal development in young females, 15 to 20 years of age, each in the lowest quartile of bone density in this age cohort and who had already sustained a fracture (Gilsanz *et al.*, 2006).

Half of the 48 enrolled subjects were subject to brief (10 min/day), low-level whole-body vibration (30 Hz, 0.3 *g*), with the remaining women serving as controls. CT performed at baseline and the end of study was used to establish changes in muscle and bone mass in weight-bearing regions of the skeleton. Using an Intention to Treat (ITT) analysis, cancellous bone in the lumbar vertebrae and cortical bone in the femoral midshaft of the experimental group increased by 2.1% ($P = 0.025$) and 3.4% ($P < 0.001$), respectively, compared with 0.1% ($P = 0.74$) and 1.1% ($P = 0.14$), in controls. Increases in cancellous and cortical bone were 2.0% ($P = 0.06$) and 2.3% ($P = 0.04$) greater, respectively, in the experimental group when compared with controls. Cross-sectional area of paraspinous musculature was 4.9% greater ($P = 0.002$) in the experimental group versus controls. When a per protocol (PP) analysis was performed, gains in both muscle and bone were strongly correlated to a threshold in compliance, where the benefit of the mechanical intervention compared with controls was realized once the device was used for at least two minutes per day ($n = 18$), as reflected by a 3.9% increase in cancellous bone of the spine ($P = 0.007$), 2.9% increase in cortical bone of the femur ($P = 0.009$), and 7.2% increase in musculature of the spine ($P = 0.001$), compared with controls plus the low-compliers (Fig. 4).

Short bouts of extremely low-level mechanical signals, several orders of magnitude below that associated with vigorous exercise, increased both trabecular and cortical

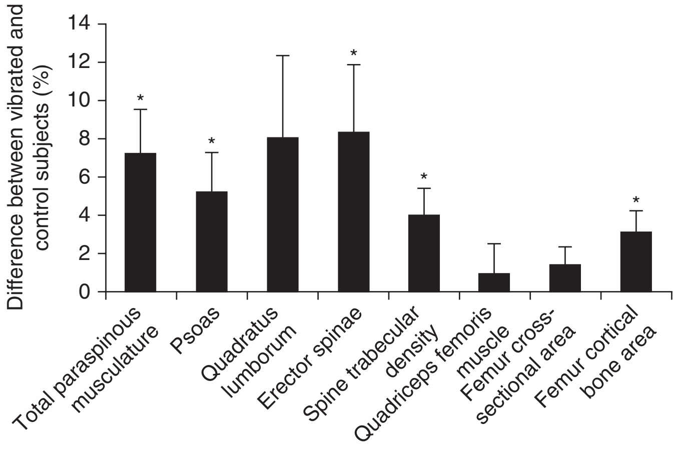

FIGURE 4 The difference in the change (mean + SE) of musculoskeletal parameters measured over the one-year period for young women who used the device for more than two minutes per day, compared with controls and women who stood on the vibrating plate for less than two minutes per day. With the exception of musculature around the femur and femoral cross-sectional area, the musculoskeleton of the experimental group benefited significantly (*) from the brief low-level mechanical intervention. Adapted from Gilsanz *et al.* (2006).

bone in weight-bearing regions of the skeleton. Further, muscle mass of the paraspinous region increased, suggesting that risk factors for fracture beyond bone quantity and quality could be mitigated. Low-level, high-frequency mechanical signals, even lower than those used in this study, have also been shown to preserve postural stability in an aging population (Gravelle *et al*., 2002), providing evidence that physical signals can serve to reduce risk factors of osteopenia, falling, and sarcopenia. Should these musculoskeletal enhancements be preserved through adulthood, this intervention may prove a deterrent to osteoporosis in the elderly.

Can Bone Differentiate between Two High-Frequency Signals?

To determine whether the responsiveness of bone to low-magnitude, high-frequency parameters is modulated by endocrine imbalance and whether one high-frequency signal may be more effective than another, ovariectomized (OVX) Sprague–Dawley rats were subjected to whole-body vibrations (WBV, $0.15\,g$) at 45 Hz ($n = 6$) or 90 Hz ($n = 6$) for 10 min/day, and compared to OVX age-matched controls ($n = 6$) (Judex *et al.*, 2007). Five additional rats were used, *in vivo*, to establish the induced bone surface strain magnitudes (and strain rates). Following a 28 day protocol, bone formation rates in the metaphysis of the proximal tibia were 159% greater in 90 Hz rats when compared with age-matched controls, but 45 Hz rats were not significantly different from controls. Bone morphology of 90 Hz rats indicated significantly greater trabecular bone volume (22% and 25%) and thicker trabeculae (11% and 12%) over either controls or 45-Hz rats in the epiphysis of the distal femur, respectively. Despite the enhanced sensitivity of the skeleton toward the 90-Hz signal, the strain magnitudes and strain rates induced by this frequency were significantly lower than during 45-Hz vibration. These data suggested that the efficacy of the low-level mechanical signal is maintained even in the absence of estrogen and factors other than matrix strain are driving the anabolic response.

How can Bone Sense a Signal so Small?

The studies above, contrasting with a more-is-better principle, suggest that matrix strains two orders of magnitude below a previously suggested threshold (Frost, 1987) can be anabolic to bone. The means by which such low-level mechanical signals can be anabolic to a tissue such as bone is not clear. If cortical matrix deformations of less than 0.001% strain, measured at the periosteum, were transduced directly to the resident osteoblast or osteocyte population, the deformation of the cell itself would be less than one Angstrom. Given that such deformations may be too small to be recognized by cells (Han *et al.*, 2004), byproducts of matrix deformation, such as fluid flow-induced shear stresses, streaming potentials, fluid drag on pericellular processes, or enhanced nutrient transport, may contribute to a cell's responsiveness to mechanical signals (Malone *et al*., 2007a). Yet even these alternative pathways are dependent on matrix deformation and therefore will be very small in magnitude during low-level mechanical stimulation.

In contrast to a matrix deformation-dependent pathway for mechanotransduction, the frequency sensitivity of the adaptive system points toward a more fundamental, perhaps unrecognized, pathway by which physical signals interact with the tissues and cells. Indeed, a mechanism that would allow a cell to sense mechanical signals directly without reliance on matrix strain would obviate the need for compensatory tissue-level amplification mechanisms, reduce complexity in the system, and may provide cells with mechanical information without the potential for damaging the surrounding tissue. Our hypothesis is that the physical acceleration of a cell may present such a signal that can transmit physical challenges to a receptive cell population in an efficient and safe manner (Garman *et al*., 2007a). By removing bone's habitual loading environment and imposing very-small-amplitude oscillatory accelerations that induce motion but no direct deformation, we have recently tested this hypothesis (Garman *et al*., 2007b).

To this end, a device was developed that can deliver high-frequency accelerations to skeletal segments in the absence of weight bearing (Fig. 5). In an anesthetized murine model, the left tibia of each mouse was subjected to oscillatory $0.6\,g$ accelerations at 45 Hz for 20min/day and the right tibia served as control. Oscillatory accelerations, applied in the absence of weight bearing, resulted in 70% greater bone formation rates in the trabeculae of the metaphysis, but similar levels of bone resorption, when compared with contralateral controls. Quantity and quality of trabecular bone also improved as a result of the acceleration stimulus, as evidenced by a significantly greater bone volume fraction (17%) and connectivity density (33%), and significantly smaller trabecular spacing (−6%) and structural model index (−11%). As expected, the matrix deformations induced by these motions are extremely small,

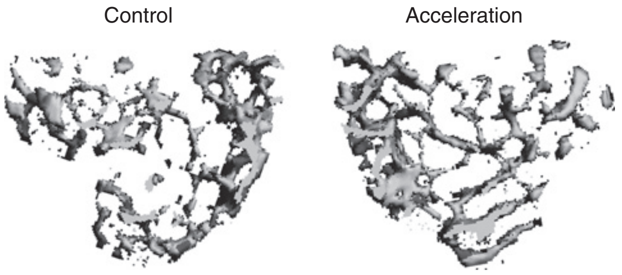

FIGURE 5 By applying very-low-level oscillatory accelerations to one leg of the anesthetized mouse while the other leg serves as a contralateral control, the efficacy of very small unconstrained motions that do not directly apply deformation to the matrix can be tested. Adapted from Garman *et al*. (2007).

however, and on the same order as those produced by postural stability. These *in vivo* data indicated that mechanosensory elements of resident bone cell populations can perceive and respond to acceleratory signals, and point to an efficient means of introducing intense physical signals into a biological system without putting the matrix at risk of overloading. Teleologically, accelerations represent a fundamentally efficient means of delivering regulatory physical information to the cell, and can be readily achieved even in the absence of matrix strains and large cellular deformations. In contrast to the previous studies in which transmission of the high-frequency mechanical signals relied on weight bearing (e.g., whole-body vibrations), the unique nature of the external applications of oscillatory motions ("shaking") may ultimately serve as an effective intervention for the prevention of bone loss in non-weight-bearing skeletal sites or in patients whose skeleton is incapable of bearing weight, such as during bed rest or space flight.

BIOCHEMICAL MODULATION OF MECHANICAL SIGNALS

Which Cells Sense the Stimulus?

It is clear that bone cells are able to sense their mechanical environment via a number of proposed mechanisms including direct matrix deformations, pressure and transient pressure waves, accelerations, fluid shear stresses, fluid drag forces, or even dynamic electric fields as interstitial fluid flows past charged bone crystals (Fig. 6). It is much less clear, however, which of the cells within a bone are the key sensory elements in mechanotransduction.

Mechanical responses of osteoprogenitor cells, including stromal cells, osteoblasts, and osteocytes, have all been documented without identifying the critical responding cell: for example, exposure to microgravity results in a decreased number of osteoblasts—but what cell senses and responds to the loss of gravity, the undifferentiated mesenchymal precursor, the differentiated osteoblast, or the distant, entombed osteocyte? Bone marrow stromal cells change their proliferation rate and gene expression patterns in response to mechanical stimulation (Li *et al.*, 2004) and alter lineage selection as well (David *et al.*, 2007). With respect to osteoclasts and bone resorption: stromal cell expression of the osteoclastogenic factor, RANKL, is sensitive to mechanical force (Rubin *et al.*, 2002c), suggesting that the number of osteoclasts present is controlled through mechanical regulation sensed by stromal cells. The osteoclast itself has also been shown to respond to mechanical signals adding another layer of control by which mechanical force might limit bone resorption (Wiltink *et al.*, 1995).

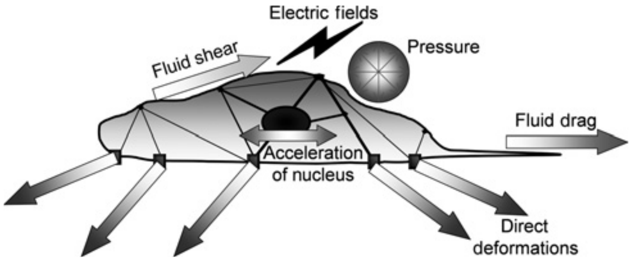

FIGURE 6 Proposed mechanisms by which bone cells may sense the application of mechanical forces to the skeleton.

Other cells present in bone, such as endothelial and smooth muscle cells in the penetrating vasculature, might also contribute to the skeleton's adaptive response to loading. After all, endothelial cells respond to shear stress and tensile strain generated by increased heart rate during exercise, by producing nitric oxide (Boo and Jo, 2003). Nitric oxide is an important humoral factor to transduction of mechanical input in vascular cells (Lane and Gross, 1999). Increased vascular release of nitric oxide is likely to regulate bone cell response: nitric oxide has pleiotropic effects in bone cells, and potently decreases resorption through decreasing osteoclast formation and activity (Fan *et al.*, 2004a). But bone stromal cells also release nitric oxide as a result of mechanical input (Rubin *et al.*, 2003) providing a secondary cell target for mechanical induction of this freely diffusible antiresorptive agent. Thus, targets of skeletal loading may include extraskeletal cells and systems.

Finally, the majority of cells in cortical bone are osteocytes, and owing to their pervasive, three-dimensional distribution throughout both trabecular and cortical bone, these cells are potentially well placed to sense the magnitude and direction mechanical strain within the tissue. Osteocytes, although enclosed in calcified tissue, are interconnected through a network of cannaliculi through which these cells cast long cell processes. Osteocytes respond to strain *in vivo* as shown by increased glucose-6-phosphate dehydrogense activity (Skerry *et al.*, 1989), or earlier response of c-*fos* mRNA (Inaoka *et al.*, 1995) after loading. As well, unloading causes osteopontin expression in osteocytes (Gross *et al.*, 2005). Dentin matrix protein (DMP1), which is a secreted matrix protein expressed in late osteoblasts and osteocytes, has been shown to increase in osteocytes after tooth movement in the jaw (Gluhak-Heinrich *et al.*, 2003).

If we consider that strain signals of even very low magnitude can stimulate an anabolic response in bone tissue, the osteocyte may be best placed to sense such a signal. This might occur through fluid flow through cannaliculi, as well as through deformation, both of which have been shown to cause changes in osteocyte function (Plotkin *et al.*, 2005). It has also been suggested that, owing to the modulus mismatch of the bone material and the lacunae, that the osteocyte within the cavity would be subject to strains as high as 30,000 microstrain, even though the bulk material was strained only to 3,000 microstrain (Nicolella *et al.*, 2005). In other words, the microarchitecture of the bone tissue could serve to indirectly (fluid pressure through cannaliculi) or directly (strain amplification via the lacunae) amplify the strain signal. Although some have suggested that osteocytes might be more sensitive to shear than, for instance, transient pressures (Klein-Nulend *et al.,* 1995), there are divergent opinions, e.g., substrate strain prevents osteocyte apoptosis. A recently developed three-dimensional model for the osteocyte process used large-deformation "elastica" theory to predict the deformed shape of the cell. Because the model predicts a cell process that is very stiff, hard tissue strains will be amplified through the cell process, indeed into the magnitudes that have been studied *in vitro* in many systems. In any case, cells contributing to bone remodeling directly (osteoblasts) and indirectly (osteoprogenitor cells and osteocytes) and distantly (muscle and vascular systems) are all known to be mechanosensitive.

Mechanoreceptors in Bone Cells

The ability of cells to read their biomechanical environment requires that their mechanoreceptors must either be in contact with the outside, through the cell membrane and its attachment to substrate, or that the mechanoreceptor be able to sense changes in a loading-induced physical intermediary such as fluid shear on the apical membrane. Although there are examples of channels that are regulated by movement of mechanosensory bristles (Sukharev and Corey, 2004), or by tension waves (Morris, 1990), a unified model of the most proximal events that lead to intracellular signal transduction in nonsensory tissues does not yet exist. Theoretical considerations may be moving toward an architectural/spatial concept that integrates positional changes between signaling proteins, scaffolds, membrane domains, and structural components of the cell. All can be perturbed by mechanical force. Whether each aspect is a mechanoreceptor in itself, or works in a holistic context combining many aspects of cell architecture and response is yet to be determined. With the multiplicity of mechanical signals presented to the cell, it is at least likely that no single mechanosensor or receptor mechanism is responsible for all of the integrated cell response to the mechanical environment.

Integrins and Integrin-Associated Proteins

Membrane deformation and shear across the membrane, as well as pressure transients, can be transmitted to the cytoskeleton and ultimately to the cell-matrix adhesion proteins that anchor the cell in place (Katsumi *et al.,* 2004). Thus, the cell represents a load transmission network whereby surface forces will affect proteins that span or are associated with the plasma membrane including the cytoskeleton, linker proteins at sites of cell attachment, and membrane-spanning integrin adhesion proteins. In this way, mechanotransduction might be expected to depend on the mechanical integrity of this network with constituents of this network serving as molecular mechanotransducers.

Indeed, in particular the β1 integrin subunit has been implicated in mechanotransduction on ostecytes and osteoblasts (Bennett *et al.,* 2001). In osteoblasts, steady fluid flow can upregulate β1 expression (Kapur *et al.,* 2003) even though blocking β3 with RGD-containing peptides did not affect ERK activation (Weyts *et al.,* 2002). Other focal adhesion proteins such as focal adhesion kinase (FAK) may be critical to integrin clustering as well as molecular signaling in bone cells (Rezzonico *et al.,* 2003). Mechanical stimulation stimulates tyrosine phosphorylation, predominantly in FAK, in osteoblasts (Boutahar *et al.,* 2004). Upon phosphorylation, FAK contributes to MAPK activation via interaction with c-src, Grb2, and the small GTPase Ras (Schlaepfer *et al.,* 1999). This is significant because MAPK activation is also one of the effectors of oscillatory flow in bone cells and this pathway has been observed in response to fluid flow in endothelial cells (Berk *et al.,* 1995). More molecular definition has lately been assigned to this pathway: involvement of an β1 integrin-linked kinase is stimulated through mechanical input via, leading to Akt activation (Nho *et al.,* 2005), a pathway that links to downstream processes regulated by canonical Wnt/catenin.

Connexins

Connexins are membrane-spanning proteins that form regulated channels that allow the direct exchange of small molecules with adjacent cells resulting in intercellular communication between cells. Intercellular communication via gap junctions has been suggested to be central to the transmission of information about the mechanical environment of a given cell and ultimately allowing a sensing cell to elicit a change in behavior at an actor cell some distance removed from the mechanosensing event (Yellowley *et al.,* 2000). Interconnected cells have also been proposed to form a cellular network that can exhibit an enhanced sensitivity to biophysical stimuli than occur in individual cells. This application might occur owing to the larger area occupied by a cell network, and therefore larger net effect of the biophysical signal, than by individual cells. Indeed, when the communication of an ensemble of cells is interrupted, a reduced sensitivity has been observed in response to biophysical signals such as electric fields (Vander Molen *et al.,* 2000) or fluid flow (Saunders *et al.,* 2001). In addition, mechanical signals may increase the expression of connexins *in vitro* and *in vivo*, suggesting that cells become better connected with their neighbors perhaps acting as a sort of positive feedback loop (Alford *et al.,* 2003).

Channels

Alterations in ion channel activity in osteoblasts have been associated with bone cell activation, whether through alteration in conductance stimulated by PTH (Ferrier *et al.,* 1986), or by stretch/strain (Duncan *et al.,* 1992). There are at least three classes of mechanosensitive ion channels in human osteoblasts (Davidson *et al.*, 1990). Through this set of channels, a mechanical stimulus could induce membrane hyper- and depolarization or a complex multiphasic response. Cyclical strain has been shown to modulate the activity of certain channels—chronically strained osteoblasts had significantly larger increases in whole-cell conductance when subjected to additional mechanical strain than unstrained controls (Duncan and Hruska, 1994). More recently, radial membrane strains of 800% were shown to be necessary to open half of the mechanosensitive channels in bone cells (Charras *et al.*, 2004). Mechanosensitive channels have also been implicated in the response of bone cells to fluid shear stress (Ryder and Duncan, 2001). In addition to direct activation of intracellular signaling cascades, influx of a charged species such as calcium can also alter membrane potential and activate voltage-sensitive channels that are not directly mechanosensitive (Li *et al.*, 2002). Taken together, it is clear that mechanically sensitive ion channels exist in bone, but the exact identification of such channel(s) remains elusive.

Membrane Structure

Cells possess a complex organizational structure that supports compartmentalization of signals within an equally complex plasma membrane. Certain proteins are integral to membrane structure while creating docking positions for signaling complexes. Caveolin 1 and 2 have been described in human fetal osteoblasts and in murine MC3T3-E1 cells along with multiple caveolar flasks in the membrane (Solomon *et al.*, 2000) and in osteoblasts in adult bone (Lofthouse *et al.*, 2001). Bone cell caveolae are associated with important signaling molecules including G proteins, Ras, nitric oxide synthase, and tyrosine kinases. These membrane domains, whether caveolae or noncaveolar lipid rafts provide a microenvironment that modulates efficiency and fidelity of mechanical signal transduction.

The organized membrane may have greater significance for mechanical response than for parsing signals arising from liganded receptors. Noncaveolar and caveolar organized membranes have been shown to be critical for mechanically induced signals in a variety of cells. In the vascular endothelium, for instance, increased flow causes the translocation of signaling molecules to caveolae; if caveolae are disassembled, both proximal and downstream signaling events, including activation of the MAPK pathway, are abrogated (Rizzo *et al.*, 1998). Stretch activation of small GTPases in cardiac myocytes has, as well, been associated with caveolae: the stretch activation of the GEFs RhoA and Rac1 fails to occur when caveolae are disrupted by treatment with methyl-β-cyclodextrin (MβCD) (Kawamura *et al.*, 2003).

The role of caveolae in processing signals in bone is being studied: caveolin-1 null mice have bigger bones, perhaps resulting from accelerated development (Rubin *et al.*, 2007). The spatial interaction between molecules of the wnt/catenin signaling system and caveolin-containing membrane (Bilic *et al.*, 2007) suggests that mechanical effects on these membrane domains will be important.

Primary Cilium

A new development in mechanical signal transduction has arisen with the reevaluation of the role of the primary cilium, a microtubule-based structure extending from almost every mammalian cell studied. The primary cilium has been known to function as a sensory organelle, but is now recognized to receive both mechanical and chemical signals from the environment (Michaud and Yoder, 2006). Epithelial cells in the kidney have been shown to recognize fluid shear stress in the lumen of the nephron through their primary cilium, and mutations leading to abnormal cilia, such as are found in polycystic kidney disease, lead to gross abnormalities in function (Nauli and Zhou, 2004). Indeed, when ciliary polycystin-1 is knocked out, besides causing polycystic kidney disease, mice have delayed endochondral and intramembramous bone formation (Xiao *et al.*, 2006). Further, interesting data have shown that bone cell primary cilia translate fluid flow into calcium signals (Malone *et al.*, 2007b).

Mechanically Activated Intracellular Signaling

Application of mechanical force to bone cells causes modulation of cell function, including changes in proliferation and function. Indeed, most aspects of cell behavior can be elucidated by a number of mechanical forces. Thus, straining osteoprogenitor cells can cause them to proliferate (Zhuang *et al.*, 1996) and to secrete extracellular matrix (Harter *et al.*, 1995). Similarly, shear stress, in addition to strain-induced effects, can induce beta-catenin signaling (Norvell *et al.*, 2004) and secretion of osteopontin (You *et al.*, 2001). To achieve these ends, it is quite clear that multiple classic signaling pathways are activated after force application.

Because the distal responses to mechanical factors are similar to those elicited by ligand–receptor pairing, and result in changes in gene expression, mechanotransduction must eventually end up utilizing similar intracellular signaling cascades. In essence, mechanical forces have been shown to activate every type of signal transduction cascade, from increases in intracellular cAMP (Lavandero *et al.*, 1993), IP3 and intracellular calcium, guanine regulatory proteins (Gudi *et al.*, 2003), and MAPK (Rubin *et al.*, 2002d). Next, rather than reviewing each mechanosignaling cascade, we provide four examples.

MAPK Signaling

Mechanical force can activate mitogen-activated protein kinases in every cell type studied to date. In bone cells many groups have shown activation of ERK1/2, in particular.

Mechanical activation of ERK1/2 is required for certain measurable responses to strain in bone stromal and osteoblast-like cells. The strained bone cell downregulates its expression of RANKL, and upregulates expression of eNOS. Although strain also activates c-jun kinase (JNK), JNK inhibition does not prevent strain effects on either RANKL or eNOS. Reduced display of RANKL by cells present in bone diminishes the local osteoclastogenic potential. As a result of increased eNOS expression, nitric oxide (NO) synthesis is enhanced.

ERK1/2 activation by mechanical stress has been linked to other specific genes such as strain induction of collagenase-3 (Yang *et al.*, 2004), as well as to proliferation. Both fosB and its spliced variant, deltafosB, which has been shown to stimulate increases in bone density *in vivo*, are induced by mechanical loading of the mouse hind limb and by fluid shear stress in mouse calvarial osteoblasts (Inoue *et al.*, 2004). The ERK1/2-dependent increase in the fos gene targets a CRE/AP-1 type element in the promoter that binds CREB. The proximal events leading to ERK1/2 activation are the subject of much continued research. This may certainly involve integrins (Whedon, 1984) as well as multiple other effectors, including small GEFs (Jin *et al.*, 2005) or changes in membrane structure (Boyd *et al.*, 2003).

Activation of Wnt/Catenin Signals

Increased expression of Wnt/β-catenin target genes is another response of osteoblasts to mechanical loading (Robinson *et al.*, 2006). Multiple known genes with catenin response elements are quickly transcribed when strain is applied to osteoblast cells in culture, or when bone cells are studied after whole-animal loading; these are likely related to the anabolic response of bone tissue to exercise. Increased canonical β-catenin signaling through a gain of function mutation in LRP5 (G171V) has further been shown to increase the response of osteoblasts to mechanical loading (Sawakami *et al.*, 2006). Mechanical activation of catenin signaling, of crucial importance to osteoprogenitor proliferation and lineage selection, will continue to be of great interest to this field.

Nitric Oxide Signaling

Nitric oxide has pleiomorphic effects on bone cells (Ralston, 1997), and may have a role in mechanical signaling in bone. Nitric oxide is released shortly after shear stress from osteoblasts and osteocytes (Smalt *et al.*, 1997) likely owing to activation of endothelial nitric oxide synthase (Klein *et al.*, 2004) similarly to known effects of shear in vascular cells. The rapid activation of nitric oxide in endothelial cells requires, in part, an intact plasma membrane, including lipid rafts (Park *et al.*, 1998) and cytoskeleton (Knudsen and Frangos, 1997), and this is likely to be true for mechanical release of nitric oxide in bone cells. Endothelial nitric oxide synthase is the predominant nitric oxide synthase isoform in adult bone (Helfrich *et al.*, 1997), and expression of this gene with subsequent increase in nitric oxide production is upregulated by strain in marrow stromal cells. Downstream nitric oxide signaling can depend on activation of guanylate cyclase or on direct actions of the molecule to nitrosylate proteins as has been shown for NO action to decrease the RANKL/osteoprotegerin ratio in stromal cells (Fan *et al.*, 2004b). Nitric oxide has also been shown to be necessary for the response to *in vivo* loading in rodents, although whether the nitric oxide derives from bone or the vasculature in bone is not yet clear (Kunnel *et al.*, 2004).

Prostaglandins

In bone's anabolic response to loading, prostaglandins may be important as indicated by their increased levels during loading (Rawlinson *et al.*, 1991). Indeed, a variety of mechanical signals, including direct stretch (Zaman *et al.*, 1997), pressure (Burger *et al.*, 1992) and fluid flow (Klein-Nulend *et al.*, 1997) can markedly upregulate prostaglandin production, whereas the disruption of the osteocyte cytoskeleton, and thus the transmission of the signal to the nucleus, completely disrupted the responsiveness of the cell (Ajubi *et al.*, 1996). Although greater detail in the mechanism of action of prostaglandins is provided elsewhere in this book, it is important to note that administration of PGE2 to rodents will increase the stiffness of trabecular bone (Akhter *et al.*, 2001), and that perhaps the EP2 receptor is most important in coordinating the prostaglandin response to mechanical signals into improved bone strength. Considering the role of prostaglandins as potent regulators of bone remodeling (Raisz, 1995), and their responsiveness to both mechanical and humoral signals, it will ultimately be important to more definitively understand the physiological responses of the skeleton to exercise and mechanical signals, and the role of biochemical messengers such as prostaglandins that can modulate bone quantity and quality.

SUMMARY

The critical contribution of mechanical stimuli in the achievement and maintenance of bone quantity and quality is clear. In contrast to systemic, pharmaceutical interventions, the attributes of such biophysical prophylaxes are that they are native to the bone tissue, safe at low intensities, incorporate all aspects of the remodeling cycle, and will ultimately induce lamellar bone. Further, these signals appear to influence tissues beyond "simply" bone, with evidence that they can help retain musculature and postural stability, thus providing a more "systems-based" intervention for osteoporosis. However, the design and widespread use of effective and clinically acceptable prophylaxes and treatments that are based on bone's sensitivity to biophysical stimuli will be delayed until we achieve a better understanding of the operative mechanisms at the levels of the tissue, cell, and gene.

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Part IV

Methods in Bone Research

Chapter 86

Application of Transgenic Mice to Problems of Skeletal Biology

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INTRODUCTION

Transgenic mice provide an excellent setting to study complex regulatory systems that cannot be modeled or fully appreciated in isolated experimental systems *in vitro* or *in cellulo*. This potential is particularly important in the analysis of the cells that synthesize the extracellular matrix because their response to regulatory signals from the external environment is dependent on their state of cellular differentiation. Because these variables cannot be reliably modeled in isolated systems, the challenge is to develop assay systems within an intact animal that can still ask a focused molecular question. This chapter is designed to illustrate examples where transgenic animals have answered subtle questions about protein structure and gene regulation affecting bone structure or bone cell differentiation that could not have been performed in any other experimental system. In addition, newly developed transgenic approaches that are now being applied to bone and have great potential in this research arena will be discussed.

ANALYSIS OF STRUCTURAL MACROMOLECULES IN BONE

Type I collagen is the major structural protein of bone, whereas type II is the primary collagen of cartilage. Mutations in type I collagen are associated with osteogenesis imperfecta (OI) while certain chondrodysplasias are the result of defects in type II collagen. Because of the multimeric structure of mature type I and type II collagen molecules, a dominant negative mechanism is associated with the pathogenesis of both classes of inherited traits. Although molecular genetic studies of humans affected with these disorders have illustrated that the spectrum of disease severity can be correlated to specific mutations (Prockop *et al.,* 1993; Prockop *et al.,* 1994), a clearer understanding of how these mutations affect protein function was obtained from transgenic mice.

A collagen transgene carrying a glycine substitution demonstrated the concept of a dominant negative mutation as these transgenic mice displayed a perinatal lethal OI (Stacey *et al.,* 1988). In these transgenic mice only relatively small amounts of the mutant protein were required to destabilize the matrix and produce a dramatic affect on the entire skeleton. Transgene expression levels as little as 10% of the normal endogenous gene production of collagen were associated with a mutant phenotype. In a series of transgenic mice bearing a large internal deletion of the collagen gene, a less severe and more variable phenotype was observed and the phenotype correlated with the level of transgene expression (Khillan *et al.,* 1991; Pereira *et al.,* 1993). In this case, the severity was more variable, predominantly less severe, and correlated with the degree of expression. Furthermore, the level of transgene expression in an inbred strain was equivalent to the endogenous gene indicating that variations in phenotypic severity were not the result of genetic background but related to the structure of the mutated gene (Pereira *et al.,* 1994). Collectively, these data indicated that the severity of the mutant phenotype is dependent upon the amount of incorporation of a mutant collagen molecule into the matrix. Thus, if the collagen gene has a major alteration, as is the case with a large internal deletion, fewer mutant molecules are incorporated into the matrix compared with the case of a mutation that has only a moderate impact of molecular assembly. In this latter case, more mutant molecules are incorporated into matrix with dramatic affects on its structural integrity. Clearly understanding how a mutation leads to the presentation of a skeletal phenotype requires a comprehension of the impact the mutant allele has on the assembly secretion and incorporation of the mutant molecule into the extracellular matrix. This concept is further highlighted by studies with a targeted deletion of the cartilage matrix associated protein (Crtap). Crtap-null mice display a severe osteopenia that is associated with a failure of prolyl hydroxylation, a post-translational step required for proper molecule maturation (Morello *et al.,* 2006). The link between defects in

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this locus in humans and OI was established by the observation that 3 out of 10 children with lethal or severe OI who lacked defects in type I collagen had mutations at the Crtap locus. This recessive form of OI was associated with a loss of prolyl 3-hydroxylation and altered production of mature triple helical collagen molecules (Barnes *et al.*, 2006). In addition to illustrating the importance of mutations that disrupt the collagen helix and lead to defective extracellular matrix formation, mutations at other sites within the molecule can demonstrate biological properties of type I collagen (Kivirikko, 1993). The turnover of collagen in the extracellular matrix begins with a site-specific cleavage by a collagenase. If this site is altered by a mutation, a resistance to collagenase degradation results (Wu *et al.*, 1990). The first step in the enzymatic turnover of collagen from the extracellular matrix is cleavage at a specific site within the helix by mammalian collagenase. Collagen molecules containing a mutation at the site of cleavage are more resistant to degradation (Wu *et al.*, 1990). Transgenic mice bearing a collagenase-resistant Col1a1 transgene display embryonic lethality; however, mice with a targeted endogenous gene with the same resistance mutation survive and develop a dermal fibrosis similar to human scleroderma (Liu *et al.*, 1995a). The role of collagenase in parathyroid hormone (PTH) stimulated bone resorption was evaluated utilizing this model. Mice homozygous for the targeted collagenase resistant allele when treated with PTH did not show elevated bone resorption compared to wild-type controls (Zhao *et al.*, 1999). Furthermore, when the resistant allele was combined with a transgene constitutively active of PTH/PTHrP receptor (CL2+) (Calvi *et al.*, 2001), trabecular bone volume was increased despite a reduction in bone formation rate. Interestingly, osteoclast number was elevated in CL2+ mice homozygous for the resistance collagen allele compared to wild types or CL2+ mice, suggesting that altered collagen cleavage impacts osteoclast function (Chiusaroli *et al.*, 2003). Finally, osteocyte and osteoblast apoptosis was elevated in homozygous-resistant mice consistent with a role of matrix degradation in the regulation of skeletal remodeling via the modulation of osteocyte and osteoblast survival (Zhao *et al.*, 2000b). Similar observations have been made for type II collagen. Changes of single amino acids in the Col2a1 transgenes have a significant impact on the skeleton. Col2a1 transgenes with glycine to cysteine or glycine to serine amino acid substitution produce the phenotype of chondrodysplasia in mice (Garofalo, 1991; Maddox *et al.*, 1997) and abnormal craniofacial morphology (Rintala *et al.*, 1993). An Arg to Cys mutation reduces the density of type II collagen fibrils in articular cartilage and a delayed skeletal development (Arita *et al.*, 2002) as well as degenerative intervertebral discs and osteoarthritic joints that were more severe in the absence of a normal endogenous Col2a1 gene (Sahlman *et al.*, 2004). A Col2a1 transgene with a small deletion (Del1) produces several skeletal defects including retarded development (Savontaus *et al.*, 1996), craniofacial abnormalities (Rintala *et al.*, 1997), and axial skeletal defects associated with abnormalities in the vertebra and discs (Savontaus *et al.*, 1997). Furthermore, as Del1 transgenic mice age a progressive degeneration of articular cartilage was observed beginning at age 3 months (Saamanen *et al.*, 2000). A transgene with a large in frame deletion lacking 12 exons in the central coding region of the gene produces a shortened procollagen chain that, when interacting with chains of normal size, leads to degradation of the chimeric molecules, termed procollagen suicide (Vandenberg *et al.*, 1991). Many of these transgenic mice display a chondrodysplasia that is affected by genetic background and older mice show an osteoarthritis phenotype associated with articular cartilage degeneration (Helminen *et al.*, 1993). Mice homozygous for a targeted knockout of the type II collagen gene develop a skeleton containing membranous and periosteal bone, lack endochondral bone, and die shortly after birth (Li *et al.*, 1995a). Homozygous mutant mice fail to dismantle their notochord during embryogenesis and thus do not develop intervertebral discs (Aszodi *et al.*, 1998). Mice heterozygous for the null allele are viable, however, have skeletal deficiencies including shorter long bones, vertebrate and cranial defects (Sahlman *et al.*, 2001) as well as ocular abnormalities similar to human Stickler syndrome (Kaarniranta *et al.*, 2006). Finally, excessive expression of a normal Col2a1 gene produces abnormally thick fibrils and mice with the highest proportion of abnormal fibrils are perinatal lethal (Garofalo *et al.*, 1993). This observation is of great significance in the design of gene therapy approaches for the management of diseases of bone. Although great strides have been made in the field of regenerative medicine, clearly careful consideration must be given for the proper regulation of a correcting gene as well as interaction with endogenous mutant alleles before application of a curative therapy.

The integrity of the skeleton relies not only on the proper structure and function of type I and II collagen but also on the interplay of minor collagens and noncollagenous proteins with the major collagen molecules. This interaction can be appreciated in transgene models bearing alterations in genes encoding minor collagens. Type X collagen functions as a homotrimer and is primarily expressed in hypertrophic chondrocytes. A transgene with a large internal deletion behaves in a dominant negative fashion and mice carrying this transgene display a phenotype similar to human spondylometaphyseal dysplasias and metaphyseal chondrodysplasias (Jacenko *et al.*, 1993). Further study of this transgenic mouse revealed moderate craniofacial skeletal abnormalities (Chung *et al.*, 1997) as well as a significant deficit in hematopoietic cell differentiation (Jacenko *et al.*, 2002). An initial study with a targeted null allele of the Col10a1 gene unexpectedly did not have any major consequences on long bone development as mice either heterozygous or homozygous for the null mutation displayed a normal phenotype (Rosati *et al.*, 1994). However, a subsequent targeting experiment that produced type X deficient mice resulted in animals that displayed abnormalities in growth plate development resulting in altered bone content. Furthermore, a reevaluation of the original knockout mice revealed a skeleto-hematopoietic phenotype and compressions in the growth plane in the proliferative zone (Gress and Jacenko, 2000). Most recently a transgenic mouse bearing a construct equivalent to a human metaphyseal chondroplasia type Schmid mutation was created. The phenotype of mice carrying this transgene paralleled the human condition and included shortened long bones and coxa vara (Ho *et al.*, 2007). Col9a1 have been the most extensively studied Type IX collagens using transgenic approaches. A mild chondrodysplasia is observed in transgenic mice expressing a truncated Col9a1 chain that predisposes these animals to a degenerative osteoarthritis (Nakata *et al.*, 1993) while a milder form of joint degeneration is observed in mice bearing Col9a1 null alleles (Fassler *et al.*, 1994; Hu *et al.*, 2006). The cartilage defect in null mice is associated with a lack of matrilin-3 in cartilage fibrils (Budde *et al.*, 2005). Furthermore, this cartilaginous failure influences bone formation in bone fracture repair as the time course of cartilaginous callus formation is impaired in type IX deficient mice (Opolka *et al.*, 2007). While genetically engineered mutant alleles of the Col11a1 gene have not been generated, a naturally occurring mutation has been observed that is associated with a frame shift mutation that results in a premature stop codon. Mice homozygous for this autosomal recessive chondrodysplasia (cho) mutation have a perinatal lethal phenotype with severe skeletal abnormalities including limbs approximately half the normal length (Li *et al.*, 1995b) whereas heterozygous animals present with an osteoarthritis-like joint phenotype (Xu *et al.*, 2003). In contrast to homozygous cho mutant mice, a mild cartilage phenotype is observed in a targeted allele that also has a premature translation termination (Li *et al.*, 2001). Type XIII is a transmembrane protein expressed in many tissues and is involved with cell adhesion. Recently it was observed that overexpression of type XIII collagen in transgenic mice surprisingly impacted on the skeleton. Although early skeletal development was unaffected, postnatally an aberrant high bone mass was detected, associated with an elevated formation rate rather than increased resorption, suggesting that type XIII collagen has a function in bone remodeling (Ylonen *et al.*, 2005). Type V collagen is another molecule that is important for type I collagen fiber formation. A targeted deletion of the N-globular region of the Col5a2 gene causes an Ehlers-Danlos type I phenotype in mice homozygous for the deleted allele. Collagen fibrillogenesis is disrupted in the dermis consistent with a regulatory role for Col5a2 in proper matrix deposition (Andrikopoulos *et al.*, 1995). The noncollagenous proteins are important in the homeostasis of connective tissue as some are likely to have a modifying role on type I and type II collagen function. However, there appears to be some redundancy in the system as knockouts of genes that are vigorously expressed in bone do not display a skeletal phenotype in the absence of gene function. Tenascin-C is strongly expressed in bone (Mackie, 1992), however, homozygous knockout mice do not have a connective tissue phenotype (Saga *et al.*, 1992; Forsberg *et al.*, 1996). Interestingly, knockout mice show neurological deficiencies including alterations in circadian rhythm control and delayed onset of odor detection (Morellini and Schachner, 2006; Cybulska-Klosowicz *et al.*, 2004). Homozygous matrilin 1, matrilin 2, or matrilin 3 knockouts develop normally without any obvious skeletal defects (Aszodi *et al.*, 1999; Mates *et al.*, 2004; Ko *et al.*, 2004), suggesting redundant function of these genetic units. However, while gross skeletal defects are not apparent, matrilin 3 knockout animals have premature chondrocyte maturation that is associated with an elevation of bone mineral density and osteoarthritis in older mice (van der Weyden *et al.*, 2006). Furthermore, both matrilin 1- and matrilin 3-deficient mice display an increase in collagen fibril diameter in the epiphysis and growth plate as well as altered ultrastructure in the knee joint cartilage (Nicolae *et al.*, 2007). Similarly, mice homozygous for a knockout of the osteocalcin gene do not present with a dramatic skeletal phenotype; however, bone remodeling is altered as osteocalcin-deficient mice have higher bone mass (Ducy *et al.*, 1996). The initial evaluation of osteonectin knockout mice did not detect any significant skeletal alterations (Gilmour *et al.*, 1998). However, subsequent analyses revealed that osteonectin-deficient mice have an osteopenia (Delany *et al.*, 2000) that is associated with decreased bone formation that is a reduction in the number of osteoblast precursors (Delany *et al.*, 2003). More recently, it has been observed that null mice display defects in intervertebral discs as mice age (Gruber *et al.*, 2005). It is obvious that as more is learned about relatively minor molecular components of skeleton, the importance of detailed analysis and the study of older animals have become apparent.

Although the functional role of the extracellular matrix contributing to the structural integrity to skeletal structures is clear, the role of extracellular matrix macromolecules in the maintenance of bone homeostasis is more clearly defined. Perhaps the best example is fibrillin 1, a large matrix glycoprotein that is a major component of extracellular microfibrils. In humans, Marfan syndrome (MFS) is an autosomal dominant connective tissue disorder that dramatically impacts the skeleton and is linked to mutations at the fibrillin 1 (FBN1) locus (Lee *et al.*, 1991; Dietz *et al.*, 1991). It was initially believed that mutant fibrillin molecules acted in a dominant negative fashion and the pathogenesis of the disease was the consequence of an alteration in microfibril structure resulting in the formation of an inferior connective tissue. Recently this paradigm has been abandoned and a new hypothesis has emerged. This new hypothesis proposes that storage of latent TGF-β involves fibrillin and that in patients with MFS there is dysregulation of TGF-β activation (Dietz *et al.*, 2005). Further data from murine models of MFS support haploinsufficiency rather than a dominant negative mechanism for the pathogenesis of MFS (Judge *et al.*, 2004). Biochemical studies have established an association between latent TGF-β binding protein(s) (LTBP) and regulation of TGF-β activity (Rifkin, 2005) and a relationship between LTBP-1 and fibrillin (Dallas *et al.*, 1995; Isogai *et al.*, 2003). Specifically, the N-terminal domain of fibrillin binds to the C-terminal end of LTBP-1, a component of the large latent complex (LLC). The LLC is involved in the regulation of TGF-β signaling as it has a role in both the sequestering and the release of active TGF-β (Kaartinen and Warburton, 2003). It has been shown that mice homozygous for a centrally deleted Fbn1 allele ($Fbn1^{mgdelta}$) who develop destructive emphysema (Pereira *et al.*, 1997) display a dysregulation of TGF-β activation and signaling in the developing lung, and treatment of these mice with TGF-β neutralizing antibody rescues the alveolar septation (Neptune *et al.*, 2003). Utilizing a targeted mutation in mouse that contained a cysteine to glycine substitution (C1039G) in the cbEGF domain of fibrillin ($Fbn1^{C1039G}$) (Judge *et al.*, 2004), Ng *et al.* documented a reversal of the development of myxomatous changes of the atrioventricular valves with the perinatal administration of TGF-β neutralizing antibody (Ng *et al.*, 2004). Finally, an extremely exciting report by Habashi *et al.* showed a correction of both aortic aneurysms and lung disease in $Fbn1^{C1039G/+}$ mutant mice with following treatment with TGF-β antagonists (Habashi *et al.*, 2006).

CIS REGULATION OF STRUCTURAL MACROMOLECULES IN BONE AND CARTILAGE

Production of major structural macromolecules constitutes the major synthetic activity of a bone or cartilage cell and can be used to define a particular stage of differentiation. Although transient and stable transfection studies can provide insight into the biochemical and molecular interaction, it is only within the intact tissue that the true biological importance of the promoter and transcriptional environment can be appreciated. This is the rationale for the extensive and expensive effort required to carry out a promoter analysis in intact mice. However, when a promoter fragment is identified that controls expression of a transgene in defined subpopulations of cells, it has great value for studies in which tissue-directed expression is required as discussed in the following sections.

The Col1a1 and Col1a2 promoters have received the greatest attention. Sequences extending a minimum of 2.3 to 3.6 kb upstream of the minimal promoter are required for high expression in most type I collagen-producing cells (Krebsbach *et al.*, 1993; Rossert *et al.*, 1995) whereas sequences as distant as 17 kb are required for strong Col1a2 activity (Antoniv *et al.*, 2001). The role of the first intron is subtle but does direct expression to a subset of type I collagen-producing cells. Specifically, a knockout of most of the intron produces a subtle reduction on type I collagen synthesis in skin and lung that was insufficient to have a physical phenotype (Hormuzdi *et al.*, 1998). However, when these transgenic mice are challenged with a stimulus for pulmonary fibrosis, an exaggerated transcriptional response from the mutant allele was observed (Hormuzdi *et al.*, 1999). Although the interpretation of this result is not fully understood, this experimental approach reveals regulatory subtleties that would not be appreciated in cell culture experiments.

A similar type of analysis has demonstrated that there are distinctly separate DNA elements that are important for high expression in different type I collagen-producing cells. The complex nature of the promoter appears to allow a single gene to be differentially regulated in different type I collagen-producing cells such as bone, tendon, and skin (Bogdanovic *et al.*, 1994). The best-defined element is the one required to regulate type I expression in bone. This "bone" element has been delineated to a 6-bp segment homeobox binding domain located 1.7 kb from the transcription start site (Dodig *et al.*, 1996; Rossert *et al.*, 1996). Mutations within this 6 bp segment inactivate the transgene in bone while transgene activity persists in tendon. The Col1a1 promoter contains another domain upstream of the bone element that is essential for high expression in skin and tendon. An element that is essential for high expression in vascular smooth muscle cells is not located within the 5' region of the gene (Bedalov *et al.*, 1994). Mapping these elements and defining the transcription factors that interact with them will be an essential step in understanding developmental pathways and signals used by cells that express the same collagen gene to varying levels in different tissues.

The osteocalcin promoter has received major attention in transient transfection studies to map transcription factor binding domains. A more complex regulatory structure becomes apparent in transgenic studies. A broader spectrum of tissue expression is seen in versions of the transgene than occurs in the endogenous gene and it has been difficult to ensure that all osteocalcin-producing cells express the osteoclacin (OC) promoter transgene (Kesterson *et al.*, 1993; Clemens *et al.*, 1997; Frenkel *et al.*, 1997). A similar complexity is seen for the bone sialoprotein promoter (Chen *et al.*, 1996; Chen *et al.*, 1999; Benson *et al.*, 2000).

The analysis of the type II collagen gene first drew attention to large fragments necessary for activity followed by identifying specific transcription factor binding domains. The importance of the first intron became fully appreciated in transgenic mice (Metsaranta *et al.,* 1995; Seghatoleslami *et al.,* 1995; Zhou *et al.,* 1995). Specific transcriptional domains that bind Sox 9 and the SRY-related high mobility group (HMG) have now been identified (Zhou *et al.,* 1998). Analysis of the Type XI promoter (Li *et al.,* 1998; Tsumaki *et al.,* 1998; Liu *et al.,* 2000) in chondrocytes and the Type X (Beier *et al.,* 1996) in hypertrophic chondrocytes has also been performed.

USE OF TISSUE-DIRECTED TRANSGENES TO ASSESS FUNCTION OF GENES AFFECTING SKELETAL DEVELOPMENT AND MAINTENANCE OF BONE MASS

Although targeted inactivation of genes by homologous recombination has contributed greatly to understanding the role of genes to embryonic development, the importance of these genes to bone and cartilage development is often obscured by the fact that other developmental systems are more severely affected and thus preclude analysis of the gene in bone. Examples include knockouts of the Wnt and notch pathways, the bHLH family of transcription factor including twist and id, and extracellular modifying factors such as noggin all have skeletal components to their embryonic phenotype indicating an essential role in skeletal biology. This section will review transgenic approaches that had been used to overcome problems associated with global knockouts. In addition, the section will review approaches for directing expression of growth factors to the bone environment.

The fundamental tool that is used in this experimental approach is a promoter that has tissue-restricted activity. This strategy creates a biological situation that rarely occurs in nature and can be considered a targeted gain of function mutation. The outcome of transgenic expression can be dramatic, but the interpretation of the biological meaning of the experiment can be complex. Uncertainties include the spectrum of cells that express the transgene. For example, the OC and bone sialoprotein (BSP) expression is specific to bones and thrombocytes, but many versions of the OC and BSP promoter have ectopic expression that includes brain. Within the lineage of bone and cartilage cells, the type I and type II collagen promoters can be designed to have preferential expression at specific stages of differentiation. However, low-level expression at other stages of differentiation or for that matter in other cell types may exist and even the low level of activity may be sufficient to disrupt cells in an unintended manner. The other uncertainty is the manner in which the expressed transgene acts, i.e., either within the cell of synthesis (cell autonomous) or on neighboring cells (cell nonautonomous). For example, OC-driven production of TGF-β will influence the entire bone cell and osteoclasts lineage (Serra *et al.,* 1997) whereas OC-driven expression on a dominant negative TGF-β receptor construct will limit its biological affect to the cells that express osteocalcin (Filvaroff *et al.,* 1999). Experimental strategies that achieve inappropriate expression of a growth or transcription factor may produce levels of the product that are never found in a normal cell and affect pathways that normally would never be utilized. This concern always arises when constructs that act in the dominant negative manner on an endogenous transcription complex or signaling pathway in which multimeric interaction exert subtle changes in gene regulation. It cannot be assumed, particularly when the dominant negative protein is highly expressed within its cell, that only the targeted partner will be affected by the interaction.

Despite these reservations, valuable insights into the role of growth and transcription factors and signaling molecules have been achieved using targeted expression techniques. For example, the global knockouts of cbfa1 dramatically demonstrated its central role for bone cell differentiation because mice deficient in this transcription factor fail to develop a mineralized skeleton. Unclear from that work was the effect of cbfa1 on the development of the hypertrophic zone of the growth plate and the role of this structure on endochondral bone formation. When cbfa1 is expressed under control of type II collagen promoter in the background of the cbfa1 knockout mouse, the hypertrophic zone does develop accompanied by vascular invasion and osteoclasts. However, osteoblasts still do not differentiate (Takeda *et al.,* 2001). This experiment is strong evidence against transdifferentiation of the hypertrophic chondrocyte to osteoblasts. In contrast, when a dominant negative cbfa1 is targeted to the chondrocyte with a different Col2a1 promoter, the cartilage remains in a proliferative stage without development and osteoblasts fail to differentiate in a pattern very similar to the global cbfa1 knockout (Ueta *et al.,* 2001). These observations suggest that the formation of the hypertrophic zone is essential for endochondral bone, but that cbfa1 expression within the osteoblast lineage is required for differentiation in the presence of an intact growth plate.

To assess the role that cbfa1 has in maintaining the osteoblastic phenotype, a dominant negative cbfa1 was expressed under control of the OC promoter. This ingenious design allows the mice to complete embryogenesis normally because OC is not expressed during prenatal life. Osteoblasts do differentiate and the dominant negative is only expressed in late osteoblast differentiation. Mice have an osteopenic phenotype with normal osteoblastic cell number and primary osteoblastic cultures derived from these mice fail to form mature bone nodules. Although this work supports the role of cbfa1 for maintenance of osteoblast lineage even in adult life, it is likely that the dominant negative can act on other members of the family of cbfa1 transcription factors, all of which can influence osteoblast differentiation (Harada *et al.,* 1999).

The role that other transcription cofactors have within the osteoblast lineage have been studied by expressing them with promoters that are more broadly expressed but that include the osteoblast lineage. The most surprising results involve the role that the fos subfamily (fra-1 and fos B) of the AP-1 helix loop-helix transcription family have in the osteoblast lineage. Even though these factors are widely expressed and their knockout either has no bone phenotype (c-fos knockout have osteopetrosis, whereas c-fos overexpressing mice get osteosarcomas and chondrosarcomas), their overexpression stimulates osteosclerotic bones by stimulating bone formation without enhancing bone resorption (Jochum *et al.,* 2000). A supporting observation is the reciprocal increase in bone mass and reduction of fat tissue in mice transgenic for FosB overexpression (Sabatakos *et al.,* 2000). Although the observations are important because this is a biological effect that has great appeal as a therapeutic agent for diminished bone mass, their interpretation of the molecular mechanism is extremely difficult. Because the factors act by forming either homodimers or heterodimers with other members of the AP-1 family and are very dosage sensitive, it is difficult to know if they work directly by partnering with another factor to stimulate an osteogenic molecular pathway or titrate out another factor that is inhibitory to an osteogenic pathway (Aubin, 2001).

Overexpression studies point to the complexities that can influence the differentiation pathway of the osteoblast. Although the role of PTHrP has received the greatest attention in the growth plate (see later discussion), it clearly has an equally important effect on the osteoblast lineage. Targeted expression of the constitutively active form of the PTHrP receptor to the osteoblast with a Col1a1 promoter results in a profound increase in trabecular and endochondral bone formation but loss in cortical bone (Calvi *et al.,* 2001). In addition, osteoclastic activity was increased in the marrow compartment. Because of its cell autonomous effect of the transgene within the osteoblast lineage, the primary effect of PTHrP in this experimental model is at the level of the osteoblast and the osteoclast response is a consequence of the activated osteoblast lineage. Although the physiological implication of the model will require further evaluation, this well-understood pathway in cartilage will have important implications for manipulation of the bone lineage.

Targeting expression of a factor secreted by osteoblast cells affects the regulation of the entire osteoblast and osteoclast lineage, making interpretation of the biological role of the factor difficult. The first example was the expression of TGFβ under control of the OC promoter producing the paradoxical outcome of high bone turnover osteoporosis with an additional component of impaired matrix mineralization. When the effect of TGFβ was limited to the osteoblasts lineage either by addition of bisphosphonates or expression of the transgene in a c-fos -/- background, enhanced differentiation of cells into mature osteoblasts was observed. When the deficiency of TGFβ was limited to mature osteoblasts by expression of a dominant negative form of the TGFβ receptor driven by OC promoter, osteoclastic activity was diminished and resulted in an increase in trabecular bone mass even though the number of mature osteoblasts was reduced (Erlebacher *et al.,* 1998; Filvaroff *et al.,* 1999). Although this experimental approach further underlines the complex interrelationship between bone formation and resorption, it does not adequately discriminate the opposing cellular regulators of bone mass. Although expression of a dominant negative or constitutively active receptor can limit the growth factor effect to a particular lineage, the subpopulation of cells within the lineage, the manner (intermittent vs. continuous) in which the stimulus is applied and age/growth rate of the mouse when the analysis is performed can all impact on the phenotype observed. The same type of experiments has now been performed with IGF1 driven either with the OC (Zhao *et al.,* 2000a) or Col1a1 promoter. Both enhance bone formation and increase bone turnover leading to a similar problem of interpretation as TGFβ forced expression. Targeted expression of noggin (producing bone loss) and the calcitonin gene-related peptide (increase bone mass) (Ballica *et al.,* 1999) are other examples of targeted secretion of a factor affecting the coupling of bone formation and resorption. Even overexpression of the TRAP enzyme results in a phenotype of high bone turnover osteoporosis (Angel *et al.,* 2000). The model utilized the SV40 enhancer and TRAP transgene and resulted in high TRAP activity in osteoclasts of bone and macrophages in non-osseous sites. Although defining the cellular control mechanisms for bone remodeling can only be fully appreciated in the integrated system of intact mouse bone, the experimental design has improved so as to limit the effect of the probing transgene to a defined subset of cells before an unambiguous interpretation of the experimental data can be made.

Because structural organization of the growth plate provides definition of the stage of differentiation of cells within the cartilage lineage, overexpression studies are more easily understood. The global knockout of PTHrP and its receptor leads to premature differentiation of hypertrophic chondrocytes whereas expression of a constitutively active form of the PTHrP receptor causes delayed maturation of hypertrophic chondrocytes. This fundamental observation has lead to a series of experiments in which targeted expression of the constitutively active PTHrP receptor in an ihh -/- or PTHrP receptor -/- background can separate the contribution of ihh and PTHrP to chondrocyte proliferation and hypertrophic differentiation (Schipani *et al.,* 1997; Karp *et al.,* 2000). A similar approach has been used to define the role of cbfa1 in the chondrocyte lineage beyond its role in supporting endochondral bone formation. Overexpression of cbfa1 via the Col2a1 promoter in an otherwise normal background is disruptive to normal joint formation and induces hypertrophic chondrocyte differentiation and vascular invasion at a site that normally never supports bone formation. Of particular interest is a disruption of pattern of tenascin expression characteristic of the chondrocytes lining an articular cartilage with the reaction of the surrounding tissues suggestive of a degenerative joint. In contrast, targeting the dominant negative form of cbfa1 to chondrocytes leads to failure of hyperchondrocyte differentiation and expression of tenascin throughout the articular cartilage (Ueta *et al.,* 2001). This work suggests more subtle levels of chondrocyte differentiation can be controlled by cbfa1 and the possibility that it might play a role in the development of degenerative joint disease (Liu *et al.,* 1995b; Ma *et al.,* 1996). TGFβ may also play a role in maintaining the articular cartilage in its appropriate state of differentiation. When the same dominant negative TGFβ receptor transgene that was used to remove TGFβ signaling to the osteoblast is targeted to the chondrocyte, a state of degenerative joint disease is observed with hypertrophic chondrocytes and synovial thickening (Serra *et al.,* 1997).

Thus, ability to assess the state of differentiation by their position or morphology adds to the interpretation of the transgenic experiments. In regards to the osteoblastic lineage, the level of differentiation is probably best appreciated in the sutures of the calvaria. Murine phenocopies of human forms of craniodystosis caused by either overexpression of the normal gene or an activated form of the transcription factor Msx2 leads to early fusion. These studies suggest that Msx2 acts to keep the cells of the osteogenic front in a proliferative and nondifferentiated state (Dodig *et al.,* 1999; Liu *et al.,* 1999). This conclusion is supported by studies of Msx2 -/- mice that fail to close their suture and demonstrate a diminished number of osteoprogenitors on the osteogenic front (Satokata *et al.,* 2000). The generalized reduction in bone mass seen in the calvaria and axial skeleton is likely to result from a similar cellular mechanism but would not have been appreciated without the analysis of the lineage within the calvarial suture. It would not be surprising that mis-expression of other molecular pathways affecting calvarial suture development such as FGF2, twist, and id are likely to act by regulating proliferation and differentiation of the osteogenic front (Rice *et al.,* 2000).

USE OF THE GFP TRANSGENE FAMILY TO ASSESS OSTEOBLAST LINEAGE

The previous section stressed the value of recognizing the level of cellular differentiation with the osteoblast and chondrocyte lineage for interpreting the effect of transgenic animal models that alter the biology of bone or cartilage by affecting the regulation of lineage progression. When position or cell morphology is not available to assist in assessing the state of differentiation with a cell population, *in situ* hybridization, immunohistochemistry, or lac Z staining for activity of stage-specific promoter-β-gal transgene can be employed (Rossert *et al.,* 1995; Antoniv *et al.,* 2001). *In situ* hybridization can localize a signal to a specific population of cells and immunohistology is best suited for extracellular molecules and both procedures are laborious and technically demanding. β galactosidase has been the traditional enzymatic marker for assessing transgene expression and has been most useful in development studies. However, its use for marking cells in adult tissue is limited because of background endogenous activity and variation of staining intensity owing to uncertainties in the diffusion of the substrate into mature tissue. The same techniques can be used to assess lineage in primary culture, but the culture has to be terminated so that progression of a specific cell to later stages of differentiation cannot be recorded.

The green fluorescent protein (GFP) family of autofluorescent proteins may provide an approach to overcome limitations of β galactosidase in both intact bone and in primary cultures derived from transgenic mice. Despite some reports that GFP can have toxic effects in transgenic mice, there are sufficient numbers of examples where viable lines have been produced and have been useful for studying cell lineage and transplantation experiments. Currently, there are fluorescent colors that can be distinguished using appropriate filter cubes. Three are isomers of GFP (topaz or yellow, sapphire, cyan) and the fourth is from another species (DS red). The most widely used form of GFP is enhanced GFP (eGFP) because its autofluorescent properties are very similar to fluorescein conjugated probes commonly used in most immunohistological studies. However, its emission spectrum spills into GFP sapphire and GFP topaz, limiting its use in multicolor experiments.

Sample preparation appears to be important for preserving GFP fluorescence in histological section. eGFP does not appear to tolerate standard paraffin embedding and instead requires frozen sections to be prepared from histological studies. However, GFP topaz, sapphire, and emerald do maintain a strong fluorescent signal in paraformaldehyde fixed, EDTA decalcified and paraffin embedded sections of bone. This allows regions of fluorescence within the histological section to be examined under optimal tissue-preserving conditions. Once the fluorescent image is recorded, standard histological staining is performed for optimal interpretation of the section. Bone has the additional problem of a high autofluorescent background in the bone marrow and to a lesser degree in the bone matrix. Use of a dual filter cube optimized for GFP and in the Texas red or rhodamine spectrum allows the green GFP signal to be separated from an orange to yellow autofluorescent color. The section can be examined by either laser confocal microscopy or standard mercury bulb illumination.

The power of the GFP transgenes in primary culture is the detection of the fluorescent signal in the same plate as it progresses from initiation of the culture to full osteoblast differentiation. In this setting, the time that the cells activate the transgene can be accurately determined and the transcription profile of that subpopulation of cells can be determined by FAC sorting for the GFP-positive cells. For example, a pOBCol3.6 promoter fragment activates GFP concomitant with the expression of alkaline phosphatase and type I collagen synthesis, which is at the stage of preosteoblast differentiation. Once bone nodules form and begin to mineralize, the pOBCol2.3 promoter fragment becomes active along with osteocalcin expression marking the stage of mature osteoblast/osteocyte differentiation. The two promoter GFP constructs have a different pattern of cellular fluorescence in sections of bone. The pOBCol3.6GFP transgenic mice show expression in the periosteal fibroblasts and lining osteoblast but with little expression in osteocytes. The pOBCol2.3GFP is not expressed in the periosteum but is found on lining osteoblasts and throughout the bone matrix. We envision building a series of promoter-GFP transgenic mice in which different stages of cellular differentiation are marked by a different color of GFP. These mice will provide the ability to recognize stages of differentiation within a complex tissue much like the cell surface markers that are used to identify precursor cells within the hematopoietic lineage. If these reagents become widely disseminated, they can provide a way to standardize the analysis of factors affecting bone biology at the level of lineage control whether in intact animals or primary culture derived from the mice.

The availability of GFP transgenes making stages of cell differentiation will have a wide variety of uses. In primary cell culture, the activation of GFP transgenes can be used to assess the tempo of differentiation and the proportion of clusters of preosteoblasts that form nodules with full bone cell differentiation. Transgenes marking differentiation of adipocytes, smooth muscle cells, and chondrocytes (Grant *et al.,* 2000) as well as vascular and endothelial cells (Kishimoto *et al.,* 2000) are available and need to be modified to a GFP that will function in paraffin-embedded tissues. These markers will allow the investigator to appreciate the complexity of primary marrow stromal cultures and factors that can modulate the culture to various lineages. Possibly its most useful application will be in microarray analysis of the osteoblast lineage. Interpretation of a complex array pattern is extremely difficult in a heterogeneous cell population. Is the change in an expression pattern a consequence of the change that occurred in a subpopulation that only represents 25% of the entire cell population? Are important patterns lost because of an increase in one subpopulation and a fall in another? The GFP also allows the isolation of a specific cell population from a heterogeneous population. The cells can be analyzed by Fluorescent-activated cell sorting (FACS) and even collected by cell sorting for biochemical or cellular analysis. This isolation may be in an essential requirement for interpreting microarray experiments.

In intact bone, direct visualizing the cells that express the transgene, which in turn reflect a level of attained differentiation during development, in a knockout model or in response to a drug intervention, will assist in the interpretation of experimental data. Building mosaic mice in which the identity of donor cells are distinguished by transgene expression can reveal cell-cell interactions that could not be appreciated in any other way. For example, mosaic mice created with β-Gal marked chondrocytes obtained from the PTHrP receptor -/-mouse produce islands of β-gal positive hypertrophic chondrocytes within an elongated zone of proliferating and β-gal negative chondrocytes. This outcome affirmed the model molecular pathway that coordinates lineage maturation within the growth plate. Loss of the PTHrP pathway induced early differentiation and these hypertrophic cells secrete ihh, which stimulates the surrounding PTHrP intact cartilage, and color osteoblastic cells into a proliferative and nondifferentiating state (Chung *et al.,* 2001). A third application is in transplantation studies of marrow stromal cells for somatic gene therapy for diseases of bone. Most of these studies to date have used nonspecific markers genes, which did not reflect differentiation into the bone lineage. Engraftment of cells expressing the OC CAT transgene has been demonstrated by *in situ* hybridization providing the most convincing data to date that osteoblastic precursor cells can engraft bone and participate in endogenous bone formation (Hou *et al.,* 1999). In the future, GFP will be a more robust and versatile marker for quantitative studies of cellular engraftment and demonstration of transplanted stem cells by direct marrow stromal cell culture of the transplanted mice.

TRANSGENES AS TOOLS TO ABLATE A SPECIFIC POPULATION OF CELLS AFFECTING BONE BIOLOGY

Defining progenitor cells that differentiated into specialized cell types is important to understanding the pathogenesis of disease as well as for the development of new therapies that incorporate tissue regenerative approaches. The skeleton is no exception to this area of biomedical research. The development of transgenic technology has permitted the production of experimental systems that remove or destroy specific types of cells. The approach is to develop a transgene that produces a peptide that is lethal or results in a lethal condition with the addition of a specific compound or reagent. The A chains of the diphtheria toxin have been exploited to created paradigms that result in direct cellular lethality (Breitman *et al.,* 1990) while conditional lethality is accomplished with the herpes thymidine kinase gene (Borrelli *et al.,* 1989; Zhang *et al.,* 2005). Although the viral thymidine kinase (tk) gene when expressed in mammalian cells shows no deleterious affects, the addition of nucleoside analogs that are only phosphorylated by the viral tk results in lethality of cells expressing the viral enzyme. However, this conditional lethality has limitations as it only impacts dividing cells that incorporated the analogs into DNA. In addition, male sterility is also a limitation; however, this has been minimized with the development of a truncated version of the gene that maintains catalytic activity but does not cause male sterility (Salomon *et al.,* 1995). Corral *et al.* (1998) employed the unmodified form of viral tk to examine the association of bone form with resorption. A transgenic line was developed using the mouse osteocalcin gene 2 (OG-2) promoter to drive viral tk expression. Treatment of transgenic mice with ganciclovir resulted in the development of an osteoporotic phenotype that recovered after the cessation of drug treatment. The osteopenia was associated with a destruction of osteoblasts; however, osteoclasts were still present. These observations were interpreted as documenting an uncoupling of bone formation and bone resorption. A second model employed a Col1a1 promoter to control the expression of the truncated form of the tk gene. This transgene contained a 2.3 kilobase fragment of the rat Col1a1 promoter that restricted transgene expression to early osteoblasts (Visnjic *et al.,* 2001). After a 2-week treatment with ganciclovir, there was a complete loss of osteoblasts; however, osteocytes were unaffected. Further osteoclasts were absent and there was substantial destruction of the hematopoietic compartment. A more detailed evaluation of hematopoietic parameters showed that in treated transgenic mice there was a loss of lymphoid, erythroid, and myeloid progenitors in the bone marrow. This bone marrow loss was complemented by an elevation of extramedullary hematopoiesis in the spleen and liver (Visnjic *et al.,* 2004). A more recent study by Boban *et al.* (2006) employing conditional ablation in a parabiotic model reported a rescue of the bone marrow destruction consistent with the circulation of hematopoietic stem cells. The significant differences observed between the osteocalcin promoter-driven transgenic model and the Col1a1 system is most likely the result of differences in the temporal expression of the transgenes in the osteoblast lineage. The osteocalcin-driven expression occurs in late lineage osteoblasts and osteocytes. These cells are not involved with the synthesis of new osteoid and thus do not have a significant impact of the activity of osteoclasts. Furthermore, early-stage osteoblasts are actively making osteoid and undergoing some cell division and are more susceptible to ganciclovir toxicity.

PROBING SKELETAL BIOLOGY WITH STRATEGIES THAT DISRUPT THE ENDOGENOUS GENES

The completion of the sequencing of the mouse genome has changed the landscape of mammalian genetics. The availability of gene sequence data has refocused research initiatives away from understanding genome structure and has directed efforts toward understanding gene function. The availability of gene targeting technologies has lead to the development of new experimental paradigms utilizing genetically engineered strains of mice with alterations that disrupt or modify the function of an endogenous gene. Tools that have been developed have made the creation of gene targeting vectors easier and more rapid (Copeland, *et al.,* 2001; Chan *et al.,* 2007). Mouse models have been developed that permit both the spatial and temporal modulation of a specific gene's function. The Cre/loxP recombinase system is the centerpiece of the new experiment models. Cre (causes recombination), an enzyme from P1 bacteriophage, directs recombination at loxP (locus of X-ing over) recombination recognition sites. A segment of DNA flanked by two directly orientated loxP sites is excised from the genetic material, resulting in a deletion of this genetic information. The application of the Cre/loxP recombinase system generally involves two genetically engineered strains of mice. One strain is a transgenic line expressing Cre recombinase under the control of a tissue-specific promoter. The other transgenic lines typically bear a targeted allele in the locus of interest. This targeted allele is generated by homologous recombination in ES cells and contains loxP sites that flank (floxed) a region of the gene in such a way that upon Cre-mediated recombination the function of the targeted allele is altered, typically gene activity is lost. The first knocking model of osteogenesis imperfecta was developed using this system (Forlino *et al.,* 1999). This experimental paradigm bearing a floxed gene, referred to as the brittle IV mouse, is a model for osteogenesis imperfecta IV that has been used to study whole bone architecture in various age groups (Kozloff *et al.,* 2004). Several Cre-expressing lines have been developed that directed Cre expression to specific skeletal cell types. Chondrocyte and hypertrophic chondrocyte specific Cre transgenic mice have been developed using the Col2a1 and Col10a1 promoter, respectively (Ovchinnikov *et al.,* 2000; Sakai *et al.,* 2001; Hao *et al.,* 2002; Yang *et al.,* 2005). An alpha2 type XI Cre transgene also is expressed in cartilage (Fujimaki *et al.,* 2005). The type I collagen promoter has been utilized to drive Cre expression in cells of the osteoblasts (Dacquin *et al.,* 2002; Cochrane *et al.,* 2007) and the dentin matrix protein gene has been used to express Cre in odontoblasts and osteocytes (Lu *et al.,* 2007). Transgenic lines bearing TRAP (tartrate-resistant acid phosphatase)-Cre and Ctsk (cathepsin K)–Cre permit rearrangement of floxed genes in osteoclasts (Chiu *et al.,* 2004). In the utilization of various Cre-expressing lines, it is important to confirm the expression of the Cre transgenes as unanticipated results have been observed. Cochrane *et al.* (2007) observed germline loxP rearrangement of a loxP-flanked Ifg1 allele by both Col3.6Cre and Col2.3Cre transgenes. Further rearrangement was seen without the inheritance of the Cre-bearing transgene. As a tool to assist in the evaluation of the Cre transgene expression several reporters transgenic lines have been developed. These lines bear transgenes consisting of a ubiquitous promoter fused to a visible reporter (i.e., GFP or lacZ) flanked by loxP sites in such a fashion as to prevent expression without Cre-mediated loxP excision (Mao *et al.,* 2001; Soriano, 1999). By crossing a Cre-expressing line with a ubiquitous Cre activity reporter bearing line an evaluation of the reporter's colorimetric expression (GFP or lacZ) in tissues of progeny bearing the Cre-expressing transgene and the reporter will be informative as to the tissue specificity of Cre expression as well as the efficiency of Cre-driven loxP site rearrangement. Finally, information of extant Cre lines, numerous floxed genes, lines under development as well as up-to-date information on Cre reporter systems can be obtained from a website maintained by Andras Nagy (http://www.mshri.on.ca/nagy/). The tissue-specific regulation of Cre recombinase expression is an effective tool to examine genetic function of a defined locus, experimental paradigms have been developed that permit both spatial and temporal control of Cre function. One experimental system involves combining the Cre recombinase with a modified version of the human estrogen receptor. This fusion protein, referred to as Cre-ERT, lacks recombinase activity without the addition of the receptor's ligand. The receptor component of the chimeric protein has been modified so that the receptor does not respond to the natural ligand, estradiol; however, the recombinase function is activated by tamoxifen, an estradiol analog (Brocard *et al.,* 1997). A second-generation, more sensitive version (Cre-ERT2) of this system has been developed. The modified Cre-ERT2 is 10 times more sensitive to tamoxifen than the original Cre-ERT (Indra *et al.,* 1999). Lines of transgenic mice bearing inducible Cre recombinase have been developed that control the expression of Cre in specific bone cell types. Specifically, a Cola1CreERT2 transgene that utilizes a 2.3 kb segment of the mouse Col1a1 promoter is expressed in skeletal osteoblasts as well as odontoblasts in teeth (Kim *et al.,* 2004). The type II collagen gene has been employed to regulate an inducible Cre in chondrocytes (Nakamura *et al.,* 2006; Chen *et al.,* 2007; Hilton *et al.,* 2007). The inducible Cre system permitting both temporal and spatial deletion of a specific gene's function also allows an investigator to study the role of a particular genetic unit in the development or homeostasis of a particular tissue or cell type that might not be possible with a non-inducible system because of embryonic lethality.

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Chapter 87

Biochemical Markers of Bone Metabolism

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INTRODUCTION

Biochemical markers of bone metabolism have been used in clinical practice for decades. Over the years, major advancements involved the development of assays to measure markers that more precisely reflect the process of bone resorption or formation, as well as sensitive, high-throughput techniques. This has made the measurement of bone markers a widely used practice in the clinical management of metabolic bone disease. In addition, their application in clinical studies and trials has provided a sound knowledge base in regard to the relationship between markers of bone turnover and specific clinical outcomes such as bone mass or fractures. In the clinical development phase of new drugs for metabolic bone disease, these established correlations are particularly important, as most markers respond rapidly to pharmacological interventions and can therefore be considered in the evaluation of drug effects.

Although bone turnover markers can be valuable in the management of patients with metabolic bone disease and in drug development, their application has caveats and limitations related both to the practical aspects of assays and sample handling and to the interpretation of data.

This chapter discusses currently available biochemical markers of bone turnover and related aspects that are relevant to correct utilization of these laboratory tests. Several examples follow to illustrate their application in clinical medicine and clinical drug development.

BIOCHEMICAL MARKERS OF BONE METABOLISM

Bone Turnover

The skeleton is continually renewed by a process called bone turnover, involving osteoclast-mediated bone resorption, osteoblast-mediated bone formation, and remineralization of newly formed bone. Bone turnover, which takes place in bone multicellular units, is usually initiated by osteoclasts eroding a mineralized surface (see Chapter 8). When osteoclasts resorb bone, they secrete a mixture of acid and neutral proteases that act sequentially to degrade the collagen fibrils into fragments. Biochemical markers of bone resorption therefore include these collagen breakdown products such as hydroxyproline, hydroxylysine glycosides, and the pyridinoline cross-links. These cross-links range in size from free amino acids to larger segments of the N-telopeptide and C-telopeptide domains. Other markers of bone resorption are enzymes secreted by the osteoclast involved in the degradation of collagen type I, such as tartrate-resistant acid phosphatase and cathepsin K, and a noncollagenous protein of the bone matrix, bone sialoprotein.

The initial step of bone resorption is followed by the recruitment of osteoblasts to the outer edge of the erosion cavity. The osteoblasts secrete new bone matrix (osteoid) that gradually fills in the resorption cavity. Biochemical markers of bone formation are products of this osteoblastic activity. These osteoblast products include the amino (N) and carboxy (C)-terminal propeptides of type I collagen, osteocalcin, and bone-specific alkaline phosphatase. Figure 1 is a schematic representation of the currently known biochemical markers of bone formation and resorption.

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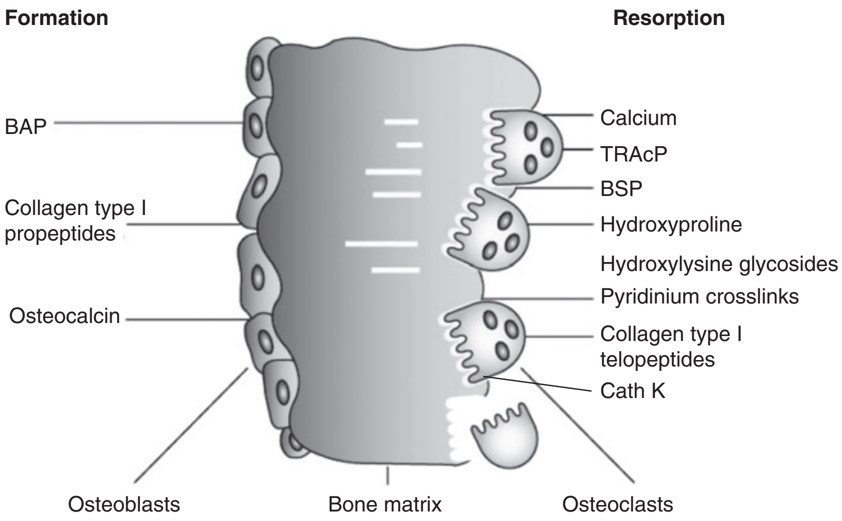

FIGURE 1 Biochemical markers of bone turnover (adapted from Seibel, 2005).

In addition to assigning bone turnover markers specifically to the process of bone resorption or bone formation, certain markers appear to reflect different stages of these cellular activities. On the other hand, bone turnover markers cannot distinguish between diseases nor do they reflect disease-specific processes. The markers are also unable to distinguish between activities occurring at cortical or trabecular bone elements of bone.

Variability

The level of any bone turnover marker in body fluids such as serum or urine depends only in part on the production rate of the marker, which can vary substantially between diseases and between individuals. In addition, concentrations of bone markers in serum and urine are influenced by factors unrelated to bone turnover, such as the rate of their elimination or clearance as well as their extraskeletal metabolism. Such factors lead to substantial intra- and interpatient variability in the measurement of markers. Finally, factors related to the analyses themselves can contribute substantially to the variability of these parameters, such as sample storage and technical assay considerations. Thus, storage-associated enzymatic, photo- and/or thermo-degradation may occur. Sample handling in which hemolysis occurs can also be a confounding issue.

Analytical Variability

At present, most markers of bone turnover are measured by either immunoassays such as enzyme-linked immunosorbant assay (ELISA), electrochemiluminescence immunoassay (ECLIA), and radioimmunoassay (RIA) or by chromatographic assays such as high-performance liquid chromatography (HPLC) or liquid chromatography/ mass spectrometry (LC/MS) (Seibel, 2005). Most immunoassays are now available on automated platforms. At relevant concentrations, the intra- and interassay variability of most assays with automated platform technology is less than 10%, a considerable improvement over previous assay methods. The contribution of analytical imprecision to overall variability in the level of the markers is relatively small, but substantial differences can exist among different methods for the same marker, which may be relevant for relating results obtained with one method to data collected with another. Therefore, new assays always need to be cross-validated, and potential discrepancies between results from one assay and results of another should always be taken into consideration (Seibel, 2001).

Biological Variability

The level of each bone turnover marker is a result of production, distribution, and elimination of the marker, all of which can be described by the kinetic parameters production rate, clearance, volume of distribution, and half-life. These parameters and thus the level of the markers and their variability depend on factors such as growth, age, gender and body weight, ethnicity and geography, pregnancy and lactation, diet, exercise and mobility, menstrual and seasonal cycles, previous fracture, the metabolic bone disease, and hepatic and renal (dys-)function (Hannon, 2000). Moreover, all bone turnover markers exhibit a circadian rhythm (see Fig. 2) (Ju, 1997). The extent of circadian variability is a function of both the variation in the production rate and the half-life of the marker. Thus, a marker with a relatively short half-life may show more circadian variability than a marker with a longer half-life. Another factor known to influence levels of some markers of bone

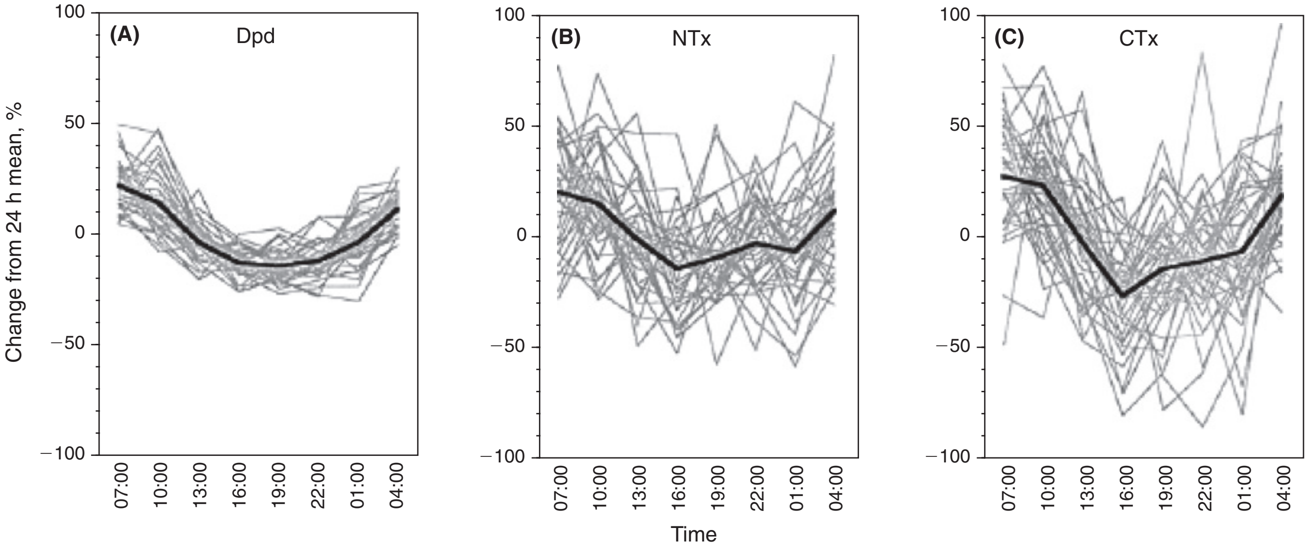

FIGURE 2 Diurnal variation in excretion of creatinine-corrected Dpd (**A**), NTx (**B**), and CTx (**C**) in 17 women and 21 men. The heavy line represents the mean; thin lines represent individuals.

metabolism is food intake (Hannon, 2000), which appears to be mediated in part by glucagon-like peptide-2 (GLP-2) (Henriksen, 2003).

A number of recent studies address the influence of factors such as renal dysfunction on the concentration range of the markers (Tsuchida, 2005), whereas other studies go even further trying to quantify the effect of potentially perturbing factors such as renal function on the kinetic parameters of the markers (Holford, 2006). Although at present no adequate guidelines exist, potentially perturbing factors should be taken into consideration when interpreting the level of a marker in a population or an individual patient (Hannon, 2000; Seibel, 2005).

In an individual patient, some of these factors can be accounted for and are therefore controllable. Examples of such factors are circadian rhythms, fasting status, menstrual status, diet, and exercise. Standardizing the timing and conditions of sample collection, for example, by always obtaining second morning void urine samples, and by asking patients to refrain from exercise 24 hours before a sample is collected are ways to control for these variabilities (Hannon, 2000). Uncontrollable factors contributing to variability are age, gender, ethnicity and geographic location, previous fractures, season, pregnancy and lactation, drugs, immobility, and comorbidities.

Bone Turnover Markers during Drug Treatment

Monitoring of treatment is one of the most important clinical applications of bone markers. It is also complicated because, in this case, not just the aforementioned factors causing variability should be taken into consideration when interpreting levels of a marker, but also the pharmacokinetics of the drug and its mechanism of action and potency are important. Finally, bone resorption and bone formation strongly differ in their kinetics. It takes osteoclasts only a few days to resorb bone, whereas the formation of bone by osteoblasts takes months.

The relevance of some of these factors is illustrated by the difference in response of bone resorption and formation markers to anabolic and antiresorptive therapy, respectively. For example, rhPTH, when given intermittently (single daily injection) is anabolic, stimulating osteoblast function. This is reflected by an increase in bone formation markers such as PINP once treatment has commenced, and a slower and later increase in bone resorption markers such as S-CTX (Fig. 3); (Bauer, 2006). This pattern is in line with anabolic mechanisms of rhPTH actions as well as with the coupling between bone formation and resorption. In contrast, potent antiresorptive agents such as the bisphosphonates induce a rapid initial decrease in the level of bone resorption markers, which is usually followed by a slower and later decrease in the levels of bone formation markers (Fig. 4); (Reid, 2002). The different patterns of change in bone markers observed between the anabolic and antiresorptive agents can be explained, at least in part, by the difference in mechanism of action as well as pharmacokinetic properties of these drug classes.

MARKERS OF BONE FORMATION

Alkaline Phosphatase

The enzyme alkaline phosphatase (ALP) belongs to a large group of proteins attached to the extracellular surface of cell

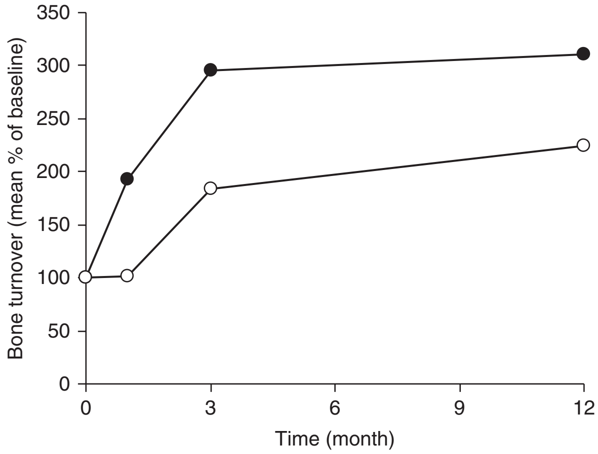

FIGURE 3 Mean levels of PINP (*filled circle*), and S-CTX (*open circle*) as a percentage of baseline in 119 osteoporotic women treated with intermittent s.c. PTH(1–84) 100 μg/day for 1 year (adapted from Bauer, 2006).

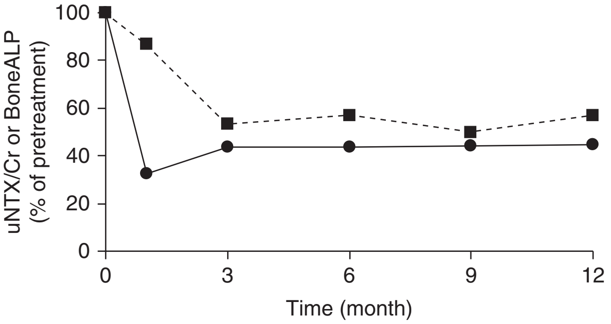

FIGURE 4 Mean levels of U-NTX/Cr (circles) and Bone ALP (squares) relative to pretreatment values after a single infusion of 4 mg of zoledronic acid in 53 postmenopausal women with low bone mineral density (adapted from Reid, 2002).

membranes via a carboxy-terminal glycan-phosphatidylinositol anchor (Low and Saltiel, 1988). Four gene loci code for ALP: three tissue-specific genes encode the intestine, mature placenta, and germ-cell enzymes; the tissue-nonspecific gene for ALP is expressed in numerous tissues (including bone, liver, kidney, and early placenta). Tissue nonspecific ALPs are the products of a single gene, but tissue-specific differences are found in their electrophoretic mobility, stability to heat, and sensitivity to a variety of chemical inhibitors. These differences are caused by variations in their carbohydrate side chains (Weiss, 1988). Because the two most common organ sources of elevated ALP levels are liver and bone, a number of techniques have been developed to distinguish between bone and liver isoforms. They rely on the differences in the carbohydrate side chains. Early methods included heat denaturation, chemical inhibition of selective activity, gel electrophoresis, and precipitation by wheat germ lectin (Calvo, 1966). Now, commercially available bone-specific immunoassays utilize monoclonal antibodies with preference for the bone isoform. They are the most commonly used method today.

A two-site immunoassay (Tandem-R-Ostase, Hybritech Inc, San Diego, CA) relies on the use of two monoclonal antibodies, both of which react preferentially with the bone isoform. This assay measures mass of the enzyme (Garnero and Delmas, 1993). Alkphase-B (METRA Biosystems, Mountain View, CA) uses a single monoclonal antibody and measures activity of the enzyme (Gomes, 1995). Comparisons of mass and activity-based immunoassays show that two methods generally provide similar clinical information. However, demonstrated variations in glycosylation patterns raise the question of immunological heterogeneity in the bone isoform which may affect the mass-to-activity ratio (Kress, 1998). Bone ALP is stable for 5 days at 2 to 8° C, for 12 months at −40° C, and for 36 months at −80° C (Metra BAP kit, package insert).

There is a great deal of interindividual variation in ALP levels, but for any one individual, values are stable over time. Bone ALP has a half-life of about 40 hours and like other glycoproteins is cleared by the liver (Crofton, 1982). Levels of bone ALP were higher in chronic kidney disease patients compared with age-matched controls and were inversely correlated with creatinine clearance (Tsuchida, 2005). Biological within-day variation of total ALP is estimated to be less than 4%. There is some evidence of a small diurnal variation in ALP activity that may be caused by the bone component (Nielsen, 1990). Like other bone formation markers, bone ALP is elevated during the luteal phase of the menstrual cycle, albeit only slightly (Nielsen, 1990). Seasonal variations have been described (Devgun, 1981; Douglas, 1996). Bone ALP levels are affected by age, gender, and hormonal status (Calvo, 1996), and levels have been reported to change in pediatrics with dramatic increases during puberty (Blumsohn, 1994; Crofto, 1992). In the third trimester of pregnancy bone ALP levels are significantly elevated (Naylor, 2000; Cross, 1995; Black, 2000). Fractures cause an increase that can last for at least one year (Bowles, 1996, 1997; Woitge, 1996).

Osteocalcin

Osteocalcin is a small protein synthesized by mature osteoblasts, odontoblasts, and hypertrophic chondrocytes. It has three residues of the calcium-binding amino acid, γ-carboxyglutamic acid (Gla). Osteocalcin is thought to interact directly with hydroxyapatite in bone through its Gla residue (Hauschka, 1989). Vitamin K is required for the post-translational gamma-carboxylation of osteocalcin (Lee, 2000).

Osteocalcin is primarily deposited in the extracellular matrix of bone, but a small amount enters the blood. Serum osteocalcin is a sensitive and specific marker of osteoblastic activity and its serum level thus reflects the rate of bone formation (Brown, 1984; Weaver, 1997; Charles, 1992).

There is some evidence that serum osteocalcin may also be derived from activities associated with bone resorption. There are several commercial kits for measuring human osteocalcin in serum, although obtained results do not correlate well between the various assays and wide variations are reported in control and patient populations (Gundberg, 1998; Gundberg and Nishimoto, 1999). Various fragments of osteocalcin can circulate (Gundberg and Weinstein, 1986; Garnero, 1994a; Taylor, 1990), and the observed assay variability is thought to be the result of differences in the ability of the various antibodies to bind these fragments. Several laboratories have developed two-site immunoassays with the intention of measuring only the intact molecule. However, rapid loss of immunoreactivity is observed with these assays when samples are left at room temperature for a few hours (Colford, 1999; Garnero, 1992; Monaghan, 1993; Parvianen, 1994).

A new generation of osteocalcin assays is based on studies which suggest that the intact molecule is degraded to a large amino(N) to midmolecule fragment in the range of residues 1–43 (OC1-43). The large N to midmolecule fragment is thought to be generated by proteolysis in the circulation or during sample processing and storage, but there is some evidence that the osteoblast is an additional source of the fragment (Gundberg, 2000). In one study, the intact molecule represents only about one-third of the circulating osteocalcin immunoreactivity. One third is composed of the N to midmolecule fragment, and another third by several other smaller fragments (Garnero, 1994a). Osteocalcin levels fall with incubation at room temperature when measured by conventional radioimmunoassays (RIAs) or by intact assays, but values are stable with an assay that recognizes both the intact and large N to midmolecule fragment. With this assay apparent instability of osteocalcin in the circulation and during sample handling is eliminated and the correlation with bone density is improved (Minisola, 1997; Dumon, 1996; Rosenquist, 1995). Immunoreactive fragments of osteocalcin have also been reported in the urine (Taylor, 1990), and recently, assays were also reported for osteocalcin fragments in urine (Ivaska, 2005). Urinary osteocalcin holds promises as a marker of bone metabolism, which is illustrated by its ability to monitor antiresorptive bisphosphonate treatment (Ivaska, 2005, 2005b).

The main route of circulating osteocalcin catabolism is renal glomerular filtration and degradation. The plasma half-life is about 20 minutes in humans. Levels of intact osteocalcin are higher in patients with chronic kidney disease compared with age-matched controls. The levels are inversely correlated with creatinine clearance (Tsuchida, 2005). Osteocalcin levels follow a circadian rhythm characterized by a decline during the morning to a low around noon, followed by a gradual rise that peaks after midnight (Gundberg, 1985). The difference between the peak and nadir in a 24-hour period can range from 10% to 20% depending on the assay used. Higher levels in the winter and spring have been reported as compared with summer and fall (Woitge, 1998. Douglas, 1996.Thomsen, 1989), and there is a significant increase in osteocalcin levels during the luteal phase of the menstrual cycle (Nielsen, 1990). Osteocalcin levels in children are higher than in adults and correlate with growth velocity, reaching a peak at puberty. Levels are higher in men than women. An age-related decline but a transient increase after the fifth decade in women have been described (Vanderschueren, 1990). Serum osteocalcin is increased after a fracture with levels remaining elevated for at least 3 months (Obrant, 1990). Although bone formation is normally increased during the third trimester of pregnancy, osteocalcin levels are not, a point that may be caused by placental degradation (Rodin, 1989).

Procollagen Peptides

All collagens contain triple-helical molecular domains. The newly translated polypeptide, a pre-pro-α chain, includes a signal sequence and amino (N)- and carboxyl (C)-terminal propeptide extensions. During collagen synthesis, the C propeptides guide the selection and association of the individual pro-αchains and prevent premature intracellular fiber formation. Specific extracellular endoproteinases cleave the procollagen molecule at precise sites in each chain. The C-terminal endoproteinase is identical to bone morphogenic protein (BMP-1) (Kessler, 1996). The C propeptide (PICP), which is a trimeric globular glycoprotein, is stabilized by disulfide bonds and circulates as a single molecule (Olsen, 1977). It has a serum half-life of 6 to 8 minutes and is cleared by hepatic endothelial cells by the mannose 6-phosphate receptor (Smedsrod, 1990). The procollagen type I amino-terminal propeptide (PINP) is a partly globular, partly helical (collagenous) 35-kDa protein. PINP circulates mainly as the intact trimeric molecule but monomers are also found (Brandt, 1999). The intact molecule is cleared from the circulation by the scavenger receptor of liver endothelial cells (Kivirikko and Myllyla, 1980). There have been suggestions that a low-molecular-weight, degraded form of PINP monomer also circulates (Melkko, 1996). PINP has a half life of 1 minute as determined in the rat model. Levels of PINP are higher in chronic kidney disease patients than age-matched controls and inversely, but not significantly, correlate with creatinine clearance (Tsuchida, 2005).

The procollagen extension peptides are cleaved from the newly formed molecule in a stoichiometric relationship with collagen biosynthesis. Therefore, they should reflect bone formation in a manner analogous to the assessment of C peptide for endogenous insulin production. However, because type I collagen is also a component of several soft tissues (fibrocartilage, tendon, skin, gingiva, intestine, heart valve, large vessels, and muscle) there is a potential contribution to circulating procollagens from soft tissue synthesis of type I collagen, as is the case for almost all collagen type I-related markers, including NTX and CTX. Nevertheless, because the rate of turnover of collagen in bone is faster than in other tissues, changes in procollagen concentrations are assumed to reflect changes primarily in bone collagen synthesis. Indeed, good correlations have been shown between PICP serum levels and the rate of bone formation (Eriksen, 1993; Hassager, 1991). Similar to other markers, PICP and PINP demonstrate a circadian rhythm with peak values occurring in the early morning hours and nadirs in the afternoon (Hassager, 1992; Saggese, 1994).

There are several assays available for the measurement of PICP and PINP, including RIA and ELISA methods. Recently, a PINP assay has become available on an automated platform (Roche Diagnostics). Some assays measure only the high-molecular-weight (trimeric) forms, whereas others also measure the low-molecular-weight (monomeric) forms (Orum, 1996; Jensen, 1998, Brandt, 1998). Serum samples are stable at 2 to 8° C for up to 5 days and for a longer period at −20° C. Repeated freeze-thaw cycles should be avoided.

Measurement of intact PINP appears to be more sensitive than total PINP and may provide more specific clinical information (Brandt, 1999; Orum, 1996; Chandani, 2000; Dominguez, 1998).

Seasonal variation in PICP levels appears to be small (Woitge, 1998; Blumsohn, 2003). PICP and PINP levels are higher in children than in adults with an increase during puberty (Blumsohn, 1994). Fractures increase levels of PINP significantly within 1 to 4 weeks, which remain elevated during at least 1 year (Ingle, 1999; Ingle, 1999b).

Markers of Bone Resorption

Hydroxyproline

Hydroxyproline (OHP) is present in essentially all tissues and all genetic types of collagen. Hydroxyproline is derived from the breakdown of collagen. The majority of the breakdown products are reabsorbed by the renal tubules and broken down in the liver, whereas only about 10% is excreted in the urine. Most are contained in di- and tripeptides (Prockop and Kivirikko, 1967; Smith, 1980). The remaining peptides in the urine are of approximately 5 kDa. There is a small amount of the free amino acid in urine. Hydroxyproline can never be reincorporated into newly synthesized collagen (Prockop, 1964), but both collagen synthesis and tissue breakdown contribute to urinary hydroxyproline. Colorimetric methods or high-performance liquid chromatography (HPLC) are commonly used to measure urinary hydroxyproline.

Urinary OHP is historically important as a marker of bone resorption, and is still a good marker when applied correctly. As a matter of fact, studies using calcium kinetics show a better correlation between bone resorption and OHP than some supposedly more specific markers. Nevertheless OHP has now largely been replaced by other bone resorption markers, mainly because of its lack of specificity for bone, including a large contribution from the C1q fraction of the complement, the need to restrict intake of gelatin and other collagen-rich foods to avoid the contributions of exogenous OHP to urinary measurements, and the contributions of OHP from the degradation of newly synthesized collagen (Calvo, 1996).

Collagen Cross-Links

Pyridinoline and Deoxypyridinoline

Newly deposited collagen fibrils in the extracellular matrix are stabilized by intramolecular and intermolecular cross-links. The main cross-links in skeletal tissues are the trivalent structures, deoxypyridinoline (DPD) and pyridinoline (PYD). The pyridinoline cross-links occur at two sites placed symmetrically at about 90 residues from the ends of the 1000-residue helical domain of the collagen fibril. Pyridinolines act as mature cross-links in fibrillar collagens of all major connective tissues other than skin (Eyre, 1984, 1988). This includes type I collagen, which is present in bone, dentin, ligaments, fascia, tendon, vascular walls, muscle, and intestine (but absent from skin). In all tissues PYD predominates, with DPD being the minor component. However, DPD is most abundant in bone and dentin and is considered the more specific bone marker because bone represents the major reservoir of total collagen in the body and turns over faster than most other connective tissues (Eyre, 1984; Seibel, 1992; Robins, 2006). This conclusion is supported by the strong correlations between DPD and bone resorption rates as determined by radio-tracer kinetics (Eastell, 1997).

Pyridinoline cross-links are released during collagen breakdown and are cleared by the kidneys. In serum and urine, PYD and DPD are present as both free amino acid derivates (about 40%) and as oligopeptide-bound fractions (about 60%) with the rate of bone turnover potentially influencing the unbound (free) fraction (Robins, 1991; Delmas, 1993; Eastell, 2003; Randall, 1996). The free form can be measured directly, whereas the conjugated form has to be hydrolyzed before assay, in which case the total amount of PYD and DPD is determined (Black, 1988; Colwell, 1993). Such acid hydrolysis is followed by solid-phase extraction, separation by HPLC, and quantitation by fluorescence (Eyre, 1984; Black, 1988, 1989; Seibel, 1989; Colwell, 1993, 1996). Good correlations between free and total cross-links are found in normal subjects and in patients with disorders associated with increased bone resorption (Robins, 1990l Abbiati, 1993) allowing direct analysis of urine samples without the need for the hydrolysis step. Specific immunoassays have been developed for DPD (Robins, 1994) and for both pyridinium cross-links (Gomez, 1996).

The cross-link markers provide distinct advantages over urinary OHP. First, they are not influenced by dietary collagen intake (Colwell, 1993). Second, they are formed only at the final stages of fibril formation and are therefore unaffected by degradation of newly synthesized collagen (Robins, 1983; Eyre, 1980). They are not reused in collagen biosynthesis, but there is evidence that some free PYD and DPD excreted into urine is produced by the kidney (Colwell, 1996), which may depend on the rate of bone turnover (Eastell, 2003). DPD excretion seems relatively unaffected by changes in renal function (McLaren, 1993), yet significant relationships have been described between creatinine clearance and serum PYD and DPD levels (Tsuchida, 2005).

Several direct immunoassays for the cross-links and a number of ELISA systems have been reported (Seyedin, 1993; Robins, 1994; Gomez, 1996). Commercial kits are available, which measure both DPD and PYD in combination (Metra PYD, METRA Biosystems) or DPD alone (Metra DPD, METRA Biosystems). Direct comparison of the immunoassays with HPLC shows a high correlation between the methods. Commercial kits are also available to measure free PYD in serum (Metra Serum PYD, METRA Biosystems), and HPLC methods for total PYD and DPD in serum have also been described (Tsuchida, 2005).

Free and conjugated forms of Figure PYD and DPD are stable in urine samples kept at room temperature for several weeks. They can be stored at −20° C and repeated freeze-thaw cycles have no effect on their concentration in urine samples (Colwell, 1996b). DPD in urine shows diurnal variation (Ju, 1997; see 2), as well as seasonal variability (Woitge, 1997). Pyridinium cross-link levels are gender-specific and also depend on age, although this relationship is complicated. In elderly subjects the proportion of free pyridinolines is smaller than in adolescents and in healthy adults, but the excretion of total pyridinoline cross-links is higher, potentially owing to a change in the molecular distribution of pyridinoline cross-links (Kamel, 1995). To circumvent the problem of the variable excretion of free pyridinolines, the total amount can be determined.

Cross-Linked Telopeptides

C-*telopeptides* Two biochemical markers reflecting degradation of the C-terminal telopeptide of type I collagen have been described: CTX, which is released by cathepsin K cleavage of intact bone collagen and can be detected by an antibody raised against the EKAHDGGR amino acid sequence, and ICTP, which is a larger fragment produced by matrix metalloproteinase (MMP) cleavage (Risteli, 1999; Garnero, 2003).

The product of the MMP degradation of collagen type I, the large C-telopeptide fragments of type I collagen in serum (ICTP of CTX-MMP) can be used as biochemical marker of bone resorption, and a RIA and an EIA (Orion Diagnostics) are available (Risteli 1993). The antigenic determinant requires a trivalent cross-link, including two phenylalanine-rich domains of the telopeptide region of type I collagen. Cathepsin K cleaves the telopeptide structure between the phenylalanine-rich region and the cross-link moiety: cathepsin K therefore abolishes immunoreactivity in the ICTP assay (Garnero, 2003).

ICTP is cleared from the blood by the kidney and circulating levels are reportedly elevated in the majority of patients with chronic renal failure (Magnusson, 2001). ICTP showed a modest but significant circadian rhythm in premenopausal women, with about 20% higher values at night than in the afternoon (Hassager, 1992), and levels vary approximately 20% over the menstrual cycle, being higher in the luteal phase (Hannon, 2000). There is a considerable age and sex influence on ICTP levels in healthy children between 2 months and 18 years (Rauchenzauner, 2007), and fractures have been reported to increase ICTP levels by 73%, which may remain elevated for one year after fracture (Akesson, 2005).

The cross-linked C-terminal telopeptide of type I collagen, or CTX-I, is a sensitive but also rather variable marker of bone resorption, and several assays for the cross-linked telopeptide are commercially available. The development of the assays for this marker is characterized by differences in ELISA design (competitive versus sandwich), antibodies (polyclonal versus monoclonal), and antigens (nonisomerized [alpha] versus isomerized [beta] synthetic octapeptide EKAHDGGR). The assays are based on antibodies raised against a synthetic eight-amino-acid peptide (EKAHDGGR) derived from a segment of the C-telopeptide of the collagen α1(I) chain, and the cross-linking molecules can contain two of such peptides.

Some antibodies specifically recognize a form of the peptide containing an β-aspartyl peptide bond (AHD-β-GGR). The isomerization of aspartyl to beta-aspartyl residues occurs over extended periods of time and is associated with the aging of proteins and peptides (Bonde, 1997) and thus bone (Cloos, 2000). The equilibrium of the isomerization reaction is reached after about 150 days (Cloos, 2000) and, thus, the assay recognizing the beta-form measures the degradation of relatively old bone (Fledelius, 1997). Another antibody binds only the nonisomerized form of this octapeptide and presumably measures the degradation of relatively young bone (α-Ctx) (Bonde, 1997).

Currently, sandwich assays are available for serum and urine with two monoclonal antibodies recognizing only a form of the peptide with two beta-aspartyl peptide bonds (beta-beta CTX, Serum Crosslaps). Another sandwich assay with two monoclonal antibodies recognizes only a form of the peptide with two alpha-aspartyl peptide bonds (alpha-alpha-CTX, ALPHA Crosslaps) and is available only for urine. The assays are available in microtiter plate form (Nordic Bioscience) and on an automated platform (Roche Diagnostics for serum beta-beta CTX only). Results of the manual ELISA are comparable to the results of the automated platform. The CTX assays measure a mixture of molecular entities rather than a single molecular entity (Fledelius, 1997). CTX has been reported stable in serum and urine, and freeze-thaw cycles have no effect on the levels (Ju, 1997).

Systemic CTX is excreted by the kidney as well as metabolized, with a renal excretion fraction of about 44% (Fall, 2000). CTX levels depend on renal (Okuno, 2003) and liver function (Seibel, 2005; Guanabens, 1998). By using modeling techniques, the half-life of serum beta-CTX in subjects with a normal renal and liver function has been estimated to be about 1 hour (Holford, 2006). Urine and serum CTX show considerable diurnal variation with a maximum at about 5 am, a minimum at about 2 pm, and a magnitude of about 40% around the 24-hour mean. Moreover, a significant influence of fasting has been described (Qvist, 2002). Urinary levels of CTX show a significant yet relatively small variation during the menstrual cycle (Gorai, 1998). Urinary excretion of CTX has been reported highest in early puberty in both girls and boys (Yang, 2006). Levels of serum CTX are highest in neonates and then markedly decrease in children after 1 year of age. A second peak is observed in girls 11 to 13 years of age and in boys who were 14 to 17 years old (Crofton, 2002; Yang, 2006).

NTX Another highly sensitive marker of bone resorption is cross-linked N-terminal telopeptide of collagen type I, also referred to as NTX. The assay is based on a monoclonal antibody raised against a peptide isolated from urine of a patient with Paget's disease of bone (Hanson, 1992). The antibody specifically recognizes the cross-linked α2(I)N-telopeptide sequence, QYDGKGVG, and in which K (lysine) is involved in the trivalent cross-linkage (Eyre, 1995). Because the rest of the molecule can differ, the NTX immunoassays actually determine a mixture of molecular entities (Hanson, 1992) rather than a single molecule. However, *in vitro* studies show that NTX can be quantitatively released from bone by the action of cathepsin K, a specific protease of the osteoclast. (Atley, 2000), and good relationships with calcium kinetics and histomorphometry data on bone resorption have been shown for NTX in urine (Weaver, 1997, Franke, 1998). Systemic NTX is excreted by the kidney and metabolized with a renal excretion fraction of 0.20±0.07 (Fall, 2000).

The NTX ELISA is available as a commercial, microtiter-plate format (Osteomark, Inverness Medical Innovations Inc, Waltham, MA) and on an automated platform (Vitros ECu, Ortho Clinical Inc., Rochester, NY). The assays measure the NTX peptide in urine, calibrated in molar equivalents of type I collagen, and results are normalized to urinary creatinine concentrations. The manual ELISA has also been developed for measuring NTX in serum (Clemens, 1997), but this assay has not been widely used. NTX levels in serum and urine appear to be stable at room temperature and at −80° C. Samples are stable below −20° C (Ju, 1997) and during freeze-thaw cycles (Woitge, 1999; Seibel, 2005). The levels in urine have been described to increase during exposure to UV light (Blumsohn, 1995), and an aspartate residue in the α2(I) part of NTX has been shown to undergo beta-isomerization (Brady, 1999).

By using modeling techniques the serum half-life of NTX in subjects with normal renal function has been estimated to be 10.8 to 11.8 hours (Holford, 2006). Serum levels of NTX as well as renal excretion of NTX are related to renal function (Hamano, 2006). uNTX/Cr has been described as elevated in patients with primary biliary cirrhosis (Guanabens, 1998).

Both urine and serum NTX levels follow a pattern of diurnal variation, although this seems less pronounced for serum NTX (Gertz, 1998; Greenspan, 1997). Variations in the level of urinary NTX have been described during the menstrual cycle (Gorai, 1995; Abrahamsen, 2003), as well as a circannual rhythm and influence of gender (Woitge, 2000). NTX was slightly decreased one year after an ankle fracture (Ingle, 1999), but increased slightly after a forearm fracture (Ingle, 1999b). Urinary excretion of NTX has been reported highest in early puberty in both girls and boys (Yang, 2006).

Galactosyl Hydroxylysine

Hydroxylysine, another modified amino acid particular to collagens, is glycosylated to varying degrees depending on the tissue type (Segrest and Cunningham, 1970). Two glycosides are formed, galactolysyl hydroxylysine (Gal-Hyl) and glucosyl galactosyl hydroxylysine (Glc-Gal-Hyl). Because of tissue-specific differences, Gal-Hyl is considered to be relatively specific to bone collagen degradation (Krane, 1977). Glycosylated hydroxylysine residues appear not to be reused or catabolized when collagen is degraded. Furthermore, they do not appear to be absorbed in significant levels from a normal diet (Segrest and Cunningham, 1970). An HPLC method has been employed for measuring urinary Gal-Hyl involving dansylation of the lysine, resolution by reversed-phase HPLC, and detection by fluorescence (Moro, 1984; Yoshihara, 1993). This assay generally shows good correlations with other resorption markers (Moro, 1997). An immunoassay has also been described (Leigh, 1998). Urinary galactosylhydroxylysine determined by HPLC showed higher intrasubject variability when compared with CTX and DPD (Plebani, 2000). The absence of an immunoassay format that is commercially available is a major disadvantage of the marker, and may be the reason why relatively few studies have incorporated the measurement of this marker.

Tartrate-Resistant Acid Phosphatase

During bone resorption, osteoclasts secrete protons and enzymes into the space between the ruffled border of the osteoclast and the surface of the bone. The enzyme, tartrate-resistant acid phosphatase (TRAcP), has been identified in both the ruffled border of the osteoclast membrane and the secretions in the resorptive space (Minkin, 1982). Six isoenzymes (types 0 to 5) of acid phosphatase have been identified by electrophoresis of human tissues (Yam, 1974). Band 5 is expressed by osteoclasts, alveolar and monocyte-derived macrophages, and the placenta (Cheung, 1995). On the basis of the catalytic and ionic properties and carbohydrate content, two isoenzymes (a and b) of band 5 TRAcP have been identified. Both band 5 acid phosphatases are resistant to tartrate inhibition. Band 5a TRAcP is sialylated, whereas band 5b, which is predominant in bone, lacks sialic acid, and the pH optima of the two isoforms differ (Whitaker, 1989; Lam, 1978, 1980; Schiele, 1988). Partly degraded type I collagen is taken up by the osteoclast into vesicles (Salo, 1996; Nesbitt, 1997; Salo, 1997). To these vesicles TRAcP5b is added intracellularly, which can degrade the type I collagen breakdown products even further (Halleen, 1999). The contents of this vesicle are ejected from the cell on the apical side (Salo, 1996; Nesbitt, 1997; Salo, 1997).

TRAcP activity in serum has been reported to reflect bone resorption rates (Yam, 1974). However, the distinction between the osteoclastic enzyme and other tissue acid phosphatases in serum and the instability of the enzyme in serum have presented the greatest technical challenge. TRAcP activity in serum has been measured kinetically (Whitaker, 1989; Lam 1978, 1980; Schiele, 1988; Lau, 1987), and more recently by immunoassay (Cheung, 1995; Kraenzlin, 1990; Halleen, 2000), but these methods vary in specificity for the osteoclast-derived isoenzyme. Recent assays employed an antibody produced against TRAcP from a bone extract. This method showed good correlation between immunoreactivity and enzyme activity, little cross-reaction with acid phosphatases from nonosteoclastic sources, and sensitivity to changes in bone turnover after 6 months of estrogen replacement therapy (Halleen, 2000). Although TRAcP has been reported to reflect the rate of bone resorption (Yam, 1974), more recent reports show that TRAcP5b mainly reflects the number of osteoclasts (Chao, 2005; Alatalo, 2004; Chen, 2004).

TRAcP5b shows a relatively small but significant diurnal variability (14%) and negligible effect of food intake (2%) (Hannon, 2004). The half-life of the marker may therefore be relatively long when compared with the half-life of a marker such as CTX, which does show considerable diurnal variability and effect of feeding (Qvist, 2002). This results in a slightly better signal-to-noise ratio for TRAcP5b when compared with the collagen type I telopeptide markers (Hannon, 2004). Because TRAP 5b does not accumulate in patients with end-stage renal disease, the marker does not seem to be cleared directly by the kidney (Hannon, 2004; Stepan, 1987). TRAP 5b has been reported stable up to 2 days at room temperature (+20/+30° C), 3 days in the refrigerator, 1 month at −20° C, and longer at −70° C or lower (Halleen, 2006).

Bone Sialoprotein

Bone sialoprotein (BSP) is an acidic, phosphorylated glycoprotein that is synthesized by osteoblasts and osteoclastic-like cells in culture. It has an unglycosylated mass of 33 kDa (glycosylated, 70–80 kDa). Although the function of BSP is still not fully understood, BSP stimulates hydroxyapatite formation *in vitro* and appears to mediate cell − cell interactions via an integrin binding site. BSP is relatively restricted to bone but it is also expressed by trophoblasts and is strongly upregulated by many malignant tumors (e.g., breast and prostate cancers). Recently, it has been suggested that BSP may play a role in angiogenesis associated with bone formation, tumor growth, and metastasis (Bellahcene, 2000). A small amount of BSP is found in the circulation and as such is a potential marker of bone turnover (Seibel, 1996; Shaarawy, 2001). An RIA kit has been described for BSP in serum (Seibel, 1996; Karmatchek, 1997; Woitge, 1997) but is presently not commercially available.

Serum BSP levels are reported to be increased in malignant bone disease (Diel, 1999; Woitge, 2001) and postmenopausal osteoporosis, and are decreased by antiresorptive treatment (Seibel, 1996; Shaarawy, 2001). BSP is stable at −80° C (Li, 1998), but little is known about the kinetics and metabolism of BSP in serum. The marker could be useful in the early detection of bone metastases and other bone disorders, and a new and improved assay for immunoreactive BSP is presently being developed (Robins S.P. and Seibel M.J., unpublished data).

Cathepsin K

Pycnodysostosis, an autosomal recessive disease characterized by osteopetrosis, is the result of mutations in the cathepsin K gene (Gelb, 1997). The enzyme cathepsin K is a member of the cysteine protease family that, unlike other cathepsins, has the unique ability to cleave both helical and telopeptide regions of collagen type I (Garnero, 1998; Kafienah, 1998; Li, 2004). The enzyme is located intracellularly in vesicles, granules, and vacuoles throughout the cytoplasm of osteoclasts and is secreted into bone resorption lacunae for extracellular collagen degradation (Goto, 2003). The enzyme is produced as a 329-amino-acid precursor ProCathepsin K, which is cleaved to its active form with a length of 215 amino acids, which *in vivo* is believed to occur in the bone resorption lacunae, having a low-pH environment. Cathepsin K has a low optimal pH and degrades many matrix proteins, including type I collagen, osteocalcin, and osteopontin (Bossard, 1996; Bromme, 1996; Garnero, 1998). To our knowledge, no data are available on the peripheral circulatory kinetics, metabolism, and/or elimination of cathepsin K.

Commercially, two kits are available for determining cathepsin K in serum, an immunocapture enzyme activity assay from Biovendor that measures enzyme activity (Biovendor, Candler, NC), and an enzyme immunoassay from Biomedica that measures the concentration of the enzyme (Biomedica, distributed in the United States by ALPCO, Windham, NH). Although cathepsin K is potentially an interesting marker of osteoclast activity, only limited data on its clinical use are available (Meier, 2006) and further research is needed for its routine clinical use. The marker may be of specific interest for the development of so-called cathepsin K inhibitors. However, the currently available assays lack sensitivity and, therefore, do not allow accurate measurements of circulating cathepsin K levels.

CLINICAL USE OF BIOCHEMICAL MARKERS OF BONE TURNOVER

In clinical practice, bone turnover markers can be used to aid in the diagnosis and prognosis of certain disease entities, and for the monitoring of specific treatment regimens. However, for each metabolic bone disorder and treatment, it is important to consider which marker, measured with what assay will provide the most relevant clinical information. In addition, knowledge of the relationship between the level of a certain marker and clinical outcome creates the potential of using this as a surrogate marker of outcome, similar to bone mineral density in osteoporosis.

Paget's Disease of Bone

Biochemical markers of bone turnover play a clear-cut role in the diagnosis of Paget's disease of bone and therapeutic monitoring. The markers are helpful in the differential diagnosis of a Pagetoid skeleton lesion, together with bone scintigraphy and radiographic techniques. Turnover markers are invariably elevated in active disease (Papapoulos, 1997; Shankar and Hosking, 2006). Alterations in the rate of bone turnover in this disease are so pronounced that bone marker measurements require relatively little sensitivity and specificity, and in most cases, total ALP and hydroxyproline are sufficient for diagnostic purposes (Papapoulos, 1997; Shankar and Hosking, 2006). More sensitive and specific bone turnover markers, such as urinary alpha-alpha CTX/Cr, uNTX/Cr, or serum BoneALP and PINP, seem to provide little additional value in Paget's disease of bone, although these markers have been shown to be superior to others (Shankar and Hosking, 2006; Alexandersen, 2005; Alvarez, 2000, 2001). These new and more specific markers may be particularly useful in cases of monostotic disease in which the elevations in bone turnover may not be marked. Although the initial response to antiresorptive treatment may be reliably monitored with a bone resorption marker, biochemical remission and relapse are often based on a bone formation marker (Papapoulos, 1997; Shankar and Hosking, 2006). Time to relapse is related to the level of bone formation at its nadir (Eekhoff, 2003; Papapoulos, 1997). Levels of bone turnover markers are related to skeletal complications from the disease, and achieving normal levels of bone turnover is the primary aim of treatment (Papapoulos, 1997; Shankar and Hosking, 2006).

Serum TRAcP5b has been shown to be significantly elevated in patients with Paget's disease of bone, with TRAP 5b activity at above normal reference values in 71% of patients (Halleen, 2001). In patients with mild biochemical disease assessed by total ALP activity, serum cathepsin K levels were significantly higher compared with healthy men and postmenopausal women (Meier, 2006).

Postmenopausal Osteoporosis

In postmenopausal osteoporosis, elevated bone markers do correlate with bone loss and fracture risk, both in treated and untreated patients. The mean levels of most of bone turnover markers may be elevated in patients with postmenopausal osteoporosis, and bone turnover markers generally show a modest inverse correlation with BMD (Melton, 1997; Garnero, 1996). However, the relationships with BMD are not strong and bone markers are never used to diagnose postmenopausal osteoporosis (Becker, 2003). In contrast to postmenopausal osteoporosis, glucocorticoid-induced osteoporosis does not show an overall elevation of bone turnover markers. In this disease, bone formation markers are generally suppressed, whereas bone resorption markers are initially increased (Reid, 2006).

With the current performance of bone markers, it appears that a single measurement of biochemical markers of bone turnover can not predict the absolute rate of bone loss in an individual woman (Stepan, 2000). However, clearly increased levels of bone markers in postmenopausal women can be regarded as a risk factor for rapid bone loss in the subsequent years (Johansen, 1988; Garnero, 1999; Garnero, 2006). Yet, if bone turnover markers are not markedly elevated, one cannot know that substantial bone loss will not occur in subsequent years.

The fracture risk for untreated patients has been shown to correlate with the level of bone markers (Van Daele, 1996; Garnero, 1996, 2000; Garnero and Delmas, 2004). This correlation is even stronger when bone markers are combined with BMD assessments (Fig. 5) (Garnero, 1996). Interestingly, data from the OFELY study, e.g., showed that 47% of the incident fractures actually occurred in nonosteoporotic patients. Among these women, the combination of bone markers and history of previous fractures was highly predictive of fracture risk (Garnero, 2000). Thus, bone markers may be used in the assessment of fracture risk in selected cases in which BMD and clinical risk factors are not sufficient to make a treatment decision (Garnero, 2004).

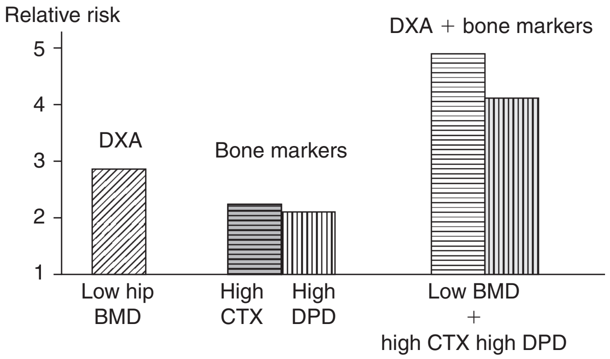

FIGURE 5 Combination of bone mineral density (BMD) at the hip assessed by dual x-ray absorptiometry (DXA) and of bone resorption to predict hip fracture risk in elderly women monitored prospectively for 2 years: The Epidos study. Low BMD was defined according to the WHO guidelines, i.e., by a value lower than 2.5 SD below the young adult mean (*T* score = 2.5). High bone resorption was defined by urinary CTX or free DPD values higher than the upper limit (mean + 2 SD) of the premenopausal range (Garnero, 1996).

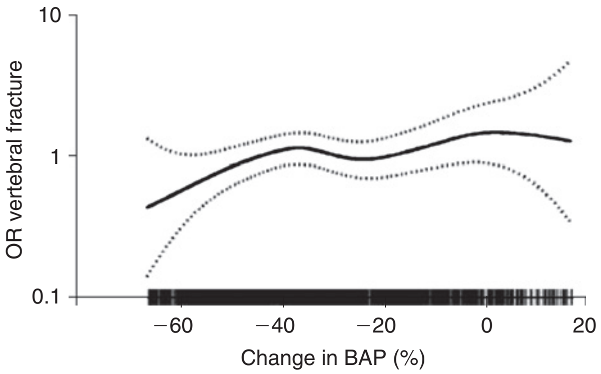

FIGURE 6 One-year change in bone ALP and spine fracture risk among alendronate-treated women. Percentage change in bone ALP and predicted risk (log OR) of spine fracture (solid line) and 95% CI (dotted lines) from logistic regression model. Individual data points represented on the *x* axis. Departure from linearity *P* value = 0.34.20 (Bauer, 2004).

Clinically, bone turnover markers are used to monitor pharmacological treatment, whether this is anticatabolic, such as bisphosphonates, hormone replacement therapy, and selective estrogen receptor modulators, or anabolic, such as teriparatide and PTH $1-84$ (see Figs. 3 and 4). The markers may be used to identify patients with drug absorption problems or patients who show lack of compliance. Pretreatment levels of bone turnover were shown to correlate both with fracture rate and with changes in BMD during antiresorptive treatment in postmenopausal women with osteoporosis. Therefore, measurement of bone turnover may prove to be useful in identifying women with osteoporosis who are likely to benefit most from therapy (Seibel, 2004; Bauer, 2006; Gonelli, 1997, 1999). In postmenopausal women without osteoporosis (by bone density criteria) measurement of bone turnover markers can potentially identify a subset of patients for whom bisphosphonate therapy to prevent fracture is cost-effective (Schousboe, 2007). Relationships between pretreatment levels of bone turnover markers and fracture risk have also been shown during anabolic therapy with teriparatide in postmenopausal women (Delmas, 2006). The observation that short-term change in bone marker levels correlate, with long-term change in bone mineral density indicates that the markers can also predict the long-term effect of treatment on bone loss (Greenspan, 1998, 2000, 2005; Iwamoto, 2005; Bjarnason, 2000; Delmas, 2000; Lane, 2000; Chen, 2005). Moreover, changes in the level of bone turnover markers have been related to fracture reduction during treatment as illustrated for alendronate in Figure 6 (Bauer, 2004). Therefore, biochemical markers of bone turnover may be useful to predict clinical outcomes, at least on a group level.

Serum cathepsin K levels were elevated in patients with postmenopausal osteoporosis (Meier, 2006). However, this finding needs to be confirmed in larger studies. Several studies have shown that TRAcP5b is elevated in postmenopausal osteoporosis (Halleen, 2001, 2002; Rosenbrock, 2002) and can predict fracture risk (Gerdhem, 2004). The marker can also be used to monitor antiresorptive treatment (Halleen, 2005). In the OFELY prospective study an increased urinary ratio between native (alpha) and isomerized (beta) CTX was found to correlate significantly with increased fracture risk independent of BMD and bone turnover (Garnero, 2002), suggesting that alterations of type I collagen isomerization may be associated with bone fragility. BSP levels were elevated in postmenopausal osteoporosis and decreased during antiresorptive treatment (Shaarawy, 2001).

Table I lists the biochemical markers of bone turnover and their relationships with clinical outcome in postmenopausal osteoporosis (Cremers and Garnero, 2006).

Male Osteoporosis

About one quarter of all osteoporotic hip fractures occur in men (Orwoll, 1995). Several hormonal and biochemical parameters known to affect bone metabolism in women, such as gonadal hormones, vitamin D and its metabolites, and growth factors, have been shown to change with age in both genders and have therefore been proposed to be involved into the age-related decline in BMD in males (Nicolas, 1994; Gray, 1991; Center, 1999). In men, similar to postmenopausal women, low BMD is associated with increased risk of osteoporotic fracture (Nguyen, 1993, 2001), and high bone resorption, as assessed by serum ICTP levels, also appears to be associated with an increased risk of osteoporotic fracture in elderly men, independent of BMD (Meier, 2005). Combining measurements of BMD and bone turnover improved fracture

TABLE I Relationships Between Biochemical Markers of Bone Turnover with Clinical Outcome in Postmenopausal Osteoporosis and Cancer Metastatic to the Bone

Marker	Matrix	Method of analysis	Relationship with risk for fracture in untreated PMO	Relationship with antifracture efficacy in PMO	Relationship with SRE without bisphosphonates in bone metastases	Relationship with SRE during bisphosphonates in bone metastases
Formation ALP	Serum	Colorimetric			+/– HRPC (Berruti,2000)	
Bone ALP	Serum	Colorimetric			+HRPC, NSCLC, other solid tumors (not BC) (Brown, 2005)	1 MM, BC, HRPC, NSCLC, other solid tumors with zoledronic acid (Coleman, 2005)
		Electrophoretic			+/– HRPC (Berruti, 2000)	
		Precipitation				
		IRMA	– Hip fracture; (Garnero 1996, van Daele 1996) – Osteoporotic fracture (Gerdhem 2004) + Osteoporotic fracture (Garnero 2000, Ross 2000)	+ Vertebral fracture with raloxifene (Bjarnasson, 2001) + Hip, vertebral, nonvertebral fracture with alendronate; (Bauer, 2004)	– HRPC (Berruti, 2000)	
		EIA				
OC	Serum	RIA	– Hip fracture (Garnero, 1996; van Daele, 1996) – Osteoporotic fracture (Gerdhem, 2004; Garnero, 2000)	+ Vertebral fracture with raloxifene (Bjarnasson, 2001; Sankar, 2004)	– HRPC (Berruti, 2000)	
		ELISA				
		CLIA				
PINP	Serum	RIA	– Osteoporotic fracture (Garnero, 2000)	+ Vertebral fracture with raloxifene (Reginster, 2004) + Vertebral fracture with alendronate (Bauer, 2004)		
		ELISA				
PICP	Serum	RIA			– HRPC (Berruti, 2000)	
		ELISA	– Osteoporotic fracture (Garnero, 2000)			
Resorption OHP	Urine	Colorimetric				
		HPLC			– HRPC (Berruti, 2000)	
DPD	Urine	HPLC	– (van Daele, 1996)		+ HRPC (Berruti, 2000)	
		ELISA	+ Hip fracture (Garnero, 1996)			

(*continued*)

TABLE I (*Continued*)

Marker	Matrix	Method of analysis	Relationship with risk for fracture in untreated PMO	Relationship with antifracture efficacy in PMO	Relationship with SRE without bisphosphonates in bone metastases	Relationship with SRE during bisphosphonates in bone metastases
			+ Osteopotic fracture (Garnero, 2000) + Hip fracture (van Daele, 1996)			
		RIA				
PYD	Urine	HPLC	– Hip fracture (van Daele, 1996)		– HRPC (Berruti, 2000)	
		ELISA				
		RIA				
ICTP	Serum	RIA				
		EIA				
TRAP5b	Serum	Colorimetric				
		RIA				
		ELISA	+ Osteoporotic fracture (Gerdhem, 2004)			
CTX (β)	Serum	ELISA (β)				
		ELISA (β –β)	+Osteoporotic fracture (Garnero, 2000; Ross, 2000) – Clinical vertebral fracture (Gerdhem, 2004)	+ Vertebral fracture with alendronate (Bauer, 2004)		
		RIA			–HRPC (Berruti 2000)	
		ECLA (β –β)	+Osteoporotic fracture (Garnero, 2001)			
CTX (β)	Urine	ELISA	+ Hip fracture (Garnero, 1996) +Osteoporotic fracture (Garnero, 2000, 2002) + all fracture (Ross *et al.*, 2000)	– Vertebral fracture with raloxifene (Bjarnasson, 2001)		
		RIA				
		ECLA		+ Vertebral fracture with risedronate (Eastell, 2003)		
CTX (α-α)	Urine	ELISA	+Osteoporotic fracture (Garnero, 2002)			
NTX	Serum	RIA				
		ELISA				
NTX	Urine	RIA				
		ELISA	– Hip fracture (Garnero, 1996)			

(*continued*)

TABLE I (*Continued*)

Marker	Matrix	Method of analysis	Relationship with risk for fracture in untreated PMO	Relationship with antifracture efficacy in PMO	Relationship with SRE without bisphosphonates in bone metastases	Relationship with SRE during bisphosphonates in bone metastases
			–Osteoporotic fracture (Garnero, 2000)			
		Vitros		+ Vertebral fracture with risedronate (Eastell, 2003)	+ ; HRPC, NSCLC, other solid tumors (not BC) (Brown, 2005)	+ MM, BC, HRPC, NSCLC, other solid tumors with zoledronic acid (Coleman, 2005) + BC, PC, other solid tumors with clodronate, pamidronate and zoledronic acid (Brown, 2003)
Cathepsin K	Serum					

+ *Relationship described in literature (reference)*; − *absence of relationship described in literature (reference)*; HRCP: *Hormone refractory prostate cancer* (PC); BC: *Breast cancer*; NSCLC: *Non-small cell lung carcinoma*; MM: *Multiple myelomal*; RMA: *Immunoradiometric assay*; EIA: *Enzyme immunoassay*; RIA: *Radioimmunoassay*; ELISA: *Enzyme-linked immunosorbant assay*; HPLC: *High-performance liquid chromatography*; ECLA: *Electrochemiluminescence immunoassay*; ALP = *(total) alkaline phosphataseBone*; ALP = *bone specific alkaline phosphatase*; OC = OsteocalcinPINP = *Amino-terminal procollagen propeptides of collagen type* I; PICP = *Carboxy-terminal procollagen propeptides of collagen type* I; BSP = *Bone sialoprotein*; OHP = *Hydroxyproline*; DPD = *Deoxypyridinoline*; PYR = *Pyridinolinel*; CTP = *Carboxy-terminal telopeptide of type* I *collagen*; CTX = *cross-linked* C-*terminal telopeptides of type* I *collagen*; NTX = *cross-linked* N-*terminal telopeptides of type* I *collagen*; TRAP5*b* = *Tartrate-resistant acid phosphatase isoenzyme* 5*b*

risk prediction in elderly men (Meier, 2005) as it does in postmenopausal women. In contrast to postmenopausal women, pretreatment levels of bone turnover markers were not related to change in BMD in men treated with alendronate. Also IGF-1 and sex steroids were not related to change in BMD, suggesting that neither biochemical nor hormonal marker allows the identification of patients who may benefit most from alendronate therapy (Drake, 2003). Much remains to be understood about the male skeleton, the pathogenesis of male osteoporosis, and the factors that determine the response to therapy.

Cancer Metastatic to the Bone

Biochemical markers of bone turnover may be helpful in the diagnosis of cancer metastatic to bone. They may also be used for predicting skeletal morbidity and monitoring treatment efficacy (Seibel, 2005).

In patients with solid tumors and bone metastases, the mean level of the bone turnover markers is elevated (Coleman, 2002). And in patients with predominantly osteoblastic skeletal lesions from prostate cancer (PC), both markers of bone formation and resorption are elevated, similar to patients with predominantly osteoclastic bone lesions such as from breast cancer (BC) (Smith, 2006).

For the detection of bone metastases from solid tumors, bALP and uNTX/Cr seem to be the most useful established biomarkers (Tanko, 2006): Approximately 75% of patients with bone metastases from solid tumors are found to have elevated uNTX/Cr and bALP levels at the time of diagnosis. In patients with multiple myeloma (MM), abnormally high levels of uNTX and bALP are seen in about 60% and 40%, respectively (Coleman, 2005).

Of the newer markers, urinary alpha-alpha-CTX and serum TRAcP5b are promising for the diagnosis of bone metastases from solid tumors, alone or in combination with other markers (Tanko, 2006; Leeming, 2006). In a retrospective study in breast cancer patients serum BSP was shown to predict development of bone metastases (Diel, 1999), and may also be a useful marker in the early diagnosis of bone metastases from prostate cancer (Jung, 2004). Currently, the assessment of a single biochemical marker is unlikely to replace bone scintigraphy for the early diagnosis of skeletal metastases from solid tumors. However, given their specificity, a combination of biochemical markers may be useful for monitoring patients between bone scintigram assessments, although this concept needs to be further explored (Voorzanger, 2006, 2007; Tanko, 2006).

In multiple myeloma biochemical markers of bone resorption, including serum TRAcP5b, are elevated and correlate with the stage of the disease (Pecherstorfer, 1997;

Terpos, 2005). In contrast, bone formation markers are usually within normal ranges, and reports on correlations with disease stage are inconclusive (Terpos, 2005).

The level of the markers reflects the severity of metastatic bone disease from solid tumors, with tumor load being well reflected by urinary alpha-alpha-CTX, uNTX/Cr, and serum bone ALP (Leeming, 2006; Garnero, 2000; Tanko, 2006).

Serum BSP levels have been reported to be associated with skeletal involvement and tumor cell burden in MM patients (Woitge, 2001). Interestingly, bone marrow plasma levels of dickkopf 1 (DKK-1), an inhibitor of the Wnt signaling pathway, which is crucial for osteoblast differentiation, has been reported as elevated in patients with multiple myeloma and skeletal involvement (Tian, 2003), and may therefore also be used as a bone marker in patients with MM. Increased serum Dkk-1 levels have also been reported in patients with breast cancer and bone metastases compared with healthy controls and patients with breast cancer with no bone metastases (Voorzanger-Rousselot, 2006)

The risk of developing skeletal-related events (SREs) from metastatic bone disease, multiple myeloma included, without and with bisphosphonate treatment, has been shown to be correlated with the level of bone turnover markers (Brown, 2003, 2005; Coleman, 2005; Vinholes, 1997). For instance, in patients ($n = 121$) with metastatic bone disease treated with bisphosphonates, those with a strongly increased urinary uNTX/Cr level (=100 nmol/mmol), determined on a monthly basis, were 19 times more likely to develop a SRE during the first 3 months than patients with uNTX/Cr <100 nmol/mmol (Brown, 2003). Normalization of bone turnover thus seems a rational treatment goal (Clamp, 2004), and may even allow individualization of dose regimens. This concept is currently being evaluated in a prospective study.

CLINICAL DRUG DEVELOPMENT

Biochemical markers of bone metabolism are a powerful tool for clinical development of drugs for bone diseases (Cremers and Garnero, 2006). They provide critically important information for key decisions in the costly drug development process, such as whether or not to continue the development of a certain drug and which dose to select during phase I/II trials. Bone markers can also assist in the optimization of dose regimens after drug approval, and in exploring the efficacy of a registered drug for other indications. For these latter applications established relationships between levels and clinical efficacy of a drug can be helpful (see Table 1). For clinical drug development in general, however, their fast response to pharmacological treatment is by and large the most important characteristic of bone turnover markers, especially when compared with the response of other biomarkers such as BMD. Such a rapid response can decrease costly drug development time significantly.

Successful application of bone markers to clinical drug development is illustrated by the various phases of the clinical development of zoledronic acid. This bisphosphonate was first developed for metastatic bone disease, followed by other indications such as postmenopausal osteoporosis. At the start of clinical development there were no data on the relationship between the level of bone turnover markers and clinical outcome. However, during early phases of development of the drug, it was found that an intravenous administration of 4 mg suppresses bone resorption markers maximally for at least 3 to 4 weeks (Fig. 7). Four milligrams every 3 to 4 weeks was further explored, and later it was shown that this dose regimen reduces the risk for SREs in metastatic bone disease (Saad, 2004; Rosen 2003, 2004). Recently, Coleman *et al.* showed that the risk for SREs during bisphosphonate therapy is correlated with normalization of bone turnover (Coleman, 2005), and partially because of concerns of the potential side effects of high-dose bisphosphonates, such as nephrotoxicity and osteonecrosis of the jaw, these data are now used to explore new, lower dose regimens (Brown, 2005). In osteoporotic women a single dose of intravenous zoledronic acid was shown to increase BMD and suppress bone turnover markers for at least one year (Reid, 2002), both to levels associated with antifracture efficacy as observed for other bisphosphonates such as alendronate, risedronate, and ibandronate (Harris, 1999; Ravn, 1996; Garnero, 1994; Devogelaer, 1996; Chesnut, 1995; Liberman, 1995). These observations suggested antifracture efficacy of the drug in this dose regimen. And indeed, recently, a single dose of 5 mg of zoledronic acid was shown to significantly decrease vertebral, nonvertebral, and hip fractures in postmenopausal osteoporosis (Black, 2007). Sufficient and continued suppression of bone turnover combined with an increase in BMD therefore seems to predict antifracture efficacy of bisphosphonates. This concept has been used successfully during the switch from daily to weekly dose regimens of alendronate and risedronate (Schnitzer, 2000; Brown, 2002), as well as for the registration of bisphosphonates for closely related indications such as glucocorticoid-induced osteoporosis. The combination of sufficient and continued suppression of bone turnover and increase in BMD may also be used in the clinical development of other antiresorptive drugs such as the RANKL antibody Denosumab (McClung, 2003) and cathepsin K inhibitors. These drugs show highly favorable effects on bone turnover markers and BMD, and are therefore likely to show antifracture efficacy. However, because these drugs have new mechanisms of action their effect on bone markers and BMD cannot be translated automatically into antifracture efficacy or an effect on SREs. Thus, bone markers are helpful, but randomized clinical trials are still needed for the development of drugs with new mechanisms of action.

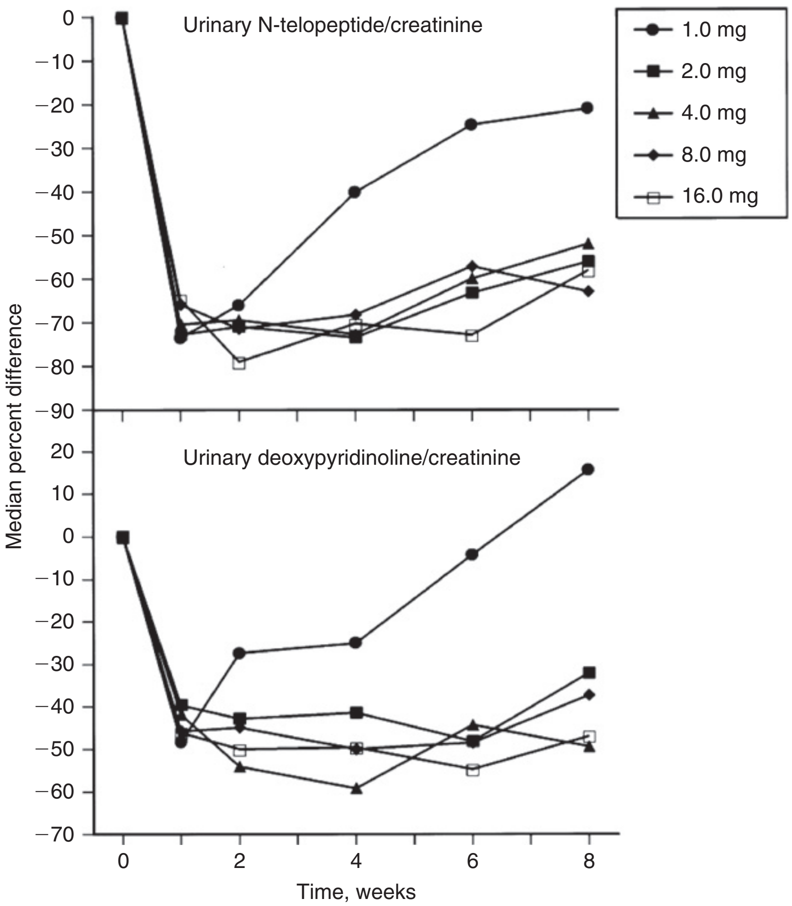

FIGURE 7 Effects of a single dose of zoledronic acid on the ratios of urinary levels of N-telopeptide of the cross-links of collagen to creatinine (*upper*) and urinary levels of deoxypyridinoline to creatinine (*lower*) in patient with cancer and bone metastases (Berenson, 2001).

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Chapter 88

Methods and Clinical Issues in Bone Densitometry

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INTRODUCTION

Osteoporosis is widely recognized as an important public health problem because of the significant morbidity, mortality, and costs associated with its complications, namely fractures of the hip, spine, forearm, and other skeletal sites (Cummings *et al*., 2002). It is estimated that every year 1.5 million people in the United States experience an osteoporosis-related fracture, including 300,000 cases of hip fracture (Surgeon General's report, 2004). One in every two white women will suffer an osteoporosis-related fracture in her lifetime, and one in six will have a hip fracture (Kanis *et al*., 2002). There is particular concern about hip fractures because these have the greatest effect on an individual's quality of life and incur the greatest cost for health services (Ray *et al*., 1997). However, other fractures are also associated with significant morbidity and costs (Melton *et al*., 2003) and both hip and vertebral fractures are associated with an increased risk of death (Cooper *et al*., 1993; Centre *et al*., 1999) and increased dependence on nursing homes and private and public care services for the basic activities of daily living. Because of the aging population and the previous lack of attention to bone health, the annual number of hip fractures in the United States is set to double by the year 2020 (Surgeon General's report, 2004).

Although for many years there was awareness of the morbidity and costs associated with fragility fractures, real progress only came with the ability to diagnose osteoporosis before any fractures occur, and with the development of preventive treatments. Bone density scanning played an important role in both these developments. Until the mid-1980s measurements of bone mineral density (BMD) were used mainly in research, and it was only with the introduction of dual-energy x-ray absorptiometry (DXA) scanners in 1987 that they entered routine clinical practice (Genant *et al*., 1996). Further significant developments included the first study showing that bisphosphonate treatment can prevent bone loss (Storm *et al*., 1990), the publication of the World Health Organisation (WHO) report defining osteoporosis in postmenopausal white women as a BMD *T* score at the spine, hip, or forearm of −2.5 or less (WHO, 1994) (Table I), and the Fracture Intervention Trial confirming that bisphosphonate treatment reduced fracture risk (Black *et al*., 1996). Since then a number of international trials have demonstrated the effectiveness of bisphosphonates (BPs) (Cummings *et al*., 1998; Harris *et al*., 1999; McClung *et al*., 2001; Chesnut *et al*., 2004; Black *et al*., 2006), selective estrogen receptor modulators (SERMs) (Ettinger *et al*., 1999), recombinant human parathyroid hormone (PTH) (Neer *et al*., 2001; Greenspan *et al*., 2007), and strontium ranelate (Meunier *et al*., 2004; Reginster *et al*., 2005; Seeman *et al*., 2006) in the prevention of fragility fractures.

THE CLINICAL ROLE OF BONE DENSITY MEASUREMENTS

Bone density measurements have an important clinical role in the evaluation of patients at risk of osteoporosis and in ensuring the appropriate use of antifracture treatment (Kanis *et al*., 1997; NOF, 1998; Genant *et al*., 1999; Kanis and Gluer, 2000). A helpful list of clinical indications for performing a bone density examination was published by the International Society for Clinical Densitometry (ISCD) and is summarized in Table II (ISCD, 2005). The most widely used method of patient investigation is DXA scanning of the lumbar spine and hip (Fig. 1A and B). BMD examinations have three principal roles, namely the diagnosis of osteoporosis, the assessment of a patient's risk of fracture, and monitoring response to treatment. The reasons for choosing to measure the hip and spine include

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TABLE I The WHO Definitions of Osteoporosis and Osteopenia (WHO, 1994)

Terminology	T-Score definition
Normal	$T \geq -1.0$
Osteopenia	$-2.5 < T < -1.0$
Osteoporosis	$T \leq -2.5$
Established osteoporosis	$T \leq -2.5$ in the presence of one or more fragility fractures

the fact that the hip is the best site for predicting hip fracture risk (Marshall *et al.*, 1996; Stone *et al.*, 2003; Johnell *et al.*, 2005), the spine is the best site for monitoring response to treatment (Eastell, 1998; Gluer, 1999), and the consensus that hip and spine BMD results should be interpreted by using the WHO *T*-score definition of osteoporosis (see Table I) (Kanis *et al.*, 1997; NOF, 1998; Genant *et al.*, 1999; Kanis and Gluer, 2000; ISCD, 2005). *T* scores are calculated by taking the difference between a patient's measured BMD and the mean BMD in healthy young adults, matched for gender and ethnic group, and expressing the difference relative to the young adult population standard deviation (SD):

$$T\text{-score} = \frac{\text{Measured BMD} - \text{Young adult mean BMD}}{\text{Young adult population SD}}$$

Other practical advantages of DXA scanning include short scan times, easy patient setup, low radiation dose, and good measurement precision. These and other advantages of spine and hip DXA are summarized in Table III. Most of the rest of this chapter is devoted to discussing these advantages in greater detail.

In addition to central DXA systems that measure the spine and hip, a wide variety of other types of bone densitometry equipment are also available (Genant *et al.*, 1996; Fogelman and Blake, 2000). These include quantitative computed tomography (QCT) measurements of the spine and hip (Guglielmi and Lang, 2002; Lang *et al.*, 2002), peripheral DXA (pDXA) systems for measuring the forearm, heel, or hand (Blake and Fogelman, 2002), and quantitative ultrasound (QUS) devices for measurements of the heel and other peripheral sites (Stewart and Reid, 2002). In principle, pDXA and QUS devices offer a quick, cheap, and convenient way of evaluating skeletal status that makes them attractive for widespread use. In practice, however, these alternative types of measurement correlate poorly with hip and spine BMD, with correlation coefficients in the range $r = 0.5$–0.7 (Lu *et al.*, 2001). This lack of agreement with measurements made using hip and spine DXA has proved a barrier to reaching a consensus on the best way of introducing these other methods into wider clinical practice (Lu *et al.*, 2001; Faulkner *et al.*, 1999).

TABLE II Indications for Bone Mineral Density (BMD) Testing (ISCD, 2005)

- Women aged 65 and older
- Postmenopausal women under age 65 with risk factors
- Men aged 70 and older
- Adults with a fragility fracture
- Adults with a disease or condition associated with low bone mass or bone loss
- Adults taking medication associated with low bone mass or bone loss
- Anyone being considered for pharmacological therapy
- Anyone being treated, to monitor treatment effect
- Anyone not receiving therapy in whom evidence of bone loss would lead to treatment

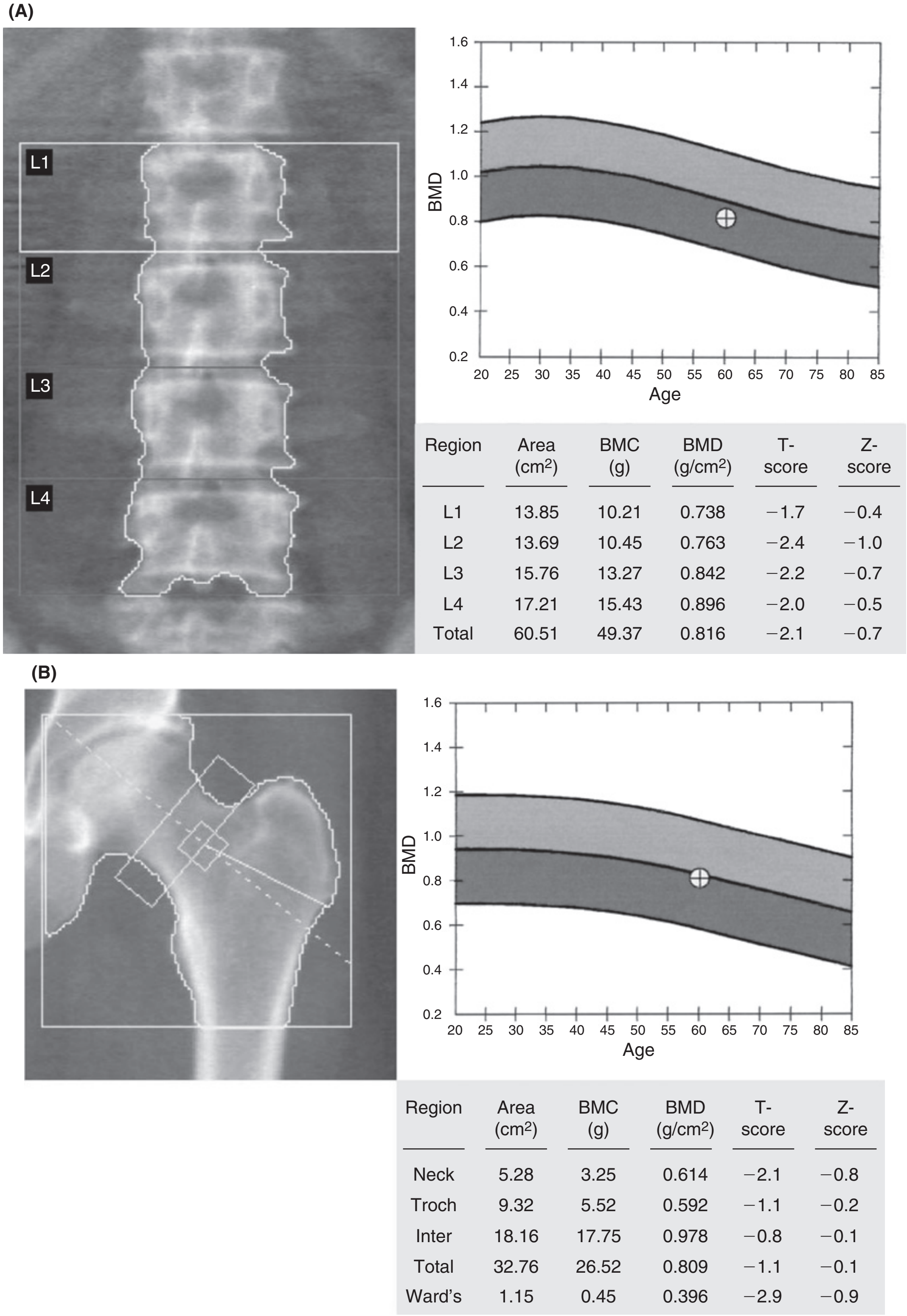
WHICH MEASUREMENT IS BEST?

Given the choice of so many different types of measurement, how do we decide which technique is the most effective for decisions about patient treatment? Fundamental to the clinical use of any type of bone densitometry examination is its ability to predict fracture risk, and the most reliable way to evaluate and compare the alternative techniques is through prospective studies of incident fractures (Marshall *et al.*, 1996). Figure 2 illustrates how data from a fracture study are analyzed to quantify the relationship between BMD and fracture risk. When the study subjects are divided into quartiles on the basis of their baseline BMD measurements, an inverse relationship is found between fracture risk and BMD. To describe this relationship the BMD figures are converted into *Z* scores. *Z* scores are similar to *T* scores except that instead of comparing the patient's measured BMD with the mean and SD for young adults, it is compared with the mean BMD and SD for healthy normal subjects matched for age, gender, and ethnic group:

$$Z\text{-score} = \frac{\text{Measured BMD} - \text{Age matched mean BMD}}{\text{Age matched population SD}}$$

Data from fracture studies are fitted by using a gradient-of-risk model in which the fracture risk increases exponentially with decreasing *Z* score (Fig. 2, *inset*). Results are usually expressed in terms of the relative risk (RR), which is defined as the increased risk of fracture for each unit decrease in *Z* score.

The larger the value of RR (or equivalently, the steeper the gradient of risk β in Fig. 2), the more effective a technique is at discriminating between patients who will suffer



Region	Area (cm²)	BMC (g)	BMD (g/cm²)	T-score	Z-score
L1	13.85	10.21	0.738	−1.7	−0.4
L2	13.69	10.45	0.763	−2.4	−1.0
L3	15.76	13.27	0.842	−2.2	−0.7
L4	17.21	15.43	0.896	−2.0	−0.5
Total	60.51	49.37	0.816	−2.1	−0.7

Region	Area (cm²)	BMC (g)	BMD (g/cm²)	T-score	Z-score
Neck	5.28	3.25	0.614	−2.1	−0.8
Troch	9.32	5.52	0.592	−1.1	−0.2
Inter	18.16	17.75	0.978	−0.8	−0.1
Total	32.76	26.52	0.809	−1.1	−0.1
Ward's	1.15	0.45	0.396	−2.9	−0.9

FIGURE 1 (**A**) Scan printout of a spine dual-energy x-ray absorptiometry (DXA) examination. The printout shows: (left) scan image of the lumbar spine; (*top right*) patient's age and bone mineral density (BMD) plotted with respect to the manufacturer's reference range; (*bottom right*) BMD figures for individual vertebrae and total spine (L1 − L4), together with the interpretation in terms of *T* scores and *Z* scores. (**B**) Scan printout of a hip DXA examination. The printout shows: (*left*) scan image of the hip; (*top right*) patient's age and total hip BMD plotted with respect to the National Health and Nutrition Examination Survey (NHANES III) reference range (Looker *et al.*, 1998); (*bottom right*) BMD figures for five different regions of interest in the hip (femoral neck, greater trochanter, intertrochanteric, total hip, and Ward's triangle), together with the interpretation in terms of *T* scores and *Z* scores by using the NHANES III reference range.

TABLE III Clinical Advantages of Hip and Spine DXA

- Proven ability to predict fracture risk
- Consensus that BMD results can be interpreted using WHO T scores
- Proven for effective targeting of antifracture treatments
- Effective for monitoring response to treatment
- Basis of new WHO algorithm for predicting fracture risk
- Many systems can perform vertebral fracture assessment
- Short scan times
- Easy patient setup
- Low radiation dose
- Good precision
- Availability of reliable reference ranges
- Stable calibration
- Effective instrument quality control procedures

a fracture in the future and those who will not. To understand the reason for this, consider a large group of subjects chosen randomly from the general population. For such a group the distribution of *Z*-score values approximates to a Gaussian curve (Fig. 3A). The distribution of *Z*-score values for the group of patients who will at some future date experience an osteoporotic fracture is found by multiplying the Gaussian curve representing the general population by the gradient-of-risk curve shown in the inset in Figure 2. When this is done the distribution of *Z*-score values for the fracture population is found to be a second Gaussian curve with the same SD as the first but with its peak offset to the left by an amount ΔZ equal to the gradient-of-risk β (or equivalently to the natural logarithm of RR) ($\Delta Z = \beta = \ln(\text{RR})$) (see Fig. 3A) (Blake and Fogelman, 2001).

To understand the importance of selecting a technique with a high RR value, consider choosing some arbitrary *Z*-score value in Fig. 3A as the threshold for making decisions about patients' treatment (for example, this might be the *Z*-score value equivalent to a *T* score of -2.5). The areas under the two curves can be evaluated to find the percentages of patients in the fracture population and the general population with BMD values below the chosen threshold. As the threshold is varied and the two percentages plotted against each other, we obtain a receiver operating characteristic (ROC) curve (see Fig. 3B) in which the percentage of true positives (those patients who will suffer a fracture in the future and were correctly identified to be at risk) is plotted against the percentage of false positives (those patients identified to be at risk but who never have a fracture). Fig. 3B is fundamental to understanding the clinical value of any type of bone density measurement used to identify and treat patients at risk of fracture. It shows that the larger the RR value of the measurement technique the more successful clinicians are at targeting preventive treatments on those patients at greatest risk of having a fracture.

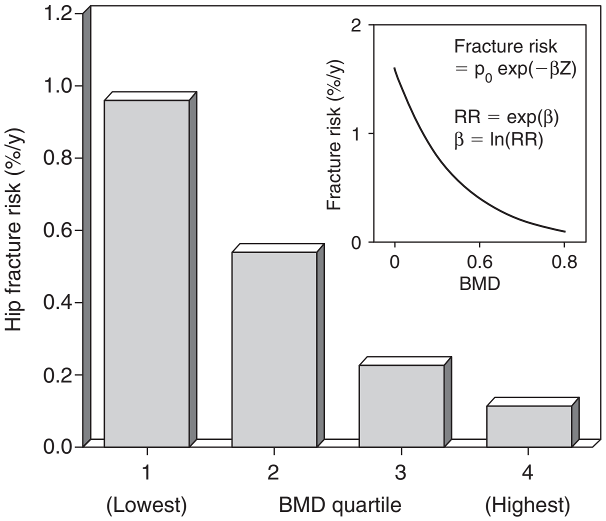


FIGURE 2 Incidence of hip fracture risk by bone mineral density (BMD) quartile for femoral neck BMD. Data are taken from the 2-year follow-up of the Study of Osteoporotic Fractures (SOF) (Cummings *et al.*, 1993). (*Inset*) Data from fractures studies are fitted using a gradient-of-risk model, in which the fracture risk varies exponentially with *Z* score with gradient β. Results are expressed in terms of the relative risk (RR), the increased risk of fracture for each unit decrease in *Z* score. The value of RR is found from β using the exponential function ($\text{RR} = \exp(\beta)$). Alternatively, the gradient of risk is found by taking the natural logarithm of RR ($\beta = \ln(\text{RR})$).

DATA FROM FRACTURE STUDIES

One of the important clinical advantages of DXA compared with other types of bone density measurements is that its ability to identify patients at risk of fracture has been assessed and proven in a large number of epidemiological studies (Marshall *et al.*, 1996; Stone *et al.*, 2003; Johnell *et al.*, 2005). The most informative studies are meta-analyses of prospective fracture studies. Two such meta-analyses have been published, the well known study by Marshall *et al.* (1996), and a more recent study by Johnell *et al.* (2005). The Marshall meta-analysis was based on more than 2000 osteoporotic fractures from 90,000 person-years of follow-up. The subjects were all women. The authors concluded that different BMD measurement sites all have a similar ability to predict fractures (RR = 1.5; 95% confidence interval: 1.4 to 1.6), with the exception of hip BMD predicting hip fractures (RR = 2.6; 95% CI: 2.0 to 3.5) (Fig. 4) and spine BMD predicting vertebral fractures (RR = 2.3; 95% CI: 1.9 to 2.8). The authors concluded that hip and spine BMD values were the best measurements for predicting

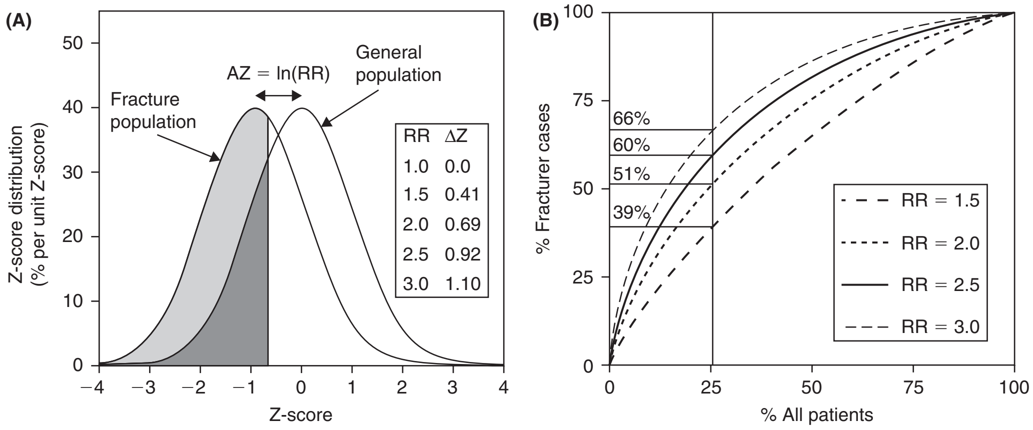


FIGURE 3 (**A**) Distribution of Z-score values in a fracture population compared with the age-matched general population. The curve for the general population is a bell-shaped curve symmetrically distributed around its peak at $Z = 0$. The corresponding curve for the population of patients who will suffer an osteoporotic fracture is a similar bell-shaped curve that is offset from the general population by a Z-score difference of $\Delta Z = \ln(\mathrm{RR})$, where RR = relative risk. The inset table lists values of RR and ΔZ. (**B**) Plot of the receiver operating characteristic (ROC) curves obtained by evaluating the areas under the two bell-shaped curves shown in A up to an arbitrarily chosen Z-score threshold and plotting the two areas against each other for different values of the relative risk (RR). The ROC curve shows the percentage of fracture cases that fall below the bone mineral density (BMD) threshold (shaded area under the fracture population curve in **A**) plotted against the percentage of subjects in the general population who fall below the same threshold (shaded area under the general population curve in **A**). It therefore shows the true-positive fraction (those patients who sustain a fracture and were correctly identified as being at risk) against the false-positive fraction (those patients identified as being at risk but who never actually have a fracture). The larger the value of RR, the wider the separation of the two curves in **A** and the more effective BMD measurements are at discriminating the patients who will have a fracture. For example, if patients in the lowest quartile of BMD are identified for treatment, then for RR values of 1.5, 2.0, 2.5, and 3.0 this group will include 39%, 51%, 60%, and 66%, respectively, of all patients who will suffer a fracture.

hip and spine fractures, respectively. A limitation of the Marshall study was that these latter conclusions were based on a relatively small number of fracture cases (80 hip fractures and 98 vertebral fractures, respectively).

The Johnell meta-analysis examined the relationship between hip fracture and hip BMD based on data from 12 different fracture studies from Australia, Canada, Europe, and Japan including both men and women (Johnell *et al.*, 2005). There were data on 971 hip fractures from a total of 168,000 person-years of follow-up. As would be expected given the much larger number of hip fractures, the statistical errors are considerably reduced and, consequently, the results are more informative. When corrected to the population SD of the female reference range of the Third National Health and Nutrition Examination Survey (NHANES III) (Looker *et al.*, 1998), the RR figure for men and women combined was 2.21 (95% CI: 2.03 to 2.41) (see Fig. 4). There was no significant difference between men and women (women: RR = 2.18; 95% CI: 1.99 to 2.39; men: RR = 2.28; 95% CI: 1.81 to 2.87). Interestingly, the relative risk figures decreased progressively with increasing age varying from RR = 3.68 (95% CI: 2.61 to 5.19) at age 50 to RR = 1.93 (95% CI: 1.76 to 2.10) at age 85. Relative risk figures for hip fracture did not vary significantly with the length of follow-up (0 to 10 years) or baseline Z score ($-4 < Z < +4$).

Among individual fracture studies, the most informative is the Study of Osteoporotic Fractures (SOF), a study of 9704 white U.S. women aged 65 years and older who

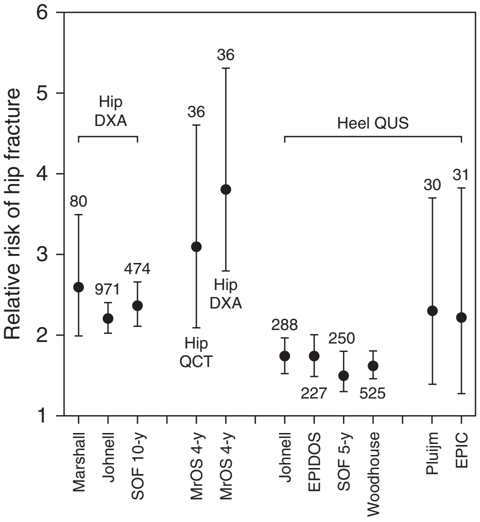


FIGURE 4 Values of the relative risk (RR) (defined as the increased risk of fracture for a 1 standard deviation decrease in bone mineral density) for hip fracture for: (1) hip DXA measurements [Marshall meta-analysis (Marshall *et al.*, 1996), Johnell meta-analysis (Johnell *et al.*, 2005), Study of Osteoporotic Fractures (SOF) 10-year study (Stone *et al.*, 2003)]; (2) QCT and DXA hip BMD measurements [MrOS study (Orwoll *et al.*, 2006)]; (3) heel QUS measurements [Johnell meta-analysis (Johnell *et al.*, 2005), EPIDOS study (Hans *et al*, 2004), SOF 5-year study (Black *et al.*, 2000), Woodhouse meta-analysis (Woodhouse *et al.*, 2000), Amsterdam study (Pluijm *et al.*, 1999), and EPIC study (Khaw *et al.*, 2004)]. The error bars show the 95% confidence intervals. The number above or below each data point shows the number of hip fractures in the study. The graph illustrates the importance of having a large number of fractures in order to reduce the error bars and allow a meaningful comparison between different bone densitometry techniques.

had baseline measurements of hip, spine, forearm, and heel BMD when the study commenced in the late 1980s (Stone *et al.*, 2003). One of the strengths of the SOF study is the large number of recorded fracture cases, with the recently published 10-year follow-up including 474 hip fractures and 2044 fractures at all sites. A second important strength is that the baseline measurements included a variety of bone densitometry sites including DXA of the hip and spine, peripheral absorptiometry of the forearm and heel, and QUS of the heel. A large number of fracture cases are essential for achieving adequate statistical power if meaningful comparisons are to be made between different bone density techniques. This is illustrated in Fig. 4, which shows RR values for hip fracture from various studies with their 95% confidence intervals and the number of fractures recorded in the study. As the SOF study has progressed the results have consistently confirmed the ability of hip BMD measurements to predict hip fracture risk with an RR value of about 2.5 (Cummings *et al.*, 1993; Black *et al.*, 2000; Stone *et al.*, 2003). The 10-year follow-up data confirm the association between BMD and fracture risk with high statistical reliability for many types of fracture and show that the prediction of hip fracture risk from a hip BMD measurement has the largest RR value and is the most effective single type of DXA examination (Fig. 5) (Stone *et al.*, 2003).

In comparison with DXA, until recently there were no prospective studies of QCT and fracture risk. However, the first results of a prospective study of QCT and hip fracture risk from the Osteoporotic Fractures in Men (MrOS) study were recently announced (Orwoll *et al.*, 2006). The MrOS study enrolled 5995 white men aged 65 and older from six U.S. centers. As well as baseline DXA scans, 3357 men had spine and hip QCT scans. The first results based on 36 hip fracture cases recorded after an average follow-up period of 4.4 years show comparable RR values for femoral neck BMD measured by QCT or DXA (see Fig. 4). As can be seen, because of the small number of fracture cases so far recorded, the statistical errors are still too large to make any meaningful comparison between QCT and DXA.

In contrast with QCT, there are a large number of published studies of QUS and fracture risk (Hans *et al.*, 1996; Bauer *et al.*, 1997; Pluijm *et al.*, 1999; Woodhouse *et al.*, 2000; Bauer *et al.*, 2001; Miller *et al.*, 2002; Khaw *et al.*, 2004; Hans *et al.*, 2004; Durosier *et al.*, 2006). The Johnell meta-analysis includes QUS data from two cohorts (EPIDOS and Sheffield) with a total of 288 hip fractures (Johnell *et al.*, 2005). RR values were 1.74 (95% CI: 1.53 to 1.97) for broadband ultrasonic attenuation (BUA) and 1.50 (95% CI: 1.31 to 1.70) for speed of sound (SOS). These and some other data for QUS are plotted in Fig. 4. Comparison of the various results plotted in Fig. 4 illustrate the importance of having studies with a large number of recorded fracture cases in order to reduce the error bars and allow a meaningful comparison between different techniques.

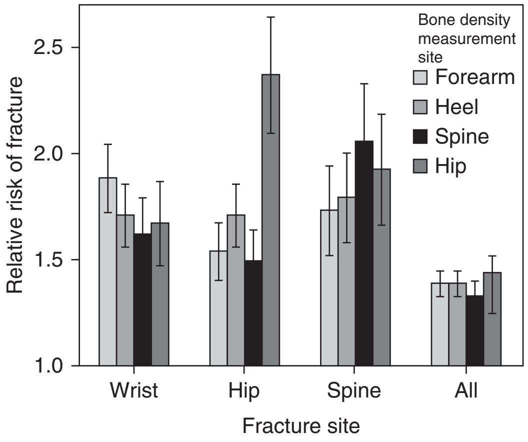


FIGURE 5 Values of the relative risk (RR) [defined as the increased risk of fracture for a 1 standard deviation decrease in bone mineral density (BMD)] for fractures at different skeletal sites (wrist, hip, spine, and any fracture) for BMD measurements made at four different sites (forearm, heel, spine, and femoral neck). The errors bars show the 95% confidence intervals. Data are taken from the 10-year follow-up of the Study of Osteoporotic Fractures (SOF) study population (Stone *et al.*, 2003). In the SOF data the largest value of RR is for the prediction of hip fracture risk from a hip BMD measurement (RR = 2.4). From the ROC curves shown in Figure 3B this means that the clinically most effective DXA scan measurement is to use hip BMD to predict hip fracture risk.

APPROPRIATE TARGETING OF ANTIFRACTURE TREATMENTS

Another of the clinical advantages of hip and spine BMD scans are their proven ability to identify patients who will respond successfully to treatments for preventing fractures. Table IV lists the principal clinical trials of the pharmaceutical agents proven to prevent vertebral and/or nonvertebral fractures. It is notable that all of the trials listed enrolled patients on the basis of study entry criteria that included a DXA scan *T* score at the hip or spine demonstrating either osteoporosis or severe osteopenia. In a number of these trials the data analysis showed that treatment was effective only in those subjects with a hip or spine *T* score of −2.5 or less (Cummings *et al.*, 1998; McClung *et al.*, 2001; Chesnut *et al.*, 2004; Reginster *et al.*, 2005). These findings have created difficulty in selecting patients for treatment using techniques other than hip or spine DXA because of the poor correlation between different techniques and the lack of evidence that patients selected by using other techniques will respond to treatment (Barr *et al.*, 2005).

AVAILABILITY OF RELIABLE REFERENCE RANGES

For the past 10 years the interpretation of DXA scans has been guided by the WHO *T*-score definition of osteoporosis (see Table I). However, if scan results are to be interpreted reliably care is necessary in the choice of reference

TABLE IV Fracture Prevention Studies That Have Selected Patients Using Central DXA

Class of agent	Name of drug	Study name[*]	T-score thresholds for patient enrollment[†]
Bisphosphonate	Alendronate	FIT 1	Femoral neck T score < -1.5[‡]
		FIT 2	Femoral neck T score < -1.5
	Risedronate	VERT NA	Spine T score < -2[‡]
		HIP	Femoral neck T score < -3.2[§]
	Ibandronate	BONE	Spine T score in range -2 to -5[‡]
	Zoledronate	HORIZON	Femoral neck T score < -2.5[‡]
Selective estrogen receptor modulator	Raloxifene	MORE	Spine or femoral neck T score < -1.8[‡]
Parathyroid hormone	PTH (1–34)	Neer study	Spine or femoral neck T score < -1[‡]
	PTH (1–84)	TOP	Spine or femoral neck T score < -2.5[‡]
Strontium	Strontium ranelate	SOTI	Spine T score < -1.9[‡]
		TROPOS	Femoral neck T score < -2.2

[*] FIT, *Fracture Intervention Trial* (Black et al., 1996; Cummings et al., 1998); VERT NA, *Vertebral Efficacy with Risedronate Therapy* (North America) *study* (Harris et al., 1999); HIP, *Risedronate Hip Study* (McClung et al., 2001); BONE, *Oral Ibandronate Osteoporosis vertebral fracture trial in North America and Europe* (Chesnut et al., 2004); HORIZON, HORIZON *Pivotal Fracture Trial* (Black et al., 2006); MORE, *Multiple Outcomes of Raloxifene Evaluation* (Ettinger et al., 1999); TOP, *Treatment of Osteoporosis with Parathyroid Hormone* (Greenspan et al., 2007); Neer study (Neer et al., 2001); SOTI, *Spinal Osteoporosis Therapeutic Intervention* (Meunier et al., 2004). TROPOS, *Treatment of Peripheral Osteoporosis* (Reginster et al., 2005).

[†] *T score thresholds are those calculated using the* NHANES III *reference range for the hip and the Hologic reference range for spine* BMD.***

[‡] *Study entry criteria also included prevalent vertebral fractures.*

[§] *Study entry criteria also included clinical risk factors.*

data for the calculation of *T* scores. For consistency, ISCD recommends the use of the NHANES III reference database (Looker *et al*., 1998) for *T*-score derivation in the hip (ICRP, 2005). This recommendation was made following the publication of a study comparing the spine and hip *T*-score results obtained on the two principal brands of DXA scanner (manufactured by GE-Lunar and Hologic) and calculated by using the manufacturers' reference ranges (Faulkner *et al*., 1996). Although good agreement was found for spine *T* scores measured on the two manufacturers' systems, a systematic difference of almost one *T*-score unit was found between the hip *T* scores. The discrepancy was resolved by all the manufacturers agreeing to use the NHANES III hip reference range (Hanson *et al*., 1997), which is based on measurements of more than 14,000 randomly selected men and women from across the entire United States. There was insufficient time in the NHANES III study to perform spine as well as hip scans, so spine BMD results are generally interpreted by using the manufacturers' reference ranges.

Comparison of reference ranges for pDXA equipment for the same anatomical site can show surprisingly large differences in the plots of mean *T*-score against age owing to factors that include the use of inappropriate populations, different conventions for deriving the reference curve from the data, and insufficient numbers of subjects (Blake *et al*., 2005). When the principal DXA manufacturers adopted the NHANES III hip BMD reference range with its large, randomly selected population this was an important factor in improving confidence in the interpretation of scan results.

INTERPRETATION OF *T* SCORES USING THE WHO CRITERIA

As explained earlier, one of the important clinical advantages of DXA is the widespread consensus that spine, hip, and forearm BMD measurements should be interpreted using the WHO *T*-score definition of osteoporosis (see Table I). However, the WHO definition should not be applied to QCT or QUS measurements, or pDXA results at sites other than the 33% radius (ICRP, 2005). The reason why this rule is so important can be understood from Figure 6. When the reference ranges for different types of bone density measurement are plotted as graphs of mean *T* score against age, the curves obtained are found to be very different for different techniques (Faulkner *et al*., 1999) (see Fig. 6). For example, the curve for spine QCT decreases rapidly with age and crosses the WHO threshold of $T = -2.5$ at age 60. This means that if QCT measurements were interpreted using the WHO definition 50% of 60-year-old women would be diagnosed with osteoporosis. In contrast, for some types of heel pDXA and QUS measurements the curve decreases so slowly with age that patients need to be age 100 before 50% of them have osteoporosis. For spine, femoral neck, and 33% radius DXA measurements the three curves decrease in a similar manner crossing the $T = -2.5$ threshold at age 75. It is clear that if care is not taken in applying the WHO criteria appropriately then cases of osteoporosis may be either seriously underdiagnosed or overdiagnosed depending on the measurement technique (Lu *et al*., 2001). In principle, measurements other

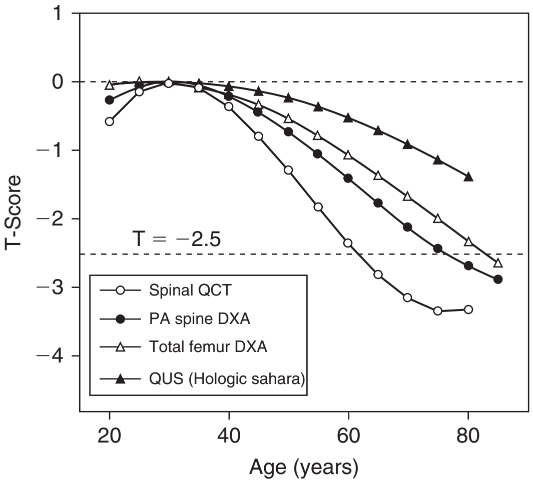


FIGURE 6 Age-related decline in mean T scores at different bone mineral density sites for healthy white female subjects. The hip DXA data are taken from the National Health and Nutrition Examination Survey (NHANES III) study (Looker *et al.*, 1998; Hanson, 1997). The DXA normative data for the lumbar spine (L1−L4) and forearm (total forearm region) were obtained from the Hologic manufacturer's reference ranges. Heel data are for the Hologic Sahara device. Spinal QCT is that used by the Image Analysis reference system. Filled circles, lumbar spine; open triangles, total hip; open circles, QCT spine; filled triangles, QUS heel.

than spine and hip DXA can be used with appropriate device-specific thresholds to identify a group of patients with high peripheral BMD that are unlikely to be at risk, and another group with low BMD and who can be treated for osteoporosis. Patients with intermediate peripheral BMD results can be referred for a central DXA examination for a definitive diagnosis. However, the clinical application of this triage algorithm requires the availability of adequate information about the device-specific thresholds (Blake *et al.*, 2005).

THE NEW WHO FRACTURE RISK ALGORITHM

Views on the best way of using the information from DXA scans to advise patients about the use of antifracture treatment continue to evolve (Kanis *et al.*, 2002; Kanis, 2002; Kanis *et al.*, 2005a; De Laet *et al.*, 2005a). As emphasized earlier, the clinical value of a BMD examination lies in the information it provides about fracture risk. An important limitation of the WHO *T*-score approach to making decisions about patient treatment is that age as well as BMD is an important factor in determining the patient's short-term risk of having a fracture (Kanis *et al.*, 2001a, 2002, 2005a). For any hip *T*-score figure, fracture risk in men and women between the ages of 45 and 85 years varies greatly according to age (Kanis *et al.*, 2001a, 2002). A new approach to the use of BMD scans to guide treatment decisions is based on the ten-year probability of the patient sustaining an osteoporotic fracture (Kanis *et al.*, 2001a, 2005a). This has a number of important advantages, including the targeting of osteoporosis treatment according the patient's risk of fracture (Kanis *et al.*, 2002), the incorporation of additional risk factors such as a history of prior fracture to refine the algorithm for estimating fracture risk (Kanis *et al.*, 2005a), and the use of health economic criteria to set intervention thresholds based on the costs of treatment, savings to health services, and the contribution of fracture prevention to patients' quality of life (Kanis, 2002).

TABLE V Clinical Risk Factors Included in WHO Fracture Algorithm (Kanis *et al.*, 2005a)

- Age
- Low body mass index
- Prior fracture after age 50
- Parental history of hip fracture
- Current smoking habit
- Current or past use of systemic corticosteroids
- Alcohol intake >2 units daily
- Rheumatoid arthritis

The value of taking account of additional risk factors that give independent information about fracture risk over and above that provided by age and BMD can be explained by reference to the ROC curve shown in Fig. 3B. With all types of bone densitometry measurement, the fracture and nonfracture patients have overlapping BMD distributions (see Fig. 3(A), leading to ROC curves (see Fig. 3B) in which, at any given *T*-score threshold, only a certain percentage of future fracture cases are identified for treatment at the cost of also having to treat a large number of patients who are not going to have a fracture. As explained earlier, the best that can be done with bone densitometry alone is to choose the BMD measurement site with the highest RR value that will optimize the ROC curve. However, by combining BMD data with age and other appropriately chosen risk factors (Table V), the ROC curve can be further improved so that treatments are better targeted on the patients at highest risk.

The new WHO fracture risk algorithm is based on a series of meta-analyses of data from 12 independent fracture studies from North America, Europe, Asia, and Australia (Kanis *et al.*, 2004b, 2004c, 2004d, 2005b, 2005c; De Laet *et al.*, 2005b). The DXA scan information required is femoral neck BMD. Because of the need to build the correct parameters into the statistical model, including the interdependence of the various risk factors, there is a specific requirement that the BMD information is provided by a hip DXA scan. The reliance on BMD information from a single skeletal site raises the important question of whether fracture risk prediction is improved by combining BMD measurements from more than one site. A meta-analysis of spine and femoral neck BMD data showed that use of the lowest *T* score did not improve the ROC curve (Kanis *et al.*, 2006). This finding

TABLE VI Comparison of Different Bone Densitometry Techniques

	Central DXA	Peripheral DXA	QCT	QUS
Compatible with WHO T scores	✓	×	×	×
Proven to predict fracture risk	✓	✓	?	✓
Compatible with new WHO fracture risk algorithm	✓	×	×	×
Proven for effective targeting of treatment	✓	?	?	?
Suitable for patient follow-up	✓	×	?	×
Stable calibration	✓	✓	✓	×
Good precision	✓	✓	✓	×
Reliable reference ranges available	✓	?	?	?

is perhaps surprising, but mathematical analysis provides the reason: although hip and spine BMD measurements are quite poorly correlated ($r = 0.5$ to 0.7), even this degree of correlation is too high for a second BMD site to provide significant additional information about fracture risk (Blake *et al.*, 2003). A further point that follows from the WHO fracture risk algorithm is that not all patients necessarily require a DXA scan (Johansson *et al.*, 2004). For some the use of age, fracture history, and the other risk factors is sufficient to place them in either the high-risk group requiring antifracture treatment, or the low-risk group who can be reassured that their likelihood of having a fracture is small. Thus, in future, a triage approach could be adopted for BMD scanning in which the fracture risk algorithm is used to select those patients for a DXA examination in whom BMD information is most likely to contribute to their management.

Another advantage of the new WHO algorithm is that it enables fracture risk thresholds for intervention to be established based on economic criteria that can be adjusted for practice in different countries (Kanis *et al.*, 2001b; Borgstrom *et al.*, 2006a). A series of health economic analyses have examined the rationale for fracture prevention and the cost effectiveness of different osteoporosis treatments (Kanis *et al.*, 2005d, 2005e; Borgstrom *et al.*, 2006b, 2006c; Zethraeus *et al.*, 2007). These analyses show that, taking account of all types of fracture, the cost-effective intervention thresholds correspond to T-score values between −2 and −3 over a range of ages from 50 to 80 years (Kanis, 2002; Kanis *et al.*, 2005a). At the present time it is unclear how quickly the new fracture risk approach will become the new paradigm for the management of osteoporosis.

MONITORING RESPONSE TO TREATMENT

Verifying response to treatment using follow-up DXA scans is widely believed to have a beneficial role in encouraging patients to continue taking their medication, and also in identifying nonresponders who may benefit from a different treatment regimen. DXA has a number of advantages as a technique for monitoring patients' response, of which one of the most important is the good precision of the measurements. Precision is usually expressed in terms of the coefficient of variation (CV) which is typically about 1% to 1.5% for spine and total hip BMD and 2% to 2.5% for femoral neck BMD (Patel *et al.*, 2000). DXA scanners have good long-term precision because, among other reasons, their calibration is extremely stable and there are effective instrument quality control procedures provided by manufacturers to detect any long-term drifts (see Table III). A second requirement for effective patient monitoring is a BMD measurement site that shows a large response to treatment. The best DXA site for follow-up measurements is the spine because the treatment changes are usually largest and the precision error is as good as or better than that at most other sites (Faulkner, 1998; Blake *et al.*, 1996).

SUMMARY AND CONCLUSIONS

Table VI compares and contrasts the clinical and technical advantages of central DXA scans compared with alternative types of bone densitometry measurement such as QCT, pDXA, and QUS. A tick sign (✓) indicates where an alternative technique is known to perform in a comparable manner to central DXA. For example, there is strong evidence that pDXA and QUS can effectively predict fracture risk, although the presently available evidence suggests that the optimum measurement is the use of hip DXA to predict hip fracture risk (see Figs. 3 and 4). A question-mark sign (?) indicates where our knowledge is limited by an absence of suitable studies. For example, it is quite possible that pDXA, QCT, and QUS can effectively target patients for fracture prevention treatment. However, because no studies have been published we simply do not know whether patients treated on the basis of these techniques achieve the same reduction in fracture risk as those treated on the basis of a central DXA examination. Finally, a crossout sign (×) indicates that alternative types of measurement are definitely unsuitable in these roles. For example, pDXA (with the exception of the 33% radius), QCT, and QUS measurements cannot be interpreted by using the WHO *T*-score definition of osteoporosis and are also unsuitable for use with the new WHO fracture risk algorithm. It is clear that there are important clinical and practical reasons why central DXA scanning should continue to be the preferred method of performing bone densitometry examinations and that as the osteoporosis community adopts the new fracture risk approach to treating patients these arguments will become even stronger.

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Chapter 89

Controversial Issues in Bone Densitometry

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Bone densitometry measurements performed by dual-energy x-ray absorptiometry (DXA) have provided the foundation for treatment of patients with osteoporosis (Miller and Bonnick, 1999; Miller *et al.*, 1999; Miller and Leonard, 2007).

DXA utilization formed the framework for the criteria to diagnose osteoporosis in the postmenopausal population (PMO) before the first fragility fracture has occurred (Kanis JA *et al.* 1994. These criteria may now be applied to men after the age of 50 years and to patients with secondary causes of osteoporosis (Lenchick *et al.*, 2002; Kahn *et al.*, 2004). DXA is also an important tool for fracture risk prediction and for monitoring the course of untreated osteoporosis or pharmacological therapy to treat this disease (Miller *et al.*, 2005; Miller, 2005; Bonnick and Shulman, 2006; Lewiecki, 2003; Miller, 2007). The international application of DXA in the clinical as well as the technical fields has been spearheaded by the International Society for Clinical Densitometry (ISCD) and the ISCD principles of quality control and clinical interpretation have been adopted by multiple medical and professional societies. In addition, the ISCD has initiated and completed four adult and the first pediatric position development conferences (PDCs) (Baim *et al.*, 2008). These intense assessments of data have set standards and guidelines for performance and interpretation of DXA. As knowledge in densitometry and osteoporosis expands and concepts evolve, controversies develop as well. In the earlier edition of this chapter, four controversial issues were discussed:

1. Discrepancies in *T* score determination among bone densitometers,
2. Prevalence versus risk in reporting bone mineral density device results;
3. Differences in ethnic and gender-specific databases among different bone density devices; and,
4. The value and limitations of serial bone mineral density measurements.

In this revised edition, I provide a brief update of the aforementioned controversies and also address new controversies in the field of bone mass measurements:

1. Using *Z* scores (age-matched) rather than *T* scores (young-normal) to define prevalence and/or risk in younger (premenopausal women, younger men, and adolescent and pediatric populations).
2. DXA as opposed to other emerging technologies to define "bone quality," bone strength, and risk prediction.
3. Utilization of vertebral fracture assessment (VFA) by DXA to define risk independent of BMD.
4. Application of the World Health Organization (WHO) 10-year absolute fracture risk algorithm in clinical practice.

DISCREPANCIES IN *T*-SCORE DETERMINATION AMONG BONE DENSITOMETERS

There has been little progress since the first edition of this chapter in standardization of young-normal reference population databases for the spine, forearm, or total body bone mineral density (BMD. In addition, despite the recognized validation and worldwide utilization of peripheral bone mass measurement devices for risk assessment, a consistent young-normal reference population database among all of these devices is lacking, leading to discrepant *T*-scores among these devices even in the same population or individual patient.

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Despite this lack of a common young-normal reference population database for central DXA of the spine between the two major DXA manufacturers (Hologic and Lunar-GE), a recently published detailed analysis suggests that there is agreement (same women classified by WHO criteria the same way) for *T* scores in 90% for the spine even with these discrepant young-normal reference population databases for the spine. Using the common reference population database (National Health and Nutrition Examination Survey III, NHANES III) for the hip (Kiebzak *et al.*, 2007) the agreement is 92% at the total hip. However, it is important to point out that in this study, all diagnostic disagreements occurred at the transition points between normal and osteopenia and between osteopenic and osteoporosis classifications. The extent of disagreement in the total hip was 0.11 SD and in the spine 0.32 SD. Although this study emphasizes diagnostic agreement in *T*-score calculation, it also points out that there may be the potential for disagreement among DXA manufacturers. In clinical practice, this may become important in the International Classification of Diseases (ICD-9) diagnostic classifications. Health care providers, health economists, and patients use the WHO diagnostic label to define disease severity as well as often determining payment thresholds for pharmacological intervention, e.g., a shift in a *T* score from 2.49 to 2.50, changes the WHO classification from osteopenia to osteoporosis and has large diagnostic but not risk implications. In addition, these observations highlight the importance of interpreting BMD or *T* scores within the clinical context of other data relevant to the patients' clinical state. In other words, the same *T* score can be interpreted quite differently depending on other factors in the patient's evaluation for other risk for fractures (Kanis *et al.*, 2001).

There are other issues, however, even if one were to have available a common database for all sites. We have an example, actually, because hip *T* scores have been standardized among the manufacturers. Although one might expect, therefore, that hip measurements among different DXA instruments would result in "perfect agreement," *T* scores at the hip still vary. Why?

The entire NHANES III database was performed on Hologic DXA machines (Looker *et al.*, 1998). The *young-normal* reference population used in NHANES III for *T*-score calculation is, therefore, derived from Hologic calibrations and software. When these Hologic data are incorporated into Lunar-GE DXA systems using different statistical software (GE Encore software, version 7.0 to 8.8), it modified the reference SDs, which, in turn, created errors of about 0.5 SD at the femoral neck and about 0.7 SD at the trochanter with the *T*-score values being lower for GE-Lunar than Hologic (Binkley *et al.*, 2005). This issue has not yet been rectified. Yet, for most people, this discrepancy is not a clinical problem. Nevertheless, these papers, as well as the following paper comparing *T* scores with *Z* scores in *young-normal* populations between DXA manufacturers, highlight the value and need for standardization, an issue recently highlighted by the joint workshop held by the National Institutes of Standardization and Technology and the ISCD (Bennett *et al.*, 2006). For clinicians, the knowledge that *T* scores may not always be identical even at the hip and hip regions between manufacturers in the same patient is important to recognize (Ahmed *et al.*, 2007; Ribom *et al.*, 2008).

For similar reasons, the *T* score and *Z* score may not be the same (as they theoretically should be) in the *young-normal* (20- to 49-year-old population) when measured even on the same DXA manufacturer. The line of identity between these standard deviation scores should be unity if the *T* and *Z* scores are calculated in *young-normal* healthy patients after peak adult bone mass is achieved and before bone loss has occurred (20 to 49 years) and if the SD score is derived from the same *young-normal* reference population database as shown in the equations for these calculations:

$$\frac{T\ \text{score} = \text{patients BMD} - \text{mean BMD of the young age-matched reference population}}{\text{SD of the aged-matched references population}}$$

$$\frac{Z\ \text{score} = \text{patients BMD} - \text{mean BMD of the young age-matched reference population}}{\text{SD of the aged-matched reference population}}$$

However, observations that the *T* and *Z* may not be identical in the same young patient even on the same DXA manufacturer has recently been suggested by a large cross-sectional study reported by Carey *et al.* from The Cleveland Clinic (Carey *et al.*, 2007). These investigators compared *T* and *Z* scores between Hologic and Lunar-GE systems in 4275 women, and mostly aged 20 to 49 years. There were significant differences between *T* and *Z* scores at the lumbar spine, and all hip regions using either DXA manufacturer ($P < 0.001$) (Figs. 1 and 2). The differences were at times large (range, -1.95 to $+1.54$ standard deviations), and remained when stratified by decade, even for the 20- to 29-year-old age group where there is agreement that this is the decade of achievement of peak adult bone mass and where there is no bone loss. The reasons for these discrepancies are not well defined but are probably multifactorial: different *young-normal* reference population databases even within the same DXA manufacturer; methods of adjustments for NHANES III in Lunar-GE or adjusting for body weight; or statistical methodology used to fit the reference population curves. The importance of statistical methodology has also been recently highlighted by several experts in this area (Shepherd and Blake, 2007a, 2007b; Kiebzak *et al.*, 2007). The solution lies in standardization of reference population databases both within the same and between DXA manufacturers and consistent statistical methods for fitting regression lines, because even

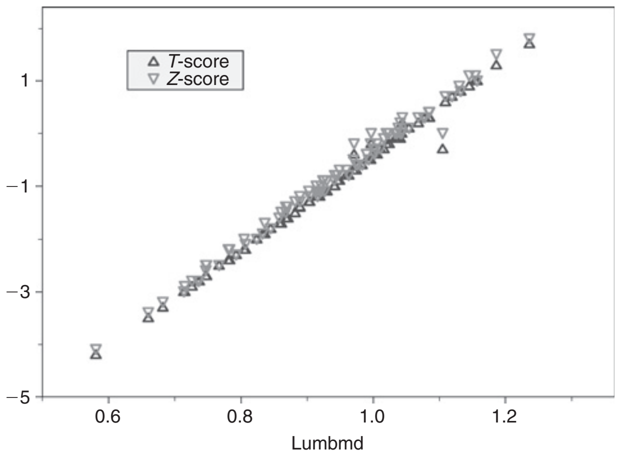

FIGURE 1 Relationship of spine *T* score and *Z* score to BMD value for 20- to 29-year-old females scanned using hologic DNA technology.

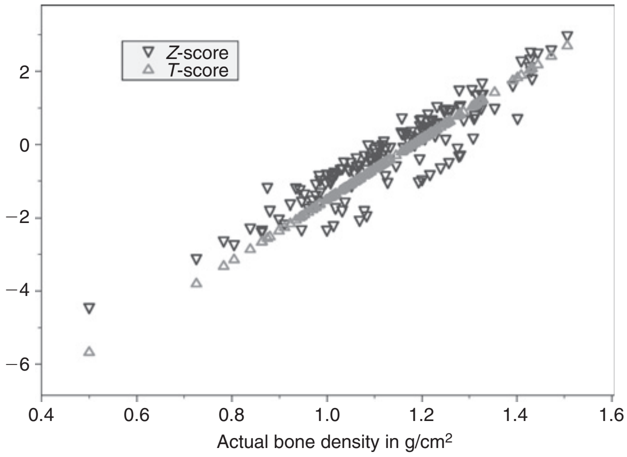

FIGURE 2 Relationship of *T* scores and *Z* scores to actual spinal BMD for 20- to 29-year-old females scanned using lunar, GE DXAs.

very small differences in the SD of the reference population can profoundly influence the calculated *T* or *Z* score. In the meantime, the ISCD Position Development Guidelines to use *Z* scores rather than *T* scores for *young-normal* and the pediatric populations to classify degrees of low bone mass is the correct position (Baim *et al*., 2008). This ISCD recommendation is, in part, based on the knowledge that the utilization of *T* scores, intended in 1994 for defining the prevalence of osteoporosis or osteopenia in the postmenopausal and aging population, but used in young-normal healthy populations that may have low peak adult bone mass but no increase in the risk for fracture, would lead to overdiagnosis of osteoporosis and overtreatment in young healthy individuals with very low fracture risk. In addition, the utilization of a *T* score in patients under the age of 50 years often classifies an otherwise healthy patient with a "disease" that is not even present.

The ISCD PDCs (including the first Pediatric PDC) have, however, provided suggestions of how to clinically employ the *Z* score for patient treatment in the young population, emphasizing that osteoporosis cannot be diagnosed based on a BMD (*Z* score) level alone. For the pediatric population the diagnosis of osteoporosis requires the presence of a clinically significant low trauma fracture in the lower extremity or more than one fracture in the upper extremity and that a *Z* score less than −2.0 can be called "low bone density" after adjusting for age, body size, and gender. In addition the ISCD Pediatric PDC wisely cautions against making therapeutic decisions based on a BMD alone. Therapeutic intervention decisions must be made in the context of the total clinical picture of the patient.

DXA AS OPPOSED TO OTHER EMERGING TECHNOLOGIES TO DEFINE BONE MASS, BONE "QUALITY," AND RISK ASSESSMENT

Single-energy x-ray absorptiometry (SXA) of the forearm was the first technology utilized in prospective longitudinal studies to show the association between degrees of low bone mineral density and fracture risk prediction in the postmenopausal population (Hui *et al*., 1998). In addition, this landmark study was the first to show that with ageing fracture risk increases at equivalent levels of BMD. Since these pivotal studies, several decades of data have been accumulated consistently documenting the reliability of determining areal BMD to predict fracture risk in postmenopausal women and in men aged 50 years and older in multiple population studies, as well as in virtually all clinical trials of osteoporosis (Black *et al*., 1992; Bonnick, 1998; Marshall *et al*., 1996; Melton, 1998; Ross *et al*., 1998; Barrett-Conner *et al*., 2005; Cauley *et al*., 2005; Kanis *et al*., 2001, 2002; Siris *et al*., 2004; Miller *et al*., 2004). The power of these DXA-derived data led to the widespread application of DXA for diagnosis and risk assessment in clinical practice. In addition, trust in the consistent capacity of DXA to treat patients with osteoporosis led to reimbursement for specific indications by most private and governmental health care authorities and to recommendations by the U.S. Prevention Services Task Force (USPSTF) and the U.S. Surgeon General's Report on The Status of America's Bone Health that DXA be used for population screening in the postmenopausal population over 60. In fact, DXA is the second medical technology embraced by U.S. policy for mass screening—the first being mammography for the detection of breast cancer (USPSTF, 2006; U.S. Surgeon General, 2006). Central DXA of the spine, hip, and forearm as well as a peripheral DXA of the forearm were the first devices that defined the relationship between prevalence of low bone mass at different cut points (e.g., the "*T* score") and the lifetime risk for hip, forearm, and clinical vertebral fracture (Miller and Leonard, 2007). This type of data has not been developed with any other bone mass measurement device [non-forearm peripheral DXA, ultrasound, central or peripheral quantitative computerized tomography (QcT)]. It is for this reason that WHO diagnostic criteria are to be used only with central or forearm DXA.

However, even though two-dimensional measurements of bone mineral density captures about 80% of the bone strength as measured by biomechanical strength testing (Blake *et al*., 2006; Riggs *et al*., 2006), there are clear observations that other factors, independent of areal DXA measurements or even true volumetric bone density (g/cm^3) contribute to bone strength. These non-BMD factors have generally been lumped under the term "bone quality" (Bouxsein, 2003). In the past 10 years many technologies have been developed to attempt to capture these non-BMD factors, including high-resolution peripheral quantitative computerized technology (HRpQCT); macro and micro ultrasound, micromagnetic resolution imaging (MRI); and hip structural analysis (HSA) (Kleerekoper, 2006; Genant and Jiang, 2006; Jiang *et al*., 2005; Hans *et al*., 2003; Krieg *et al*., 2008; Hans *et al*., 2008; Wehrli *et al*., 2006; Kazakia, 2007; Prevrhal *et al*., 2008; Bonnick, 2007; Yates *et al*., 2007).

The challenge for all of these methodologies is to determine whether they are also useful in predicting fracture risk and whether measurements by these different technologies are independent of BMD measurements performed by DXA. Both ultrasound of different skeletal sites, peripheral DXA (pDXA), peripheral quantitative computerized tomography (pQcT), and spine QCT have been shown in head-to-head clinical studies to predict fracture risk in specific populations as well as central (spine and hip) DXA (Genant and Jiang, 2006; Krieg *et al*., 2008; Hand *et al*., 2008). In the National Osteoporosis Risk Assessment (NORA) longitudinal population study, peripheral devices predicted the relative risk for all (global) fractures with the same predictive value and area under the curve in receiver operating analysis (ROC) as central DXA (Miller *et al*., 2002). Although micro-MRI and micro-CT can quantitative the extent of bone properties such as trabecular architecture and separation, there are no head-to-head fracture studies in human beings comparable to central DXA. Although hip structural analysis (HSA) has been shown in clinical trials to be an independent measure of hip fracture risk (independent of hip DXA), there are no data in population studies to validate HSA to predict risk. Moreover, there are inconsistencies in the results obtained by several currently available HSA software programs.

A major challenge is to develop practical technologies to measure components of bone strength that are independent of BMD. If this challenge can be met, many paradoxes such as fractures occurring at "normal" DXA-derived *T* scores could be explained. In addition, means to study and apply measurements of bone quality (and the more relevant equivalent, bone strength) will help explain how our osteoporosis therapies reduce fracture risk in ways that appear to be independent of DXA-determined changes in BMD.

ETHNIC AND GENDER DIFFERENCES: PREVALENCE VERSUS RISK

The WHO criteria for the diagnosis of osteoporosis were established in Caucasian postmenopausal women (Miller and Leonard, 2007). However, as bone mass measurement technologies have evolved, manufacturers of DXA equipment have incorporated various ethnic as well as gender-specific reference population databases for both *T*- and *Z*-score calculation. It is therefore important to consider how the initial WHO criteria can be applied to non-Caucasian female and in male populations. Currently the ISCD suggests using a non-race-adjusted (Caucasian-only) reference population database for risk assessment (Binkley *et al*., 2002). Much of this recommendation is based on historical knowledge that for many years in the early development of densitometry, the manufactures only had Caucasian reference databases; and that in the NORA head-to-head multiethnic fractures study (the only head-to-head long-term population-based fracture study available among multiple ethnicities of postmenopausal women) the relative risk (RR) for global fracture risk per SD reduction in BMD from the *young-normal* Caucasian reference database (*T* score) was similar among five U.S. ethnicities: Caucasian, Hispanic, Asians, African Americans, and Native Americans (Barrett-Conner *et al*., 2005). There are valid arguments for and against a common single ethnicity reference population database (Leslie. 2006). If race/ethnicity is simply a proxy for other determinants of health (such as socioeconomic status, latitude, or diet) then approaches should focus on the specific factors that are directly implicated in the causal chain rather than on race/ethnicity. Arguably, race/ethnicity should only be used when it is the only available proxy for important unmeasured variables (Miller and Bonnick, 1999; Outram and Ellison, 2005). Continentally defined populations are not homogeneous and can be subdivided along national, religious, linguistic, and other lines. Many still adhere to the system of continental population groups, but recognize that this is an imperfect approach. The need for population-specific reference data in order to calculate *T* scores is controversial (Leslie, 2006). In part, this derives from differences in fracture rates and bone density that have been observed between countries and different ethnic groups, as well as different countries with similar ethnic groups but with a concern that *T* scores derived from a single reference population (usually taken to be whites) may be inappropriate. In addition, any dissimilar reference population data will yield differences in prevalence of osteoporosis among different ethnicities owing to the effect of small differences in the SD of the mean BMD of different young-normal reference populations. On the other hand, the need to develop specific reference data for every combination of country, race/ethnicity, and gender on every bone density instrument is virtually impossible. In fact, even within a relatively homogeneous population, variation in bone density and fracture rates can be observed (Miller and Bonnick, 1999). To follow this argument to its logical conclusion would require reference data for each subgroup within a country that shows a different average bone density and/or fracture rate. How daunting and impractical a task this would be is best illustrated in The People's Republic of China that officially recognizes 56 nationalities. The economic implications cannot be ignored, given cultural differences in health priorities and regional inequities in health care resources (Leslie *et al.*, 2006).

Individual population data suggest that vertebral fracture prevalence as a function of age may not be dissimilar in Hispanic (United States, Mexico, Spain, South America), Asian (United States, China, Japan, Hong Kong), or Native American (United States and Canada) populations compared with Caucasians (Riggs and Melton, 2008). However, for nonvertebral fracture risk, especially hip fracture risk, the absolute fracture risk (prevalence and incident) is lower worldwide in Asians and African-Americans than it is in Caucasians, and Hispanics (Barrett-Conner E *et al.*, 2005). It follows that population-specific reference data cannot resolve the inherent discordance in fracture rates, since there may be socio-economic, nutritional, geographic and other bone-specific issues (geometry) that may also contribute to ethnic differences in fracture rates that are not captured by BMD, BMI or other "bone" factors. Meanwhile, use of a common reference population enhances consistent reporting and reduces confusion. White reference data are the most complete and best validated as a tool for fracture prediction and treatment initiation, and by default is the most convenient. While prevalence of WHO classifications of osteopenia and osteoporosis will differ among ethnicities and between genders using a Caucasian-only female reference database for T-score calculation, differences in relative fracture risk calculated from a Caucasian-specific female reference data may not differ enough among most ethnicities and between genders to justify the enormous expense of developing a multiethnic and gender specific reference databases to sub classify prevalence(s) linked to prospective risks. Absolute fracture risk captured from head-to-head gender comparative population studies (Cummings SR *et al.*, 2006; Bergstrom U. *et al.*, 2008; Bischoff-Ferrari HA *et al.*, 2007; Rivedeneria *et al.*, 2007) suggest that risk in men may be similar as in women when BMD is measured by DXA from a female reference population database. Thus, Although at the current time the ISCD PDC suggests using gender-specific reference population databases for WHO criteria and risk assessment, arguments could be advanced to justify using a non-gender-specific database for risk prediction.

USING BONE DENSITOMETRY FOR MONITORING

Bone densitometry, either with central (spine and hip) DXA or spine OCT, can be used for monitoring the effects of disease on bone mass (such a celiac, Cushings disease, etc.); for monitoring the effects of drugs that can negatively effect bone strength or BMD (aromatase inhibitors, depoprovera, glucocorticoids, etc.), or for monitoring the effect of treatments designed to treat osteoporosis (Miller *et al.*, 2005; Miller, 2005, 2007; Bonnick and Shulman, 2006; Lewiecki, 2003).

There are no other accepted modalities available to measure and help guide clinical decisions in the area of osteoporosis other than serial measurements with DXA or QCT. Many experts feel strongly that clinical treatment of patients with osteoporosis cannot be achieved without serial BMD measurements.

The controversies surrounding serial BMD measurements are:

1. The frequency of measurements.
2. The adequacy among DXA/QCT measuring centers in performance of both *in vitro* as well as *in vivo* quality control to know their precision (or the opposite, precision error) in order to know their least significant change (LSC) (Bonnick, 1998).
3. The misinterpretation by some health care insurance carriers on the intent of the Medicare bone mass measurement act of 1997 on the allowed frequency of reimbursements of serial BMD measurements.
4. The relationship between the magnitude of change in BMD and the magnitude of change in bone strength.

The frequency of serial BMD measurements is not a fixed interval. Frequency of BMD measurements is determined by the disease the physician is treating, the anticipated rate of bone loss (or gain), the *in vivo* least significant change of the DXA/QCT machine (LSC), and the results of other aspects of the patient's evaluation.

Disease processes or the use of medications that are associated with rapid bone loss: post solid organ transplantation, high-dose glucocortiocoid use, aromatase inhibitor use, immobilization, thyroid "storm" or severe primary hyperparathyroidism, for instance, all often require annual and at times every-6-months determination. Postmenopausal women or men initiating treatment with FDA-approved therapies for osteoporosis should have a BMD measurement 12 months after initiating treatment. If the treatment is successful, then the monitoring frequency is recommended to be every 2 years, unless a change in management of other important factors that could impact bone health intervenes. Such an intervention could be a change in the pharmacological agent [e.g., a different bisphosphonate, or switching to teriparatide or a selective estrogen receptor modulator, or route of administration (oral to intravenous or transdermal)], the development of a new fracture on treatment, or the development of a new disease state that can be associated with bone loss. In these circumstances, the repeat BMD should be done within 12 months, not after 2 years.

The bone mass measurement act (BMMA) statute stating an every-23-month BMD testing interval was intended to be language directed only at a specific population: stable postmenopausal women (Department of Health and Human Services, 1998. It is both medically as well as ethically wrong to restrict reimbursement to other less stable and more ill populations where clinical decisions may change rapidly and require the knowledge of the bone mass determination.

Bone density testing centers that do not know their own *in vivo* precision cannot competently interpret changes in serial BMD measurements. *In vitro* (phantom) scanning is inadequate because patients move and phantoms have a fixed region of interest (ROI). Each facility that performs bone mass measurements should do correct *in vivo* precision studies to determine their LSC (Gluer *et al.*, 1995; Shepherd and Lu, 1997). Guidance on how to perform precision studies and calculate the LSC can be found on the ISCD website (ISCD.ORG).

In addition, because precision differs from manufacturer to manufacturer, it is ideal to perform serial BMD measurements on the same DXA or QCT machine. Although there are equations and calculations to compare serial BMD changes from one manufacturer to another (Hui *et al.*, 1997), cross-calibration is also associated with a substantial loss of precision making comparative studies on change in BMD problematical. In the ideal world the patient should be measured on the same DXA/QCT machine. It is very difficult to have confidence in serial measurements when compared between different manufacturers.

DETERMINATION OF VERTEBRAL FRACTURES BY DXA (VFA)

In the past several years research and clinical observations have documented the importance of assessing not only clinical (painful) but also asymptomatic vertebral compression fractures (VCFs). The prevalence of VCFs is high in all populations especially over the age of 60 years. Moreover, these asymptomatic VCFs predict future fractures at both vertebral and nonvertebral sites risk (Schousboe *et al.*, 2002; Vokes *et al.*, 2006; Lewiecki and Laster, 2007; Laster and Lewiecki, 2007; Genant *et al.*, 2000; Doboeuf *et al.*, 2007; Chapurlat *et al.*, 2006; Greenspan *et al.*, 2001). This increased risk of fracture is independent of the prevailing BMD (*T* score). The risk is higher as either the severity (grade 1 to 3) or the number of vertebral fractures increase (Genant *et al.*, 2007; Grigoryan *et al.*, 2003). A prior fragility fracture "trumps" all other risk factors as a predictor of future fracture risk.

Routine anterior-posterior (AP) and lateral spine x-rays have been the gold-standard for detecting VCF. But DXA technology has advanced to the point that now VFA by DXA is a recognized methodology for detecting VCF and at far lower radiation than routine x-ray. In addition, there is now a Medicare code for VFA reimbursement. The ISCD Position Development Conferences have documented this development (Vokes *et al.*, 2006). Utilizing the best available evidence the ISCD has suggested specific indications for VFA (Table I). Yes, there is no universal agreement across all Medicare carriers as to which indications for VFA will be reimbursed. In addition, although VFA by DXA has sensitivity and specificity similar to routine spinal x-rays for the detection of VCF, VFA loses some sensitivity for mild (grade I) fractures between T4 and T8 (Doboeuf *et al.*, 2007; Chapurlat *et al.*, 2006). Hence, it is suggested that if a clinician is uncertain about a Grade I VCF assessed by VFA-DXA at those vertebral levels, a follow-up x-ray should be considered.

Wider utilization of VFA by DXA in the right population will detect a large number of patients who have osteoporosis independent of their prevailing *T* score.

WORLD HEALTH ORGANIZATION (WHO) VALIDATED 10-YEAR ABSOLUTE FRACTURE RISK PROJECT

Published in March 2008, the long-awaited validated WHO absolute fracture risk model will help put osteoporosis risk assessment into a clinical arena the equivalent of The Framingham study for cardiovascular risk factors. This 10-year project spearheaded by John Kanis is an enormous scientific effort to validate individual risk factors for fracture (hip as well as global all-fracture risk) in the untreated postmenopausal population and how these risk factors interact to facilitate risk prediction (Kanis *et al.*, 2008). In addition, the WHO effort facilitates treatment (intervention) decisions by utilizing disutility calculations to assess the cost of fractures for any specific population and what that country can afford in order to reduce risk according to their individual gross domestic product and cost of treatments. For example, in the United States at the current costs of most osteoporosis therapies it becomes cost-effective to treat at a 10-year absolute fracture risk of 10% or greater. In addition to helping physicians and health economists decide on appropriate levels of treatment, the WHO absolute risk model will remove some

TABLE I Indications for VFA (ISCD Official Position)

Consider VFA when the results may influence clinical management
When BMD measurement is indicated, performance of VFA should be considered in clinical situations that may be associated with vertebral fractures. Examples include:

- Documented height loss of greater than 2.0 cm (0.75 in) or historical height loss greater than 4 cm (1.5 in) since young adult.
- History of fracture after age 50 years
- Commitment to long-term oral or parenteral glucocorticoid therapy
- History and/or findings suggestive of vertebral fracture not documented by prior radiological study

From Vokes et al. (2006).

of the confusion and debate surrounding the treatment of "osteopenia" because the *T* score level will be incorporated into the total risk model to come up with a 10-year risk for both hip and global fracture risk. The model can be applied to both postmenopausal women and elderly men.

The model is published both in its totality and the U.S. adaptation of the models (Hughes *et al.*, 2008). In addition, access to the formulas to calculate the risk is available through several websites (ISCD.ORG, NOF.ORG, and FRAX.ORG). Implementation of the WHO absolute fracture risk in the United States is being shepherded by The National Osteoporosis Foundation in collaboration with many other professional organizations with interest in osteoporosis as well as the bone mass measurement manufacturing companies. The U.S. NOF Clinicians Guide is intended to be an adaptation of FRAX that is unique to the U.S. clinical practice and reimbursement system. The NOF Clinicians Guide will clearly allow use of *T* score at the spine for WHO diagnosis and the use of gender-specific reference population databases for diagnosis as well, whereas all risk calculations from FRAX are based on hip-only measurements with Caucasian female reference population databases. In addition the U.S. Guide will be more flexible on the recommendations of the frequency of repeated BMD measurements in special clinical circumstances that require closer BMD monitoring and the use of morphometric vertebral deformities as a independent risk for high future fracture risk. It is important to stress that the models will not remove individual clinical judgment and individual patient treatment and specific treatment recommendations can still be personalized through shared decision making between patient and physician.

It should be pointed out that the WHO validation captured risk factors known to be predictive of fracture risk during the term of the population studies used for validation. Since then, other risk factors not captured in the WHO model have been identified as risk factors for fracture and will need to be utilized by clinicians for management decisions: bone turnover rates as measured by serum and/or urinary bone turnover markers, hip structural analysis, cortical porosity, fall rates, etc. Nevertheless, the WHO risk model will greatly facilitate both clinical and health-economic decisions with regard to timing of and need for osteoporosis intervention.

CONCLUSIONS

Bone mass measurements have paved the way for the clinical management of patients with either low bone mass and/or fragility fractures. The place of this measurement technology is ingrained in their applications and supported by consistent scientific evidence and clinical data. Bone mass measurements are still underutilized for the detection of osteoporosis and at-risk patients. The devices and their data output are still being improved. The need for the use of other non-DXA bone mass measurement devices to compliment DXA measurements for assessing bone strength and quality is great—and devices are currently either in research and development or are currently available to perform such additional analysis.

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Chapter 90

Macro- and Microimaging of Bone Architecture

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INTRODUCTION

Bone and therefore bone architecture is a generic term used for many different entities. At the macroscopic level the term bone is used for the organ bone as a distinct entity of the skeleton. At this level bone architecture describes the overall shape and geometry of bone as well as the differentiation into cancellous (also referred to as trabecular) and cortical bone. Typical parameters describing architecture are cortical thickness, moment of inertia and other geometrical measures. Bone mineral density (BMD) of the whole bone or of well-defined subvolumes is another important parameter. At this level bone architecture is typically assessed *in vivo* with x-ray-based imaging modalities either using planar methods such as conventional radiography and dual x-ray absorptiometry (DXA), or volumetric methods such as quantitative computed tomography (QCT). DXA and QCT are also preferred methods for the determination of BMD.

At the microscopic level, i.e., if the spatial resolution of the acquired images is better than 100 μm, bone architectural assessment is predominantly associated with trabecular structure, i.e., the interconnecting lattice of bone tissue filled with marrow (Fig. 1). Typical parameters are trabecular thickness and separation of trabeculae and parameters comprehensively describing the network architecture, such as the structure model index (SMI). Methods of choice are three-dimensional (3D) microcomputed tomography (μCT) and less frequently three-dimensional micromagnetic resonance imaging (μMRI). Excised bones, bone specimen, or complete small laboratory animals such as mice or rats can be investigated. Historically, trabecular structure has been measured by two-dimensional (2D) stereological methods applied to thin sections using optical microscopes or x-ray based microradiography. However, the preparation of thin (≈10 μm) bone sections typically embedded in epoxy resin is tedious and therefore these methods have been widely replaced by 3D μCT.

Owing to the high radiation exposure associated with this level of spatial resolution, *in-vivo* investigations with μCT are limited to small animals and cannot be carried out in humans. Although μMRI does not use ionizing radiation, its low signal-to-noise ratio is a limiting factor for large sample volumes, which also precludes *in vivo* measurements in humans. If the resolution is further increased to levels below 10 μm, cortical architecture, which can, for example, be characterized by porosity, can be accurately assessed. At even higher spatial resolution, bone architecture describes the structure of the compound of minerals embedded in the organic collagen matrix. This is the domain of techniques such as electron microscopy and fourier-transform infrared microscopy which is beyond the scope of this chapter.

Although it is not precisely defined, the range between macro- and microimaging i.e., from approximately 100 μm to 500 μm, is usually denoted as high-resolution CT (hrCT) and MRI (hrMRI) imaging for CT and MRI (see Fig. 1). These methods push existing such clinical scanners to their technical limits. Their main aim is the 3D assessment of trabecular architecture in humans *in vivo*; however, the spatial resolution is not high enough to accurately separate

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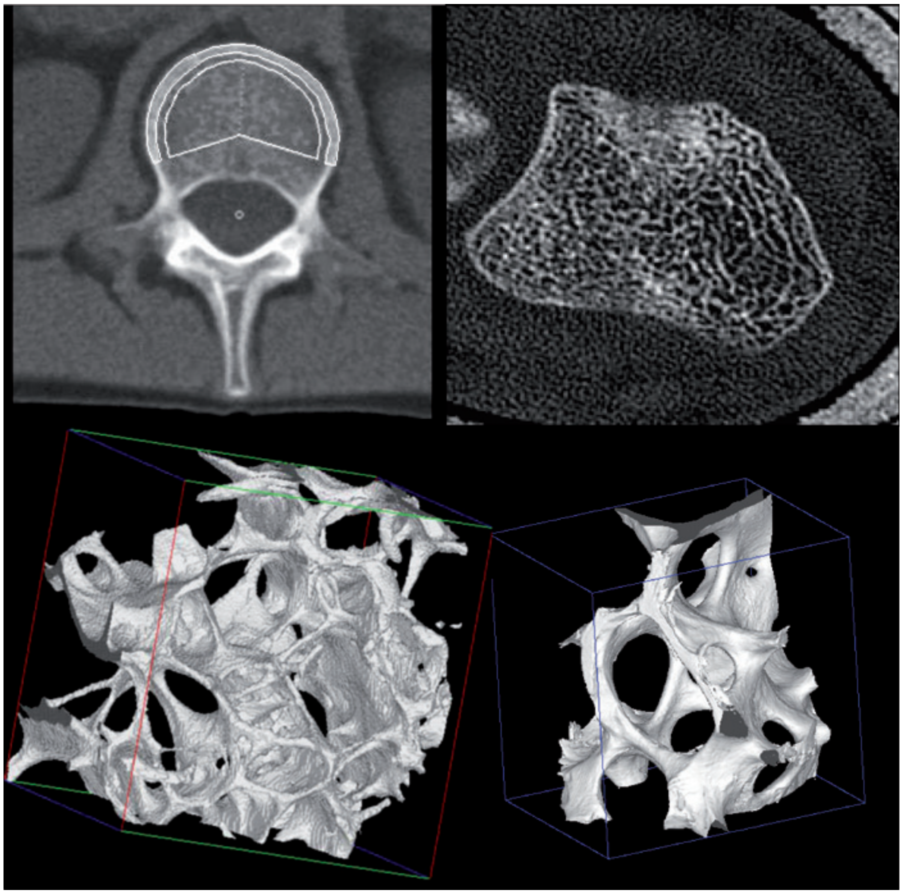


FIGURE 1 Visualization of bone architecture using x-ray tomography. (*Top left*) CT of vertebral body (in-plane pixel size, 550 μm; slice thickness, 10 mm) to determine BMD and macrostructure (details in sections 4.1 and 4.2). (*Top right*) hrCT of ultradistal forearm (inplane pixel size, 200 μm; slice thickness, 0.5 mm) to determine texture and structure of the trabecular network (details in section 4.3). (*Button*) μCT of vertebral spongiosa (isotropic voxel size, left, 30 μm; right, 10 μm) to determine structure of the trabecular network (details in section 4.4). (See plate section)

and quantify individual trabeculae. Thus, the use of standard stereological analysis methods in these images is problematic. As a consequence, advanced image-processing methods that quantify different parameters of the trabecular network such as texture or statistical features have been developed. Some of these methods have also been used to quantify trabecular architecture by using plain radiographs.

From the clinician's point of view the determination of bone architecture is a tool to improve the assessment of bone strength in order to more accurately predict fracture risk and its dependence on age, disease, and treatments. Bone mineral density is currently the most important predictor of fracture risk because it correlates well with bone strength and because it can easily be measured *in vivo* in humans with high precision and accuracy. However, BMD values in normal and osteoporotic people overlap widely, and many osteoporotic fractures occur in osteopenic subjects that still have relatively high BMD. Obviously bone strength and fracture risk cannot be fully explained by a global measurement of bone mineral density. The spatial distribution of BMD or, in other words, the bone architecture at the macroscopic and microscopic levels and their combination with BMD in finite-element analysis methods as well as multivariate models is thought to perhaps ultimately be a better predictor of bone strength than BMD and further improve our ability to understand the pathophysiology and progression of osteoporosis and other bone disorders.

RADIOGRAPHY

Conventional Radiography

Conventional radiography is the most widely available noninvasive technique of visualizing bone structure. The term osteopenia, "paucity of bone," is used to describe visually decreased bone density—or radiolucency—on radiographs. Thus, it also provides qualitative information on bone density. However, plain films are rather insensitive to changes in bone mineral density. In the spine, for example, it has been estimated that as much as 20% to 40% of bone mass must be lost before a decrease in bone visual density can

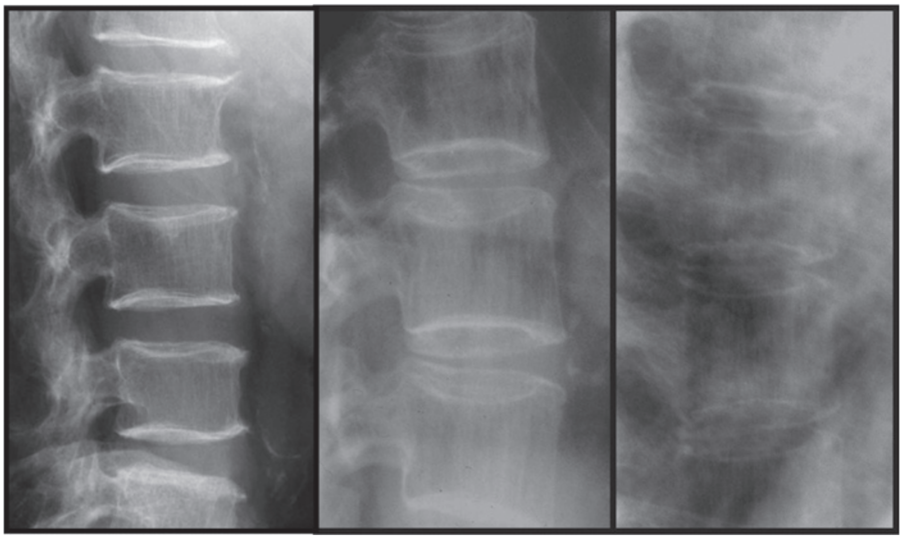


FIGURE 2 (*Left*) General decreased visual bone density results in increased radiolucency of the vertebrae with relative accentuation of the cortical rim, or “picture framing.” (*Center*) Increased vertical striation owing to the proportionally greater loss of horizontal trabeculae and compensatory hypertrophy of the vertical trabeculae. (*Right*) Severe osteoporosis: the vertebrae can hardly be distinguished on the lateral lumbar spine radiograph. There are multiple endplate and compression fractures.

be seen in lateral spine radiographs. The decrease in density is primarily a result of a decrease in mineralized bone volume rather than a decrease in mineralization of remaining bone; in osteoporosis and osteopenia, the amount of calcium per unit of mineralized bone volume remains constant at about 35%. Alone or in conjunction with other advanced imaging techniques, conventional radiography is still widely used to diagnose osteoporotic fractures and to differentiate various disorders associated with osteoporosis and osteomalacia.

Changes in the axial skeleton and the ends of the long tubular bones are most prominent because these sites have a relatively greater proportion of trabecular bone that has more surface and responds more quickly to overall shifts in bone metabolic rate than cortical bone. As bone is lost, non-weight-bearing connecting trabeculae are resorbed first, leaving the remaining weight-bearing trabeculae more widely separated. Some may undergo compensatory thickening in the direction of mechanical stress. Trabecular anisotropy increases, resulting in distinct patterns on radiographs, such as the organized sequential trabecular changes along the compressive and tensile stress in the proximal femur, and an appearance of vertical striation in the lumbar spine in early stages of osteopenia caused by rarefaction of the horizontal trabeculae and a relative reinforcement of the vertical trabeculae (Fig. 2). Vertebrae may appear on radiographs as a “picture frame” or as an “empty box” because of accentuation of the cortical margins surrounding the lucent trabecular center. Osteopenic vertebrae may also demonstrate increased biconcavity of the vertebral endplates owing to protrusion of the intervertebral disk into the weakened vertebral body.

Vertebral fracture, the hallmark of osteoporosis, has a wide range of morphological appearances from increased concavity of the endplates and anterior wedging to a complete destruction of the vertebral anatomy in vertebral crush fractures (Fig. 3). Fractures can be graded by radiologists or experienced clinicians and quantified with morphometric methods to reduce the subjectivity inherent in grading (Genant *et al.,* 1996; Wu *et al.,* 2000). The Genant scoring method (Genant *et al.,* 1993; Lentle *et al.,* 2007) shown in Figure 4 grades 0 for normal, 1 for mildly deformed (20% to 25% reduction in anterior, middle, and/or posterior height, and 10% to 20% reduction in the projected area of the vertebral body), 2 for moderately deformed (25% to 40% reduction in height, and 20% to 40% in area), and 3 for severely deformed (40% or greater reduction in height and in area). This method offers high reproducibility on conventional as well as on digitized radiographs (Wu *et al.,* 2000). It has been widely used to evaluate fracture endpoints in the majority of epidemiological studies and of clinical trials investigating the efficacy of osteoporotic drugs (Ettinger *et al.,* 1999; Harris *et al.,* 1999; Szulc *et al.,* 2001; Grados *et al.,* 2004; Genant *et al.,* 2005).

Of most concern in osteoporosis are fractures of the hips, vertebral bodies and wrists. Changes in the bone cortex or spongiosa may make such injuries more likely to occur. Fractures of the vertebral bodies are a result of predominantly axial compressive loads. One reason for the decreased load-bearing capacity is the increased absorption and removal of the horizontal trabeculae or lateral support crossties (Mosekilde *et al.,* 1985). The vertical trabecular bone, therefore, behaves as a column and is prone to critical buckling loads (Townsend *et al.,* 1975). A 50%

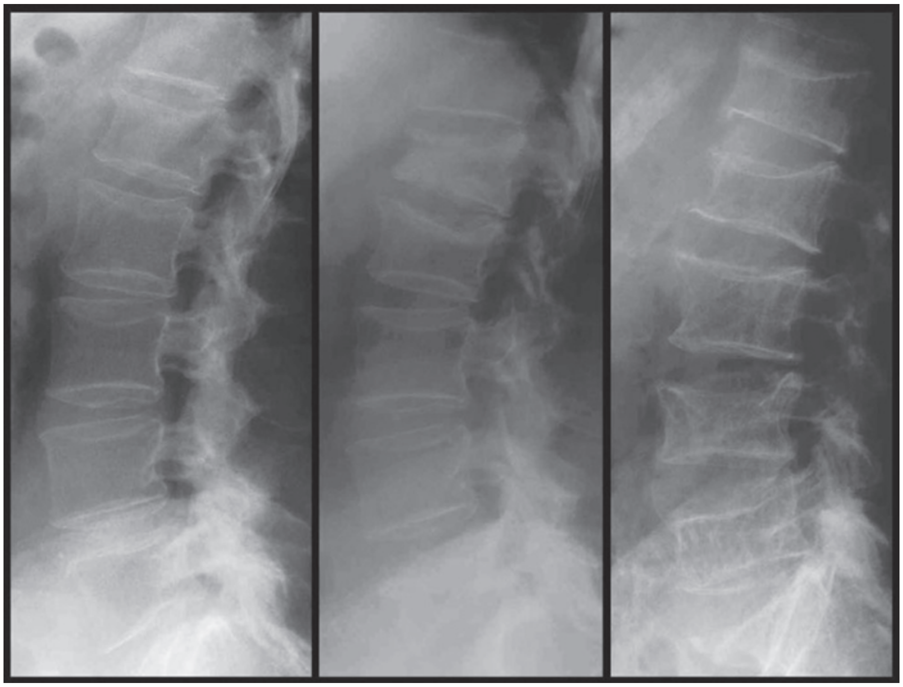
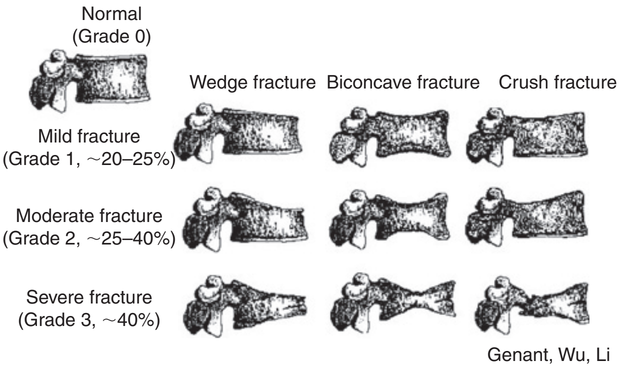


FIGURE 3 Multiple fractures in the thoracic spine in advanced involutional osteoporosis, including endplate, wedge, and compression fractures.

decrease in cross-sectional area as a result of absorption of the horizontal trabeculae is associated with a 75% decrease in the load-bearing capacity of the vertebral body (Einhorn, 1992).

In the hip and the forearm, radiographs are typically used to diagnose and classify a fracture and to develop a strategy for a potential surgical intervention. Quantitative radiographic grading of osteoporosis has not gained much attention, although in the hip the Singh index has been developed to grade differences in the trabecular pattern (Singh *et al.,* 1973). The trabecular compartment is of interest because osteoporotic fractures of the hip and the forearm typically occur in trabecular-rich locations: at the femoral neck and trochanter in the hip and at the ultradistal site of the forearm, whereas osteoporotic fractures are much less frequent in the shaft where bone strength only depends on compact bone (Smith *et al.,* 1976).

The Singh index grades the trabecular pattern of the proximal femur from I to VII. Grade VII denotes individuals with dense trabeculae even in the region of the Ward's triangle. Grade VI down to grade I denotes normal pattern of all compressive and tensile trabeculae (VI) to marked reductions in the principal compressive trabeculae (I). Although in several cadaver studies the Singh index correlated with $r > 0.5$ with bone strength (Delaere *et al.,* 1989; Krischak *et al.,* 1999; Wachter *et al.,* 2001) the diagnostic value of the Singh index has been criticized because it has been shown to suffer from high interobserver variation (Koot *et al.,* 1996) and different results regarding its relationship with bone mass (Disen *et al.,* 1979; Eriksson *et al.,* 1988; Kawashima *et al.,* 1991; Barondess *et al.,* 2002) and vertebral or femoral fracture (Heneghan *et al.,* 1997).



Genant, Wu, Li

FIGURE 4 The Genant grading method for a semiquantitative evaluation of osteoporotic vertebral fracture grades; fracture severity from normal (grade 0) to severe deformity (grade 3). The scoring scheme illustrates reductions of the anterior height that correspond to the grade of deformity. Reductions of the middle or posterior heights or combinations thereof can be evaluated accordingly.

Radiogrammetry

By common definition, radiogrammetry refers to the morphometric assessment of tubular bones in the hands or wrists from projection radiographs; i.e., dimensions of structures such as the cortical thickness of finger bones are assessed. The method can be applied more generally to any bone suitable for such an evaluation. Several authors have applied radiogrammetric techniques to radiographs of the humerus (Meema *et al.,* 1963; Bloom *et al.,* 1970) and the femur (Barnett *et al.,* 1960; Saville *et al.,* 1976; Horsman *et al.,* 1982; Glüer *et al.,* 1994) as well as to DXA scans of the proximal femur (Faulkner *et al.,* 1994).

In radiogrammetry, a nonscreen acquisition using fine-grain film is most often used but some investigators have used high-detail industrial film (Meema, 1981). Periosteal (D) and endosteal (d) diameters at the midshaft are the primary measurements; they are directly determined on the x-ray film using a simple ruler or caliper (Fig. 5). From these measurements, indices of bone shape can be calculated, such as combined cortical thickness, the metacarpal index $\mathrm{CCT} = \mathrm{D} - \mathrm{d}$ or the metacarpal index $\mathrm{MCI} = (D - d)/D$, with the latter accounting for skeletal size differences. Annual decreases in CCT of 0.4% before menopause and of 1.3% after menopause have been reported (Falch *et al.,* 1990), which is comparable to a decline of 1.45% in untreated women after oophorectomy (Genant *et al.,* 1982).

Under the assumption of a circular bone cross section, cortical area can be defined as: $A_{\mathrm{cort}} = A_{\mathrm{tot}} - A_{\mathrm{med}} = (\pi/4)(D^2 - d^2)$, where A_{tot} denotes the total area of the metacarpal and A_{med} denotes the area of the medullary space. Another parameter is percent cortical area: $\mathrm{PCA} = A_{\mathrm{cort}}/A_{\mathrm{tot}} = 1 - (d^2/D^2)$. Because the absolute measurement of area is not important to the clinical interpretation of these results, the scaling factor of $\pi/4$ is typically disregarded.

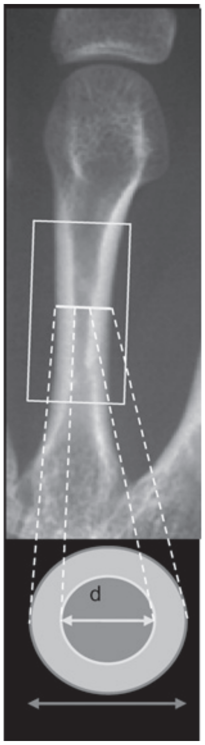


FIGURE 5 Radiogrammetry of the second metacarpal; originally, only the midshaft, as indicated by the horizontal line, was used for measurements. Newer direct digital x-ray methods use a larger ROI covering a large part of the diaphysis, as indicated by the box.

Digital Radiogrammetry

Newer direct digital x-ray methods like computed radiography (Rico *et al.,* 1994) combined with computer-assisted analysis that allowed for analysis of continuous segments of the metacarpal bone rather than single measurements at the midshaft (see Fig. 5) (Cosman *et al.,* 1991; Derisquebourg *et al.,* 1994; Matsumoto *et al.,* 1994; Adami *et al.,* 1996; Jorgensen *et al.,* 2000; Rosholm *et al.,* 2001) reduced precision errors from about of 6% to 11% (Adams *et al.,* 1969; Naor *et al.,* 1972) in film-based implementations to about 1% to 2% (Dey *et al.,* 2000).

In the late 1990s, digital x-ray radiogrammetry (DXR) was introduced as an extension to radiogrammetry. This technique estimates areal BMD from radiogrammetry and cortical porosity. It employs a fully automated approach in a total of five regions in the metacarpals, radius, and ulna (Jorgensen *et al.,* 2000; Rosholm *et al.,* 2001). The innovation was to use the results to estimate a mean bone volume per projected bone area (VPA) to gain higher independence of bone size. In DXR, areal BMD ($\mathrm{BMD_a}$) is estimated by empirically calibrating VPA to $\mathrm{BMD_a}$ as measured in the midregion of a DXA forearm scan and correcting for cortical porosity p: $\mathrm{BMD_a} = c \bullet \mathrm{VPA}(1 - p)$, where c is a constant obtained empirically from the calibration process. In principle, this is the same as assuming a constant mass density of compact bone. DXR takes into account that porosity increases with age. An estimate of porosity is made by a simple texture analysis in the medullary space derived for each individual measurement (Rosholm *et al.,* 2001). The value of p is an average porosity estimated in the five bone regions and typically has a value of less than 2%. Precision of $\mathrm{BMD_a}$ as measured by DXR was reported to be in the range of 0.5% to 1% (Jorgensen *et al.,* 2000; Rosholm *et al.,* 2001; Ward *et al.,* 2003).

High-Resolution Radiography and Microradiography

High-Resolution Radiography

In order to improve the spatial resolution of conventional radiographs for a better visualization of bone structure, contact radiography with high-resolution films or magnification radiography with fine- or microfocus x-ray tubes have been used (Genant *et al.,* 1975; Doi *et al.,* 1976; Fodor *et al.,* 1987; Pruneau *et al.,* 1987; Jonsson *et al.,* 1995; Boyce *et al.,* 2006). Actually radiogrammetry, where the hand is typically placed directly on the film, is one example of high-resolution contact radiography. However, historically, for *in-vivo* applications, magnification radiography, a technique in which the size of the focal spot of the x-ray tube determines the spatial resolution, has been more widely used than high-resolution contact radiography, where the spatial resolution is determined by the film. Advanced techniques of magnification radiography use electronic detectors and μ-focus x-ray tubes with focal spot sizes down to 10 μm (Link *et al.,* 1994). Advanced systems for contact radiography use high-resolution digital detectors and can be used for the calcaneus and the forearm (Wilkie *et al.,* 2004; Lespessailles *et al.,* 2007), which ensures that the object–detector distance is small.

Microradiography versus Histomorphometry

In bone and mineral research, the most commonly applied microradiography technique is contact microradiography of prepared sections of excised bone tissue with a thickness of about 50 to 100 μm. It has been used for decades to determine bone mineralization with an in-plane spatial resolution of less than 10 μm (Cosslet *et al.,* 1960; Engstrom, 1962). The images are recorded on film or spectroscopic plates (Conlogue *et al.,* 1987) and are then analyzed using optical microdensitometry. Results are typically calibrated with an aluminum step wedge imaged simultaneously with the bone specimen. Newer systems digitize the microradiogram for further computer-aided analysis (Kalebo *et al.,* 1988; Strid *et al.,* 1988; Boivin *et al.,* 2002).

As an alternative, the technique of histomorphometry has been developed. It facilitates the very time-consuming and difficult x-ray exposure but requires even thinner sections (≈10 μm), which are stained and then investigated using an optical microscope. A "stained thickness" of 10 μm can be achieved either by grinding or by special surface-staining techniques (Hahn *et al.,* 1991). Owing to a decrease of partial volume artifacts in histomorphometry, the accuracy of the quantification of bone structure is improved compared with microradiography (Engelke *et al.,* 1993). Because histomorphometry is based on principles of stereology, some basic assumptions of the underlying structure of the trabecular network are required. Typically either a rod- or a plate-like structure is assumed (Parfitt, 1983; Parfitt *et al.,* 1987). Relative bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular spacing (Tb.Sp), and trabecular number (Tb.N) are most prominent among the long list of standard histomorphometric parameters.

Mineralization of the trabecular bone can be divided into primary and secondary mineral apposition. Primary mineral apposition is mineralization of the unmineralized organic matrix (osteoid). Secondary mineral apposition is a slow and gradual maturation of the mineral component, including an increase in the amount of crystals and/or an augmentation of crystal size toward their maximum dimensions. Increased bone turnover, as in postmenopausal osteoporosis, results in hypomineralization, because resorption begins prematurely, before mineralization is complete. The least mineralized bone is highly radiotranslucent and is the most recently formed, or has just achieved its primary mineralization, and represents about 75% of the complete mineralization. It contrasts with the adjacent fully mineralized interstitial bone, with low radiotranslucency. Thus, the differentiation between the degree of mineralization and the volume of the mineralized bone is important. For example, oral administration of alendronate for 2 or 3 years in osteoporotic women increases BMD, but in iliac crest bone biopsies does not increase trabecular bone volume, which can be explained by an increase in secondary mineralization as a result of reduced bone turnover (Meunier *et al.,* 1997).

Histomorphometry of stained grindings is the method of choice to quantify bone architecture and BV/TV, whereas contact radiography is superior in quantifying bone mineral density. With histomorphometry, areas of primary and secondary mineral apposition can be distinguished, but for a finer differentiation of mineralization, x-ray based techniques such as microradiography are required. Alternative techniques to microradiography are backscattered electron imaging and small-angle x-ray scattering imaging and, of course, μCT (see section below), although the spatial resolution of most μCT systems still does not reach that of microradiography.

Analysis of Trabecular Texture using Radiographs

On radiographs, the projection of the 3D trabecular architecture results in a 2D texture. The binarization of such a texture and a subsequent 2D stereological analysis to quantify bone structure is not very promising. Sophisticated image-processing techniques based on digitized grayscale images have been designed to extract a large variety of parameters such as fractal dimension, morphological gradients, Fourier descriptors, or bone anisotropy. For *in-vivo* studies radiographs of the calcaneus are well suited because the effect of overlying soft tissue is minimized (Benhamou *et al.,* 1994; Lespessailles *et al.,* 1996; Brunet-Imbault *et al.,* 2005; Chappard *et al.,* 2005), although other skeletal sites such as the spine (Caligiuri *et al.,* 1994), the femur (Geraets *et al.,* 1998; Gregory *et al.,* 2004), the wrist (Geraets *et al.,* 1990; Lespessailles *et al.,* 1996; Majumdar *et al.,* 2000), or the knee (Lynch *et al.,* 1991; Podsiadlo *et al.,* 2007) have been used as well. For *in vitro* studies excised vertebrae are preferred (Link *et al.,* 1997; Ouyang *et al.,* 1998; Veenland *et al.,* 1998; Majumdar *et al.,* 1999; Dougherty 2001). Two studies have also been reported of the femur (Jennane *et al.,* 2007; Pulkkinen *et al.,* 2007). In the calcaneus *in vivo* precision errors of below 1.5% have been reported for a variety of texture measures (Lespessailles *et al.,* 2007).

A combination of 2D texture parameters determined in radiographs simulated from 3D μCT images of femoral neck and calcaneal bone specimen predicted up to

93% of the variance of microarchitecture parameters other than BV/TV (Apostol *et al.,* 2006). Similar results were reported for iliac crest specimen (Guggenbuhl *et al.,* 2006). However, it remains unclear whether this highly interesting result can improve the prediction of fractures in clinical practice. So far, one *in vivo* study has found that the combination of fractal and anisotropy parameters measured in radiographs of the calcaneus and BMD as measured by DXA at the spine or femoral neck could better discriminate between groups with and without spinal fractures (Benhamou *et al.,* 2001; Chappard *et al.,* 2005) than BMD alone, but no prospective data have been published so far.

DUAL X-RAY ABSORPTIOMETRY

Hip Geometry

Several investigations have found that projectional measurements of parameters describing femur geometry can predict hip fractures independent of BMD. One prominent example is hip axis length, determined from conventional radiographs or with DXA. It predicts hip fracture, because a longer hip axis length is associated with an increase in femoral neck and trochanteric fracture risk (Faulkner *et al.,* 1994; Glüer *et al.,* 1994). Instead of varying with age after midadolescence, the hip axis length seems to be mainly influenced by genetic factors (Flicker *et al.,* 1996), which supports other reports that differences in the hip axis length may partially explain racial differences in hip fractures (Cummings *et al.,* 1994).

Hip axis length adds information to BMD and appears to be useful for cross-sectional studies of risk factors for osteoporosis, but has little value in longitudinal studies. The neck-shaft angle as determined from DXA scans was also associated with hip fractures in two studies (Gnudi *et al.,* 2002; Pulkkinen *et al.,* 2004), but one *in vivo* study (Bergot *et al.,* 2002) showed negative results. This was supported by an *in-vitro* study of femoral cadavers where no association was found between neck-shaft angle and breaking strength. In this study the neck width correlated with breaking strength (Cheng *et al.,* 1997).

Hip Structural Analysis

A more comprehensive approach to estimate geometrical and mechanical properties from DXA of the hip is hip structural or hip strength analysis (HSA), which was first applied to cross-sectional bone mineral absorption curves generated by single-photon absorptiometry at the forearm (Martin *et al.,* 1984). This early work has been extended to DXA at the proximal femur to automatically estimate cross-sectional moments of inertia (CMSI) and cross-sectional areas (CSA) of the neck, the trochanter, and the shaft, as well as the femoral neck-shaft angle, bending moments, and the hip axis length (HAL) (Beck *et al.,* 1990; Yoshikawa *et al.,* 1994).

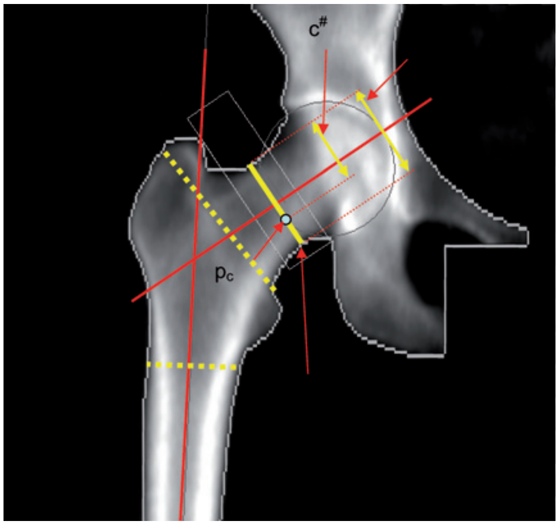


FIGURE 6 HSA analysis: p_c center of mass of cross section. *c as defined by hip structure analysis (Beck *et al.*, 1990); #c as defined by hip strength analysis (see text). In hip structure analysis, CSMI is estimated along the three yellow profiles; in hip strength analysis CSMI is only estimated along the solid yellow profile. (See plate section)

For HSA one-dimensional profiles are extracted from narrow regions across the neck, the intertrochanter, and the shaft by averaging pixel values perpendicularly to the profile directions (Fig. 6). For each pixel along the profile, areal BMD (BMD_a) is obtained from the DXA measurement, and bone mineral content (BMC) is determined using the known pixel dimensions. The BMC distribution along the profile is used to calculate *pc*, the position of its center of mass. Under the assumption that the specific axis through *pc* which is perpendicular to the plane of the DXA image is also the main bending axis, the CSMI of the cross section defined by the profile can (apart from a multiplicative constant) be accurately calculated from the DXA images by

$$CSMI = \frac{1}{BMD_{cort}} \sum_i BMC_i[\Delta(p_c - i)]^2$$

BMD_{cort} is the areal density of cortical bone and a value of 1.051 g/cm^2 is assumed. Δ denotes the length of a profile pixel.

From the CSMI the section modulus $Z = \text{CSMI}/c$ is estimated, where c denotes the distance from the bending axis to the furthest edge of the section and is defined as half the periosteal width is taken from the DXA image (Beck *et al.,* 2001). The section modulus is an important physical property that relates the bending moment M to the bending stress F by $Z = M/F$. The buckling ratio B is another parameter estimated from CSMI and assumes a circular cross section of the femur. It is computed as $B = r/t$, where r is the radius of the cross section and t the cortical thickness. Beck and coworkers estimated mean cortical thickness by using simple models of neck and shaft cross sections as hollow annuli (Beck *et al.,* 2001). Obviously the estimation of the variables c, r, and t form a projected density profile of a 2D cross section, which, in particular for the femoral neck and the trochanter, is not circular, shows limitations of the HSA analysis of DXA images.

Currently two different implementations of HSA are more widely used. The first one termed hip *structure* analysis was implemented by Beck and coworkers and is available on some Hologic scanners (Beck *et al.,* 1990). The second version of HSA termed hip *strength* analysis, which is implemented on GE Lunar DXA devices, additionally estimates a femur strength index calculated as the ratio of estimated compressive yield strength of the femoral neck to the expected compressive stress of a fall on the greater trochanter. The compressive forces are calculated from the bending moments and the distance d between the center of the head and the extracted profile. The bending moment caused by a fall on the greater trochanter is estimated from an individual's height and weight (Robinovitch *et al.,* 1991). In this implementation, c is defined as distance from the superior aspect of the femoral neck to the center of mass of the extracted profiles (see Fig. 6). Thus, in essence, the femur strength index is a combination of neck length, neck diameter, and the regional distribution of BMC across the selected neck profiles combined with the height and weight of the patient.

HSA was successfully used in several studies to better understand changes of femoral strength and geometry under pharmaceutical treatment (Beck *et al.,* 2001; Greenspan *et al.,* 2005; Uusi-Rasi *et al.,* 2005; Burnham *et al.,* 2007; Knapen *et al.,* 2007), to investigate population (Crabtree *et al.,* 2000; Nelson *et al.,* 2000, 2004) or gender (Kaptoge *et al.,* 2003; Nissen *et al.,* 2005) related differences, or to improve fracture risk prediction (Karlamangla *et al.,* 2004; Ahlborg *et al.,* 2005; Faulkner *et al.,* 2006). However, only a few studies adjusted HSA results for BMD and anthropometric parameters or used multivariate statistics (Crabtree *et al.,* 2002; Karlamangla *et al.,* 2004; Faulkner *et al.,* 2006). In Faulkner et al's cross-sectional study, the diagnosis of prior fractures was improved only slightly. The area under the ROC curve increased from 0.71 using BMD alone to 0.74 when including hip axis length and femur strength index in a logistic model (Faulkner *et al.,* 2006).

Further detailed comparisons with 3D QCT should be performed to assess the value of HSA. From these studies it should be possible to determine optimized parameters and analysis volumes of interest (VOIs). For example, it has been shown *in vitro* that in DXA a novel neck region of interest (ROI) which only included the upper part of the neck better predicted bone strength than the standard DXA regions (Boehm *et al.,* 2005). Also, a circular ROI in the central neck better discriminated hip fractures than the standard regions (Prevrhal *et al.,* 2004). Thus, an appropriate regional analysis of BMD may have a larger effect than combining BMD and HSA.

VXA

Volumetric DXA, also termed VXA, is a further step toward a volumetric assessment of BMD and 3D geometry is (Ahmad *et al.,* 2007) or 3D-XA (Kolta *et al.,* 2005; Le Bras *et al.,* 2006; Kolta *et al.,* 2007). In the 3D-XA approach developed by Skalli and coworkers, orthogonal 2D projections of 3D contours extracted from a CT dataset of an arbitrary femur are fitted to two orthogonal DXA images of the femur under investigation. The resulting deformed 3D contours define a personalized 3D model of the proximal femur. In a study on excised femurs, comparison of these 3D models with real 3D CT data showed a mean difference of only 0.06 ± 1.02 mm, although maximum errors were as high as 7.8 mm in the greater and lesser trochanter (Kolta *et al.,* 2005). However, the *in-vivo* situation is more complicated because both femurs and potentially bone from the acetabulum overlap in the lateral DXA projection. As a consequence, an *in-vivo* application of this technique so far has only been reported for the spine (Kolta *et al.,* 2007).

For the femur, the VXA approach is more promising than 3D-XA (Ahmad *et al.,* 2007): Several (less than 10) 2D projections are taken from the proximal femur using different projection angles by rotating the C-arm of the DXA scanner. In contrast to the 3D-XA method the total angle covered by all projections is considerably smaller than 90 degrees, thus avoiding the overlap with other bone structures. The VXA projections are fitted to a statistical 3D atlas of the femur generated from volumetric CT data of a larger number of persons. It was shown that individual anatomical variations from the atlas can be described by 10 to 20 different parameters, the so-called Eigenvectors. This demonstrates that a limited number of 2D projections is sufficient to obtain reasonably accurate 3D geometrical and densitometric information of the femur. Figure 7 shows a comparison between one of the measured DXA images and the 2D projection obtained from the 3D atlas-based VXA dataset using the same projection angle.

COMPUTED TOMOGRAPHY

The term Quantitative computed tomography (QCT) denotes the specific technique to quantify bone mineral density in the cross-sectional x-ray computed-tomography (CT) images. Originally, QCT was restricted to measurement of trabecular bone mineral density in single transverse CT slices at the lumbar midvertebral levels and the distal forearm (Rüegsegger *et al.,* 1976; Genant *et al.,* 1982), but with the advent of multidetector row spiral CT

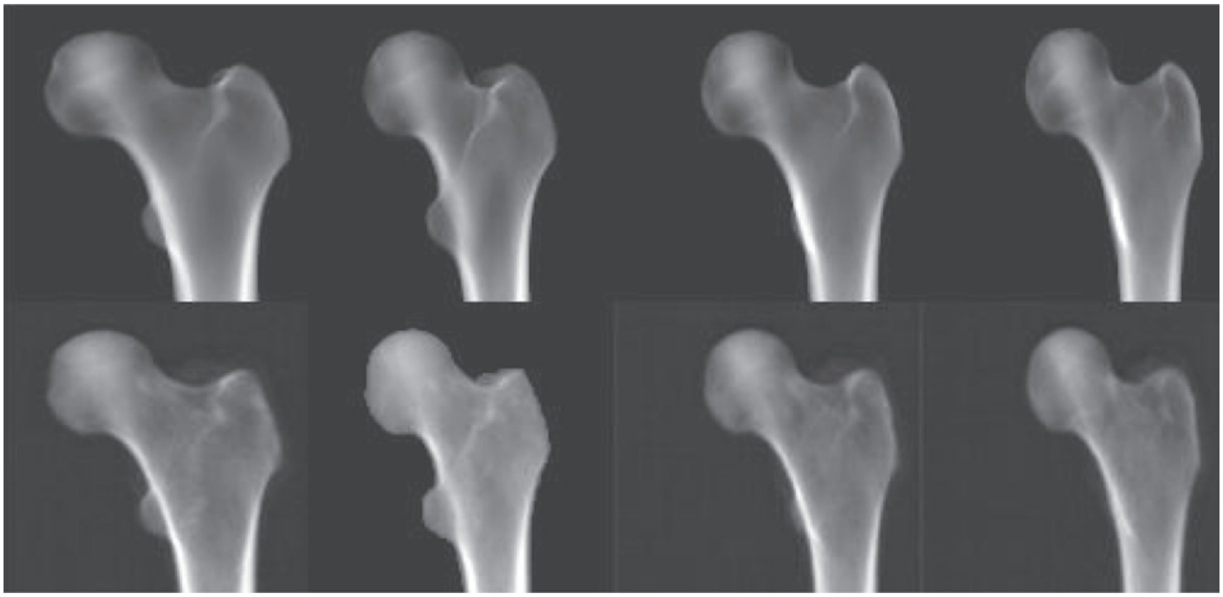


FIGURE 7 VXA of the proximal femur. (*Top*) 2D projections of 3D personalized results obtained form the statistical atlas based on measured DXA projections. (*Bottom*) Corresponding measured DXA projection. Results for four excised femora are shown (Ahmad *et al*., 2007).

technology, newer, fully three-dimensional QCT techniques (vQCT) have now been developed that encompass the entire object of interest (Lang *et al.,* 1999, 2002; Kang *et al.,* 2003; Mastmeyer *et al.,* 2006) (see Figs. 1 and 2). The term peripheral QCT (pQCT) was, and still is, sometimes used to refer specifically to dedicated peripheral scanners (Rüegsegger *et al.,* 1976). However, pQCT measurements can also be performed on clinical whole-body CT scanners, and therefore the term pQCT covers all peripheral QCT measurements.

Volumetric QCT of Spine and Femur

Whereas trabecular bone, in particular at the spine, is metabolically more active and may therefore serve as an early indicator of treatment success, cortical bone, in particular at the hip, may be more important to estimate fracture risk (Bousson *et al.,* 2006). Therefore the unique ability of QCT to separately analyze BMD of the trabecular and cortical compartments was advocated from the beginning as an advantage over projectional methods such as DXA. State-of-the-art spiral CT provides almost isotropic spatial resolution offering new opportunities for the 3D assessment of the cortex (Fig. 8). While the spatial resolution is not high enough to give accurate results of cortical thickness below values of approximately 1.0 to 1.5 mm, even below these values a 10% to 20% change of thickness can still be measured accurately, as shown by Prevrhal *et al.* (1999). In general, it is easier to measure cortical thickness in the femur than in the spine where thicknesses of as thin as 200 to 500 μm are encountered frequently, especially in the elderly.

The partial volume artifact caused by the limited spatial resolution also leads to an underestimation of cortical BMD on the order of 10% to 30%. Some analysis programs use empirical correction factors based on phantom measurements, but it should be cautioned that these correction factors vary among different scanner types. In particular in multicenter trials, the use of such factors seems to be questionable. The assessment of the cortex also highlights the importance of selecting adequate scan protocols. Slice thickness, reconstruction kernel, and reconstructed field of view are important parameters in order to optimize measurements of the cortex. In addition to the cortex, vQCT is a sophisticated tool to determine geometrical parameters of mechanical relevance such as cross-sectional moments of inertia.

At the spine, the cross-sectional area of the vertebral bodies is a macrostructural parameter of interest because it is likely that larger vertebrae can sustain loads better than smaller ones, albeit bigger individuals with larger cross sections also bear greater loads. Periosteal apposition, which may occur at the spine and the femur, has the potential to offset the increase in fragility caused by loss of bone mass by increasing cross-sectional area. A cross-sectional vQCT study by Riggs and coworkers showed that women not only start out with smaller vertebrae and lose bone mass faster, but also increase cross-sectional area slower than men (Riggs *et al.,* 2004). Although the magnitude of the changes reported by vQCT are inconsistent with DXA findings (Duan *et al.,* 2003), the study indicates that spinal cross-sectional area measurement with vQCT may provide additional predictive power for fracture risk.

Because the geometry of the proximal femur is much more complex than that of a vertebral body, macrostructural

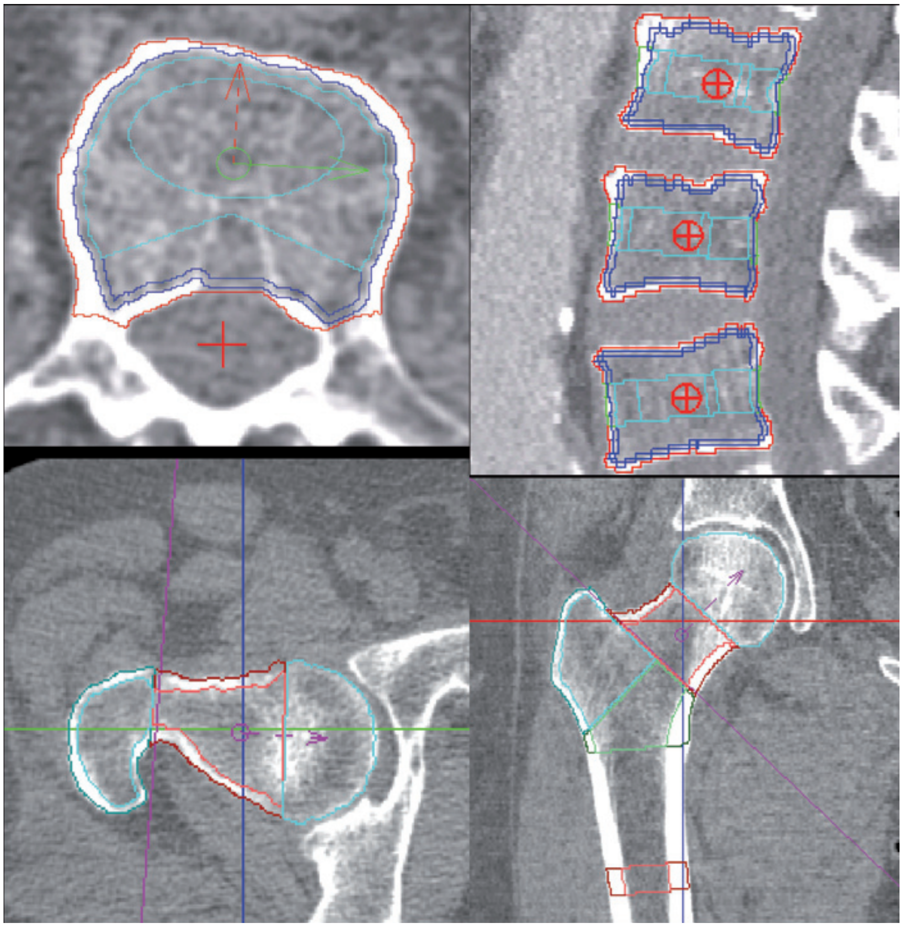


FIGURE 8 Advanced 3D QCT at spine and hip (slice thickness, 1 mm; in-plane resolution, 300 μm) to analyze regional BMD and geometry such as cortical thickness; left axial, right coronal MPRs of 3D datasets (Kang *et al*., 2003; Mastmeyer *et al*., 2006). (See plate section)

parameters of interest include cross-sectional areas at the neck and greater trochanter, hip axis length, and simple mechanical measures such as cross-sectional moment of inertia (a measure of how bone is radially distributed) and section moduli at various cross sections along the femoral neck axis. As in the spine, periosteal apposition causes the cross-sectional areas of the femoral neck and shaft to expand with age (Riggs *et al.,* 2004). The large Osteoporotic Fractures in Men Study (MrOS) study confirmed this and also found cortical thinning with age. However, whereas the neck seemed to exhibit net cortical bone loss, periosteal expansion seemed to offset cortical thinning in the shaft to maintain cortical cross-sectional area (Marshall *et al.,* 2006). This study also showed ethnic differences with higher femoral neck and lumbar spine volumetric BMD but lower cross-sectional areas in African Americans, which might contribute to some of the ethnic difference in hip and vertebral fracture epidemiology.

Lang and colleagues showed in a specimen study that these parameters explain femoral strength partially independently of BMD (Lang *et al.,* 1997). Interestingly, Cheng and colleagues found in a retrospective vQCT study of Chinese elderly women that fracture cases had larger femoral necks than nonfractured controls, which was interpreted as an adaptation to their lower femoral neck BMD (Cheng *et al.,* 2007). Volumetric QCT measurement was also employed to investigate bone loss during space flight. In the 14 crew members of the International Space Station, vQCT demonstrated that total femur integral BMC, but not integral volumetric BMD or trabecular BMD, recovered to its preflight value, 12 months after flight lasting 4 to 7 months. Recovery of bone mass involved increasing both bone density and bone size. Incomplete recovery of BMD in the hip in the year after long-duration spaceflight was observed. As shown by an increase in the minimum femoral neck cross-sectional area and integral tissue volume, the proximal femur appears to adapt to resumption of load bearing by periosteal apposition (Lang *et al.,* 2004).

Treatment effects on cortical volume using vQCT were reported from the PaTH study, investigating parathyroid hormone (PTH) and Alendronate treatment alone or in combination. The study found that femoral cortical volume increased with one-year treatment with PTH followed by one year with Alendronate (Black *et al.,* 2003, 2005). Similar results on cortical bone were observed in another PTH study (Greenspan *et al.,* 2007). The potential relevance of other geometrical parameters for the determination of bone strength has been shown in *in vitro* studies measuring bone failure loads. In the spine the endplate area (Biggemann *et al.,* 1988; Brinckmann *et al.,* 1989), cross-sectional area (Mosekilde *et al.,* 1989), or vertebral surface area (Eriksson *et al.,* 1989) improved the correlation of BMD with failure loads. In the femoral neck cross-sectional area, neck axis length (Lang *et al.,* 1997) and cortical thickness were identified as independent predictors of failure load.

Peripheral QCT

With respect to bone architecture pQCT has predominantly been used to assess the cortex of the distal and mid radius. At the ultradistal site in some studies cortical parameters were more successful than total or trabecular BMD, e.g., for fracture prediction (Grampp *et al.,* 1997), age-related bone loss (Hernandez *et al.,* 1997), or monitoring treatment (Ruegsegger *et al.,* 1995; Prince *et al.,* 2006). At the ultradistal site other treatment studies (Dambacher *et al.,* 1997; Fujita *et al.,* 2000; Merki-Feld *et al.,* 2003) showed better effects for trabecular or total BMD. Although this discrepancy may be explained in part by the particular pharmaceutical intervention, it is more likely that partial volume artifacts and differences in image-processing techniques, such as the selection of thresholds, have a large impact.

These effects probably also explain some of the controversial findings on differential age-related bone loss in cortical and trabecular bone. In cross-sectional studies of healthy women some authors reported higher trabecular, than cortical, losses (Boonen *et al.,* 1997; Nijs *et al.,* 1998; Qin *et al.,* 2000), whereas others showed comparable losses (Grampp *et al.,* 1995; Gatti *et al.,* 1996; Hasegawa *et al.,* 1997). If cortical parameters are of interest, then a more proximal site toward the shaft should be used because here the cortex is thicker and can more accurately be quantified. Such a location has indeed been used in several studies (Vico *et al.,* 1992; Nijs *et al.,* 1998; Schneider *et al.,* 1999; Tsurusaki *et al.,* 2000; Neu *et al.,* 2001; Clowes *et al.,* 2005; Rittweger *et al.,* 2005; Russo *et al.,* 2006). In *in vitro* studies a high association of geometry-based parameters with bone failure loads (Louis *et al.,* 1995; Augat *et al.,* 1996, 1998; Lochmuller *et al.,* 2002; Muller *et al.,* 2003) has been demonstrated. Moment of inertia, section modulus or moment of resistance, and stress-strain index (SSI) (density-weighted section modulus) demonstrated the strongest correlations with failure load.

In the radius, cortical thickness of the shaft significantly contributes to strength. However, studies that directly compared parameters failed to provide clear evidence that geometry-based parameters perform significantly better than measurement of bone mass (BMC) alone (Spadaro *et al.,* 1994; Augat *et al.,* 1996; Lochmuller *et al.,* 2002; Muller *et al.,* 2003; Ashe *et al.,* 2006). The combination of multiple pQCT parameters, however, tended to improve the prediction of failure loads versus single variables, but the multiple regression coefficients were generally not significantly higher than those obtained by DXA alone (Lochmuller *et al.,* 2002; Muller *et al.,* 2003) because most of these *in-vitro* studies did not adjust appropriately for bone size.

In the forearm, the scan location that best predicts mechanical competence of the radius has not yet been identified. Some studies found higher correlation coefficients for measurements at the shaft compared with measurements at the distal end of the bone, whereas others reported similar correlations for both sites (Lochmüller *et al.,* 2002; Müller *et al.,* 2003). Compression tests of the ultradistal region revealed the highest correlations between ultimate strength and BMC ($r = 0.83$ to 0.87). The loads to produce distal radius fractures by simulating a fall on to the outstretched hand (Myers *et al.,* 1993, 1994) have been accurately predicted by density measurements and geometric measurements. The most accurate predictors of fracture strength were BMC at the ultradistal site ($r = 0.94$) (Spadaro *et al.,* 1994), area of cortical bone at the shaft site ($r = 0.84$ to 0.89) (Myers *et al.,* 1993; Spadaro *et al.,* 1994), and combinations of BMD with moments of inertia ($r = 0.93$) (Augat *et al.,* 1996). In *in-vitro* studies, BMC instead of BMD was often the more important variable, but it must be considered that larger bones can withstand larger loads than smaller bones. At comparable BMD smaller bones thus would contain less BMC. However, big bone/little bone confounders have been rarely accounted for *in vitro*.

In *in-vivo* studies the SSI showed a similar dependence on the measurement site as in *in-vitro* studies discussed earlier. Nijs and coworkers found in a cross-sectional study that SSI at the proximal radius was almost as diagnostically sensitive to prevalent vertebral fracture as trabecular and total BMD at the ultradistal radius (ROC = 0.72 vs. 0.75). However, the SSI showed a much weaker diagnostic power at the ultradistal site (Nijs *et al.,* 1998). Augat and coworkers measured the 4% ultradistal site in hip fracture cases and controls and found that SSI ranked behind all other pQCT parameters, although none of them had a significant odd's ratio (Augat *et al.,* 1998). In another study, at the same site, geometrical parameters such as cross-sectional moment of inertia and cortical area discriminated less well than trabecular BMC and BMD between patients with and without Colles fractures (Schneider *et al.,* 2001). In patients receiving alendronate treatment, the bone strength index that is similar to the SSI showed a borderline significant ($P = 0.04$) increase of 4.7% versus baseline compared with a highly significant increase of 6.8% in total BMD after 24 months (Schneider *et al.,* 1999).

High-Resolution Computed Tomography (hrCT)

With appropriate acquisition and tomographic reconstruction protocols modern whole-body CT scanners for measurements of the axial skeleton achieve almost isotropic spatial resolution of approximately 0.5 mm. Dedicated single-slice pQCT scanners have improved in-plane resolution by about a factor of 2 to 3 but use slice thicknesses of 1 to 2 mm, although some pQCT research scanners also provide 0.5-mm-thick slices (Gordon *et al.,* 1996). Given typical trabecular dimensions (100 to- 400 μm) and trabecular spaces (200 to 2000 μm), this resolution is still insufficient for an accurate determination of standard stereological parameters. This is illustrated in Figure 9 where high-resolution CT images were simulated from a stack of 21 sagittal grindings (9 × 7 mm^2) of a trabecular network from a human calcaneus. The thickness of each grinding was 5 μm and the distance between two adjacent grindings was 51 μm owing to preparation requirements. All grindings were digitized, resulting in a stack of binary images (pixel area: 12 × 12 μm^2). Additional slices obtained by interpolation between adjacent binary images were added to simulate a slice thickness of 12 μm. Thus, the final dataset consisted of isotropic voxels.

The images of the stack were averaged along the direction indicated in Figure 9. The first row of simulated images

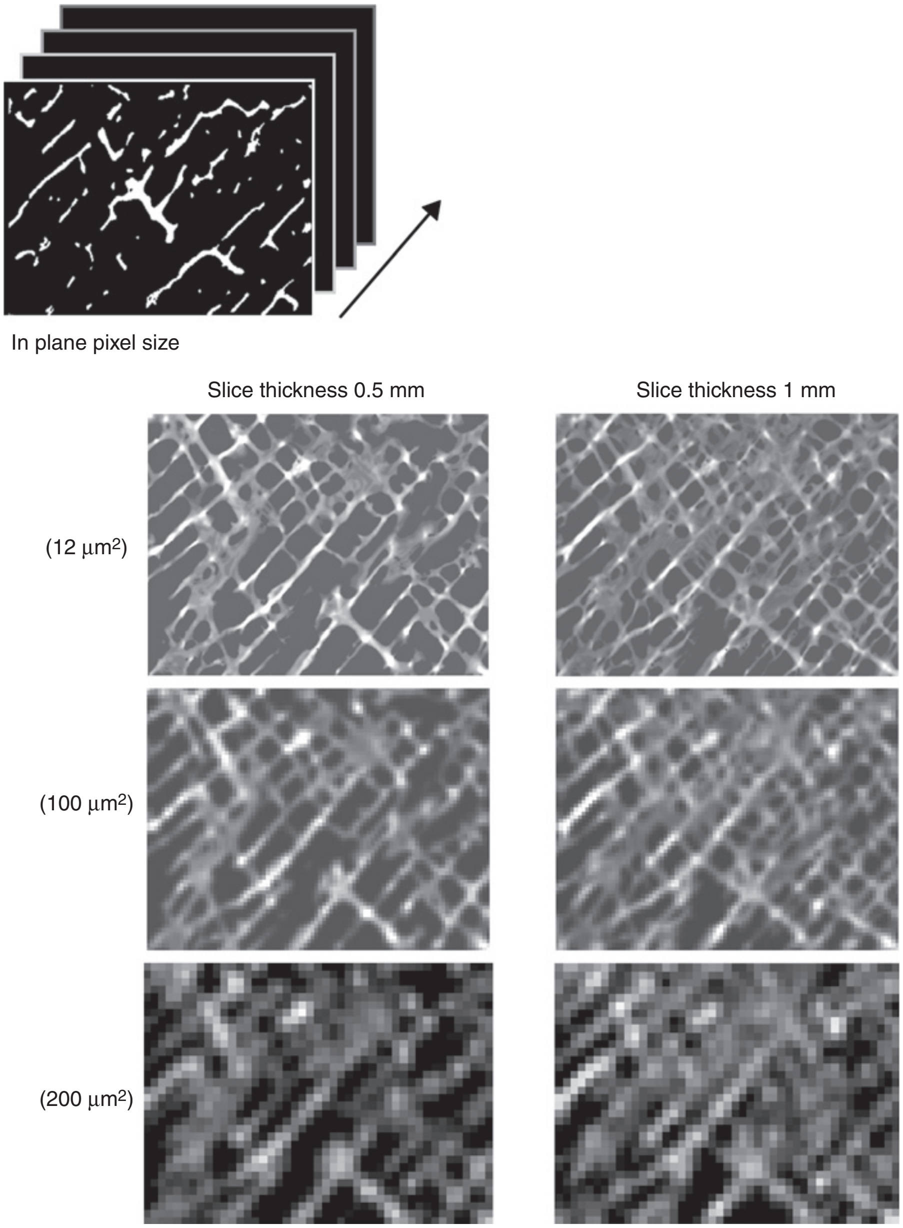


FIGURE 9 Effect of slice thickness and in-plane pixel size on trabecular structure. Simulation of radiographic and hrCT images based on a stack of stained grindings. (*Top*) Stack of grindings. (*Bottom*) Simulated radiographic and hrCT images (Engelke *et al.*, 2001).

shows two radiographic type images. One resulted by averaging half the stack and the other by averaging all images of the stack (see Fig. 9). Thus, a total slice thickness of 0.51 mm and of 1.02 mm was simulated, respectively. The in-plane pixel area in these radiographic-type images is unchanged at $12 \times 12\,\mu m^2$. There are still artificial discontinuities in the gray-value variations. They would be avoided by averaging more images. The second and third rows show simulated hrCT images with pixel sizes of 100 μm and 200 μm. The images were blurred with a Gaussian filter to give a more realistic impression of what can be expected *in vivo*.

The simulations demonstrate that in hrCT images only the dominant structures are preserved; details of the trabecular network cannot be extracted. The texture in the CT-like and in the radiographic-like images is similar and a major deterioration of the network will be noticeable in the hrCT images. However, a determination of parameters typically used in histomorphometry is problematic. It is evident that parameters like Tb.Th, Tb.Sp, or Tb.N change drastically with resolution. In order to indicate that structural parameters measured in hrCT and hrMRI images differ significantly from those measured by histomorphometry of thin section, it is recommended to call them apparent trabecular thickness, etc., and to use the prefix “app” such as appTb.Th, appTb.Sp, or appTb.N.

Laib and coworkers compared different structure extraction and morphometric evaluation techniques to measure stereological parameters in high-resolution images. They proposed to first extract the skeleton of the structure and then to apply a network model independent 3D technique to calculate appTb.N. The assumption of a plate- or rod-like trabecular structure is no longer necessary. appTb.Th and appTb.Sp can finally be calculated from densitometric bone volume fraction and appTb.N. Traditional histomorphometric analysis methods based on bone surface and volume ratios and assuming a plate model performed less well (Laib *et al.*, 1999). The 3D techniques also resulted in a number of new parameters to quantify the trabecular network, like ridge number density (Laib *et al.*, 1997) and the structure model index (SMI) (Hildebrand *et al.*, 1997). The SMI is based on a differential analysis of the triangulated bone surface. The SMI value is 0 for an ideal plate structure and 3 for an ideal rod structure, independent of the physical dimensions. For a structure with plates and rods of equal thickness the value lies between 0 and 3, depending on the volume ratio of rods and plates. Parameters quantifying network connectivity, such as Euler number, node-to-terminus strut count, or terminus-to-terminus strut count, have also been used (Cortet *et al.*, 2002).

In several publications, approaches of measuring structural parameters in binarized images (Klotz *et al.*, 1986; Durand *et al.*, 1991; Ito *et al.*, 1995) were replaced by textural or statistical descriptors to characterize the trabecular architecture without requiring segmentation to separate bone from marrow and other soft tissue. The mathematical principles used to describe texture in hrCT as well as in high-resolution MRI images (see section below) are often similar to those used for texture analysis of 2D radiographs (see section above).

Textural parameters still based on binary images used for instance the trabecular fragmentation index (length of the trabecular network divided by the number of discontinuities) (Chevalier *et al.*, 1992), a run-length analysis (Ito *et al.*, 1995), a parameter reflecting trabecular hole area, analogous to star volume (Gordon *et al.*, 1996, 1998; Showalter *et al.*, 2006), and co-occurrence texture measures (Showalter *et al.*, 2006). Newer approaches prefer gray-level analyses and use, for example, Minkowsky functionals (Saparin *et al.*, 2006) or Gabor wavelets (Xiang *et al.*, 2007) to quantify trabecular topology.

HrCT of the Spine

In the past, hrCT applications of the spine have often yielded ambiguous results because either a segmentation step was used for binarization during the analysis or because CT scanners providing slice thicknesses below 1 or 1.5 mm were not available at that time. CT reconstruction parameters such as the reconstruction kernel, a parameter that can be chosen to balance spatial resolution versus noise in the tomographic image, or the reconstructed field of view (FOV), were often not optimized. For example, Laval-Jeantet and colleagues (Chevalier *et al.*, 1992) used 1.5-mm thin spinal CT images but a larger-than- necessary FOV. Their trabecular fragmentation index separated women with spinal fracture from those without but not as well as BMD. In another *in-vivo* study, Ito and coworkers used CT of the spine with 2-mm slice thickness. Trabecular separation significantly increased with age in women 50 years of age or older, whereas women with vertebral fractures had significantly lower BMD and significantly higher trabecular separation (Ito *et al.*, 1995; Ito *et al.*, 1997). Trabecular separation was a better discriminator than BMD in 60- to 69-year-old but not in 50- to 59-year-old women. Gordon and coworkers showed that trabecular hole area discriminated vertebral fractures even after adjustment for BMD (Gordon *et al.*, 1998).

Some *in-vitro* studies using QCT of bone specimens (Jiang *et al.*, 1998; Link *et al.*, 1998; Bauer *et al.*, 2004) or whole excised bones (Link *et al.*, 2004; Bauer *et al.*, 2006; Saparin *et al.*, 2006) also indicate the potential to improve fracture discrimination by using structural parameters. However, even with identical acquisition protocols *in-vitro* scans typically have better image quality than *in-vivo* scans because the signal-to-noise ratio is increased considerably owing to the smaller size of the samples and removed soft tissue (Bauer *et al.*, 2007). Thus, there should be some caution generalizing results of structure analysis techniques in hrCT images from *in vitro* to *in vivo* studies.

If the structural analysis is based on binary images, then segmentation is the most crucial step (Dufresne 1998; Cendre *et al.,* 2000; Engelke *et al.,* 2001; Elmoutaouakkil *et al.,* 2002), and it is advisable to evaluate accuracy with the help of high-resolution data, e.g., taken from μCT scans which can be sub-sampled to the resolution of the hrCT image (Engelke *et al.,* 1996; Elmoutaouakkil *et al.,* 2002). In order to avoid segmentation and its problems, the application of gray-level analysis methods such as those mentioned above seems to be very promising. *In-vivo* studies using these methods in CT or MRI have not been published yet. Interestingly, the two most recent *in vivo* studies applying trabecular structure analysis of vertebral bodies using hrCT images have applied rather simplistic global thresholds to segment trabecular bone (Ito *et al.,* 2005; Graeff *et al.,* 2007), but both used 0.5-mm slice thickness and 0.2-mm in-plane voxel size and applied a volumetric analysis by using several slices per vertebra.

In Ito's cross-sectional study (Fig. 10), vertebral trabecular structure parameters measured with hrCT better distinguished between fractured cases and non-fractured controls than BMD measurements with DXA, but not better than with site-matched BMD as measured by vQCT (Ito *et al.,* 2005). Glüer and colleagues (Graeff *et al.,* 2007) carried out a longitudinal study using teriparatide and claimed that structural changes under treatment, in particular, those for appBV/TV and appTb.N, were partly independent of BMD. However, it is not entirely clear whether the decoupling of BMD and appBV/TV may at least in part be caused by the segmentation process. With a fixed threshold a slight increase in mineralization from just below to just above the threshold may overproportionally increase the appBV/TV. If treatment effects do not substantially affect mineralization, as seems to be the case with teriparatide, then appBV/TV changes should approximate site-matched BMD changes.

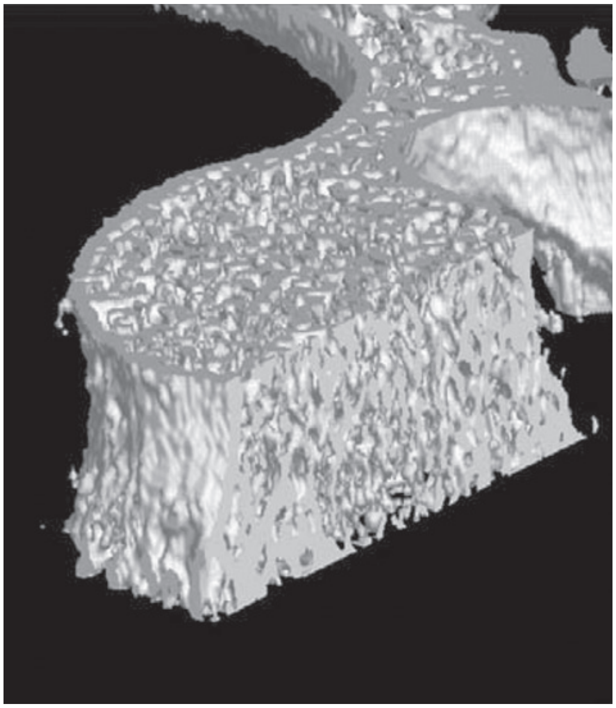


FIGURE 10 High-resolution *in vivo* CT image of the spine. The 3D rendering reveals the connectedness of the trabecular network but also reveals that stereological parameters cannot be determined accurately. Courtesy of Masako Ito.

HrCT of the Forearm

Because the assessment of trabecular structure *in vivo* is difficult, special-purpose peripheral CT scanners have been developed to assess the distal forearm, where trabecular thickness ranges from 60 to 150 μm and trabecular separation ranges from 300 to 1000 μm. The trabecular dimensions are similar to the spine, but owing to the much smaller cross section of the forearm compared with the central body, the absorption decreases significantly and, therefore, the spatial resolution can be increased without a radiation dose penalty. On the contrary, because the forearm tissue is less radiation sensitive than that at the spine for forearm hrCT the effective dose is typically well below 100 μSv.

The first to pursue hrCT successfully at the distal forearm were Rüegsegger and colleagues, who built a thin-slice high-resolution laboratory pQCT scanner for *in vivo* applications with an isotropic voxel size of $(170\,\mu m)^3$ (Durand *et al.,* 1992), which was successfully decreased further to below $(100\,\mu m)^3$. Müller reported a high *in vivo* reproducibility of about 1% achieved by careful registration of the acquired three-dimensional datasets (Muller *et al.,* 1996). When *in vitro* pQCT structure measurements of iliac crest biopsies were compared with μCT, the correlation of various three-dimensional structural parameters between the two systems was $r^2 > 0.9$, despite the lower resolution of the high-resolution pQCT system. For a very precise analysis an isotropic spatial resolution of approximately 10 μm was required. Up to about a resolution of 100 to 150 μm, changes of these parameters could be described by a linear relation. Therefore a dedicated segmentation threshold can be obtained for pQCT by calibrating the pQCT bone volume fraction to the μCT bone volume fraction. That is, appropriate calibration techniques can restore an accurate value of BV/TV (Müller *et al.,* 1996). A similar study of cadaveric specimens of the calcaneus that were scanned with hrCT at a slice thickness of 1 mm and histomorphometry showed that, at this resolution, correlations for BV/TV, Tb.N, and Tb.Sp dropped to $r = 0.7$ to 0.9 and were even lower for the Euler number and the number of nodes (Cortet *et al.,* 2004). Thus, the calibration approach suggested by Müller would not work for standard pQCT or hrCT of the spine.

The laboratory efforts of the Swiss group cumulated in the XtremeCT, a commercially available *in-vivo* pQCT scanner for the forearm and the tibia (Boutroy *et al.,* 2005).

As proposed by Laib *et al.* (1999), histomorphometric parameters are determined from a measurement of bone volume fraction and apparent trabecular number. The spatial registration of baseline and follow-up scans with a very small accuracy error (lower than 100 μm) is a critical step in the analysis of follow-up scans in order to detect longitudinal changes of bone structure within a given subject. Thus during these scans, even slight motions of the forearm must be avoided, which is not an easy task, given a scan time of several minutes. Currently, a 2D slice-by-slice based area matching between baseline and follow-up scans is used for XtremeCT scans. A full 3D registration may reduce motion artifacts and may improve precision, but will expand the time required for image processing.

The first indication that peripheral trabecular structure assessment is indeed useful to differentiate women with an osteoporotic fracture history from controls better than DXA at hip or spine came from Boutroy and colleagues (2005). Apart from trabecular, cortical, and total BMD, apparent trabecular spacing significantly discriminated osteopenic women with and without a variety of different prior fractures. In a second age-matched case control study of 101 women of the OFELY study, radial DXA BMD_a-adjusted differences between cases with fragility fractures at various sites and controls remained borderline ($P = 0.06$) significant for trabecular BMD, appBV/TV, and appTb.Sp as measured by XtremeCT. BMD_a adjusted ORs ranged from 1.32 to 1.5 (Sornay-Rendu *et al.,* 2007). At the tibia, adjusted ORs for cortical thickness and appTb.Th also ranged from 1.80 to 2.09 after adjusting for BMD_a as measured by DXA of the hip. Khosla *et al.* (2006a, b) examined age- and sex-related bone loss cross-sectionally and speculated as to the different patterns of bone loss in men and women.

Microcomputed Tomography (μCT)

Since the pioneering work for the use of μCT in medicine by using either x-ray tubes (Elliott *et al.,* 1984; Feldkamp *et al.,* 1989; Kuhn *et al.,* 1989) or synchrotron radiation (Engelke *et al.,* 1987; Flannery *et al.,* 1987; Engelke *et al.,* 1989), μCT has greatly matured technically and is now widely used in various medical areas such a vessel and tumor research. Most of the initial work on μCT was dedicated to the investigation of trabecular bone architecture. As will be discussed in this section, this a still a major focus today but other areas in bone research such as fracture healing (Ibiwoye *et al.,* 2004; Gauthier *et al.,* 2005; Shefelbine *et al.,* 2005), tissue engineering (Jaecques *et al.,* 2004; Van Cleynenbreugel *et al.,* 2006; Peyrin *et al.,* 2007), bone implants and scaffolds (Hiu-Yan *et al.,* 2005; De Smet *et al.,* 2006; Otsuki *et al.,* 2006; Cowan *et al.,* 2007; Jones *et al.,* 2007; Stoppie *et al.,* 2007), or oral applications (Van Oosterwyck *et al.,* 2000; Verdonschot *et al.,* 2001; Butz *et al.,* 2006; Cheung *et al.,* 2006; Nomoto *et al.,* 2006; Takada *et al.,* 2006) are increasingly benefiting from μCT. Unfortunately, a description of these applications is beyond the scope of this review, but the cited literature, although far from being complete, may provide a stimulating entry point for further research.

A broad range of commercial and research scanners for 3D μCT is available today. Scanners using x-ray sources offer spatial resolution from about 10 μm to 100 μm, whereas synchrotron radiation (SR) is still the method of choice for higher spatial resolutions. Several commercial devices integrate sophisticated software for the analysis of bone structure including finite-element modeling (see section below). Most earlier μCT studies aimed at technical improvements of the equipment, at the development of new 3D analysis methods for the quantification of trabecular structure (Odgaard *et al.,* 1993; Hildebrand *et al.,* 1997, 1997), and at the characterization (Müller *et al.,* 1996; Ding *et al.,* 1999) and establishment of μCT as a valuable research tool (Bonse *et al.,* 1996; Borah *et al.,* 2001; Dalstra *et al.,* 2001). After this period, in particular, in conjunction with the introduction of genetically engineered laboratory animals like mice and rats, many studies started to focus on medical questions.

For example, iliac crest bone biopsy specimens were analyzed from women participating in a placebo-controlled risedronate trial. After one year, in the control group, BV/TV decreased by 20% and Tb.N decreased by 14% compared with baseline. Tb.Sp increased by 13% and marrow star volume by 86%. In the same period, lumbar spine BMD_a as measured by DXA decreased by only 3.3%. In the risedronate-treated group the architectural parameters did not significantly change during the same period (Dufresne *et al.,* 2003). In another study of paired biopsies taken before and after treatment with human PTH, μCT showed increased 3D connectivity density and confirmed the preservation of 2D histomorphometric BV/TV, Tb.N, and Tb.Th (Dempster *et al.,* 2001). Similar results for PTH were reported recently in a third biopsy study. After 19 months of PTH treatment compared with placebo, BV/TV increased by 44%, Tb.N by 12%, Tb.Th by 16%, and connectivity density by 25%. Tb.Sp decreased by 10% and SMI by 50%, demonstrating the usefulness of 3D parameters obtainable from μCT (Fox *et al.,* 2005). In a study in ovariectomized baboons, bisphosphonates preserved the microarchitecture in thoracic vertebrae (Hordon *et al.,* 2006).

As it is rather difficult to obtain human bone biopsies, studies investigating drug and disease effects are typically performed during the preclinical phase using laboratory animals. In an investigation of rat tibiae 16 weeks after ovariectomy (OVX), BV/TV decreased by 69% and Tb.Th by 30% compared with a sham-operated control group. Tb.Sp increased by 100% and SMI by 48%. This showed that with estrogen deficiency the trabecular network consisted of more rod-shaped trabeculae (Yang *et al.,* 2003). Treatment of OVX rats with residronate maintained the plate-like trabecular structure and network connectivity (Ito *et al.,* 2005). A study with either cathepsin K- or rolipram-treated OVX BALB/c mice showed that, compared with the sham-operated control group, in both treatment arms a decrease of BV/TV and deterioration of trabecular structure were prevented (Xiang *et al.,* 2007). Another study with ovariectomized rats showed that PTH and elcatonin (ECT), a synthetic derivative of eel calcitonin, preserved bone architecture by different means. After 12 weeks of treatment BV/TV was greater in the ECT and PTH groups than in the OVX group. The number of nodes per volume (N.Nd/TV) and Tb.N were significantly greater in the ECT group, whereas Tb.Th was greater in the PTH group (Washimi *et al.,* 2007). 3D μCT has also been used to quantify trabecular architecture in osteoarthritis (Ding *et al.,* 2003; Patel *et al.,* 2003; Batiste *et al.,* 2004a, 2004b; Wachsmuth *et al.,* 2004; Chappard *et al.,* 2006).

In contrast to hrCT and hrMRI techniques, the aim of μCT is to measure structural parameters accurately. The question of what spatial resolution is required to do so has been discussed in several publications in which 3D μCT was compared with standard 2D histomorphometry of thin sections. Using histomorphometry as a gold standard may be questionable if just one or two slices are extracted from the volume assessed by μCT, because there may be considerable variations of 2D slice-based structural parameters within the 3D volume analyzed by μCT (Engelke *et al.,* 1996). Nevertheless, the comparison of μCT and histomorphometry allows characterizing some potential weakness in using μCT for structural analysis.

One study comparing the two techniques in human bone biopsies found that BV/TV was slightly overestimated by 3% in μCT. However, Tb.Th was overestimated by almost 50%. The discrepancy of Tb.Sp was smaller (25%) but depended on the absolute value of Tb.Sp (Chappard *et al.,* 2005). For μCT a voxel size of $(14\,\mu m)^3$ and a dual-threshold segmentation was used. A discrepancy of 50% for Tb.Th seems to be very high at this voxel size; however, it must be cautioned that most studies only give voxel sizes in the reconstructed μCT dataset and not a true spatial resolution that, for example, can been determined with the help of the modulation transfer function. Without knowledge of the acquisition parameters, such as detector pixel size and magnification factor, it is impossible to judge the true spatial resolution from the reported image voxel size. Indeed, the voxel size in the μCT dataset can be selected during the tomographic reconstruction and can easily be decreased below the true spatial resolution of the tomographic scanner. A study comparing voxel sizes of $(21\,\mu m)^3$, $(50\,\mu m)^3$, and $(110\,\mu m)^3$ showed that subsampling data during tomographic reconstruction in order to improve spatial resolution does not give the same results as scanning with higher resolution (Kim *et al.,* 2004). Unfortunately the true spatial resolution of a given scanner and scan acquisition protocol is rarely known and therefore just the voxel size is reported. Also this is often advantageous from a marketing perspective.

In contrast to Chappard's results a different study also comparing 2D histomorphometry and 3D μCT found only a 22% overestimation of Tb.Th by μCT. BV/TV was overestimated by 4% and Tb.N by 5%; Tb.Sp was underestimated by 10% (Fox *et al.,* 2005). The good agreement for BV/TV with histomorphometry was confirmed in a third study with μCT by using a voxel size of $(20\,\mu m)^3$ (Thomsen *et al.,* 2005). As the correlation of results between μCT and histomorphometry is high, e.g., $r = 0.95$ for BV/TV, $r = 0.75$ for Tb.Th, and $r = 0.81$ for Tb.Sp (Chappard *et al.,* 2005), some authors argue that with linear correction factors μCT is a substitute for histomorphometry when determining trabecular structure (Thomsen *et al.,* 2005). However, caution should be exercised if Tb.Th is determined directly in μCT images instead of being indirectly derived from a measurement of Tb.N and BV/TV. Direct measurements are very sensitive to the segmentation algorithm (Lublinsky *et al.,* 2007).

For the comparisons between histomorphometry and μCT discussed earlier human or bovine bone biopsies were used. To our knowledge, a comparison in excised bones of mice and rats has not been published so far. The imaging and image processing of those bones pose new challenges for μCT because dimensions are smaller compared with human bone biopsies. A typical voxel size of (20 to $30\,\mu m^3$) resolution may be acceptable for connectivity determinations of human trabeculae, but is insufficient for accurate measurement in rats or mice where the trabecular widths average about 50 μm and trabecular separations average 150 μm or less (Kinney *et al.,* 1995).

As an example, Figure 11 shows the 3D segmentation of a mouse knee (Wachsmuth *et al.,* 2004). Owing to partial volume artifacts, the results are still not perfect in small pores in the trabeculae, in sclerotized regions and for thinner trabeculae, which has significant impact in particular on the accuracy of Tb.Th. A further significant increase in spatial resolution down to 1 μm can be achieved by using Synchrotron radiation (SR) (Peyrin *et al.,* 1998, 2001; Martin-Badosa *et al.,* 2003; Ito, 2005). Compared with μCT systems equipped with x-ray tubes, SR offers magnitudes higher x-ray intensity and monochromatic radiation, among other advantages. Higher intensity can be used to increase spatial resolution or signal-to-noise ratio or to decrease scan times or a combination of the three parameters. With spatial resolution better than 10 μm, the investigation of cortical porosity and of the Haversian system becomes feasible (Bousson *et al.,* 2004; Matsumoto *et al.,* 2006; Schneider *et al.,* 2007). Spatial resolution between 1 and 10 μm can also be achieved with micro-focus x-ray tubes, but then scan times are in the order of several hours. Nevertheless, such systems have also been used for the investigation of cortical bone beyond cortical thickness and volume (Cooper *et al.,* 2003, 2004; Basillais *et al.,* 2007; Cooper *et al.,* 2007).

The use of monochromatic synchrotron radiation avoids beam-hardening artifacts of the tomographic reconstruction, which pose a major obstacle for the determination

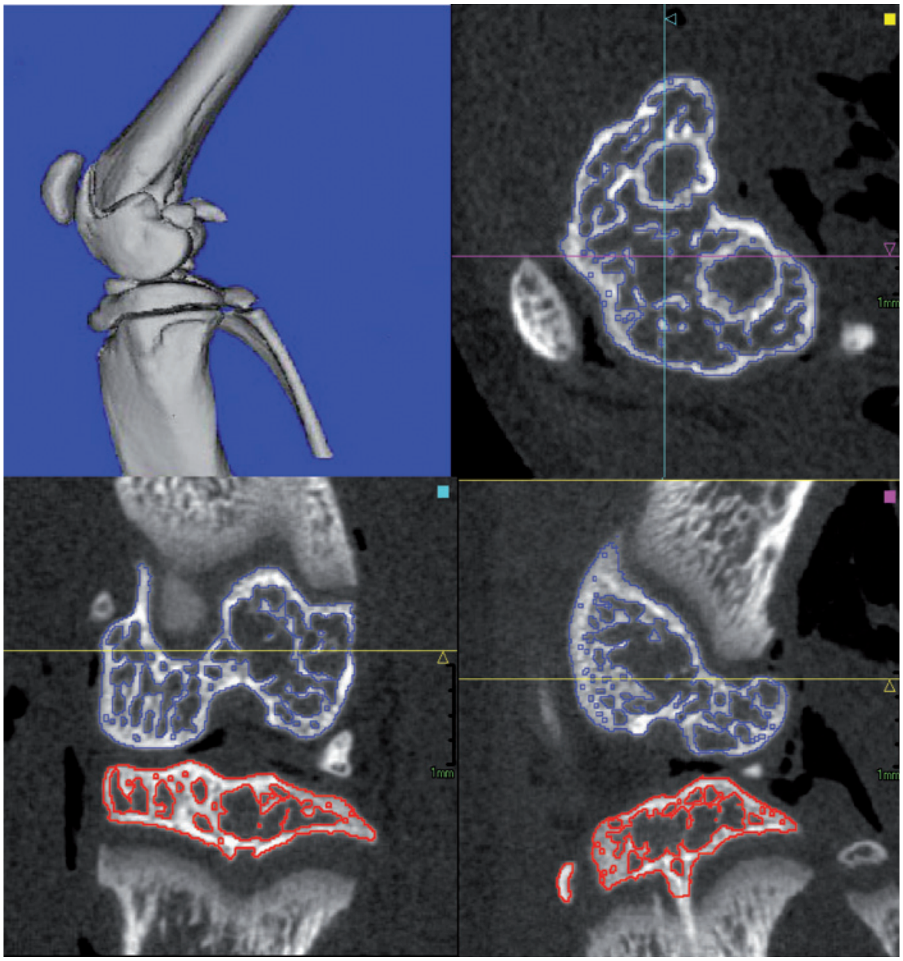


FIGURE 11 μCT dataset with (11 μm)^3 voxel size of a knee joint of a 30-day-old CBA mouse. A 3D segmentation of the subchondral bone is shown in axial, coronal, and sagittal multiplanar reconstructions using advanced multistep gradient-based segmentation algorithms (Wachsmuth *et al*., 2004). (See plate section)

of bone mineral density in μCT. Techniques for x-ray-tube-equipped μCT scanners have been reported (Mulder *et al.,* 2006; Schweizer *et al.,* 2007), but the calibration of CT values to BMD remains a challenge if a polychromatic x-ray spectrum is used, whereas monochromatic radiation can easily be obtained from synchrotrons. Therefore, a number of studies have employed SR to measure local mineralization or the Ca/P ratio in μCT images (Nuzzo *et al.,* 2002a, 2002b; Martin-Badosa *et al.,* 2003; Borah *et al.,* 2006; Matsumoto *et al.,* 2006; Tzaphlidou *et al.,* 2006).

Another avenue to use the high intensity of SR has been pioneered by Kinney and coworkers, who first reported serial measurements in rats *in vivo*. In order to limit the radiation exposure only one leg fixed in a stretched out position was scanned (Kinney *et al.,* 1995). Meanwhile the development of x-ray-tube-based μCT scanners for *in vivo* investigations of small anesthetized laboratory animals has gained considerable attention and a number of *in vivo* studies to monitor the bone-protective effects of zoledronic acid and 17-alpha ethinylestradiol in rats (Gasser *et al.,* 2005), bone architecture alterations in hind-limb-unloaded rats (David *et al.,* 2003), bone loss in OVX rats (Waarsing *et al.,* 2004; Boyd *et al.,* 2006), subchondral bone adaptations in a rodent model of osteoarthritis (OA) (McErlain *et al.,* 2007), or age-related changes of trabecular microarchitecture in rabbits (Voor *et al.,* 2007) have been reported. In order to position the analysis volumes reproducibly a 3D registration of baseline and follow-up scans has been suggested (Boyd *et al.,* 2006). Waarsing reported an *in vivo* precision of 3% for BV/TV and of better than 1% for Tb.Th (Waarsing *et al.,* 2004). Voor obtained values of about 2% for BV/TV, Tb.Sp, and Tb.Th (Voor *et al.,* 2007).

MAGNETIC RESONANCE IMAGING

MRI depicts trabecular bone as a negative image by virtue of the strong signal generated by the abundant fat and water protons in the surrounding marrow tissue, whereas bone mineral lacks free protons and generates no MR signal. Similar to CT, MRI can be used to determine geometrical measures such as the cross-sectional area of the femoral neck (Hong *et al.,* 2000; Arokoski *et al.,* 2002; Hogler *et al.,* 2003; McKay *et al.,* 2004; Manske *et al.,* 2006) or cortical thickness (Saha *et al.,* 2004). Another avenue to employ MRI that has been explored even earlier (Davis *et al.,* 1986) is the use of susceptibility differences between trabecular bone and the surrounding bone marrow that affect the marrow signal intensity by using, for example, gradient echo or asymmetric spin echo sequences. The susceptibility differences cause local inhomogeneities in the main magnetic field which result in changes in the apparent relaxation time T_2*. Similar to a BMD measurement typically T_2* is analyzed in a larger ROI or VOI. The use of the macroscopic measurement of the relaxation rate $1/T_2$* for diagnostic purposes, its correlation with BMD and with bone structure, has been summarized in a number of review articles (Genant *et al.,* 1996; Link, 2008).

In the context discussed in this review, however, the more interesting application of MRI is the determination of trabecular architecture either *in vivo* by using methods of high-resolution MRI (hrMRI) or *in vitro* by using micro MR (μMR) technology. Both techniques have received considerable attention as research and potential clinical tools over the past decade. Although scan times with MR are much longer than with CT and BMD cannot be quantified and the analysis and the interpretation of MR images is more complicated than for CT, MR is a highly interesting approach because of the lack of ionizing radiation, which is a fundamental limitation to achieve higher spatial resolution in *in-vivo* investigations using CT.

Both spin-echo (Jara *et al.,* 1993; Magland *et al.,* 2006) and gradient-echo sequences (Majumdar *et al.,* 1995) can be used to generate hrMRI images. The appearance of the trabecular network on these images is affected by many factors beyond spatial resolution, including the field strength and specific pulse sequence used, the echo time, the signal-to-noise achieved, and the marrow compositional changes (Majumdar *et al.,* 1995; De Bisschop *et al.,* 1996; Newitt *et al.,* 1996; Selby *et al.,* 1996; Fransson *et al.,* 1999; Banerjee *et al.,* 2005; Techawiboonwong *et al.,* 2005). For example, fast 3D spin-echo approaches are preferable to gradient echoes because they are less sensitive to local T_2* differences between bone and marrow. As a consequence, overestimation of trabecular dimension is more pronounced in gradient-echo images (Boutry *et al.,* 2004), especially when the echo-time TE is increased. In contrast, gradient echo sequences are advocated for their higher signal-to-noise characteristics allowing for shorter scan times, which is an important factor for *in vivo* imaging (Majumdar *et al.,* 1995).

High-Resolution Magnetic Resonance Imaging (hrMRI)

With hrMRI using standard 1.5-Tesla (T) clinical MR scanners, an in-plane spatial resolution of approximately 0.3 mm can be achieved, which is approximately twice as high as for hrCT, whereas the slice thicknesses of 0.5 mm is comparable in both methods. In a direct comparison of cadaver specimens between hrMRI and hrCT with contact radiographs (Fig. 12), apparent histomorphometric parameters derived from hrMRI better predicted the values

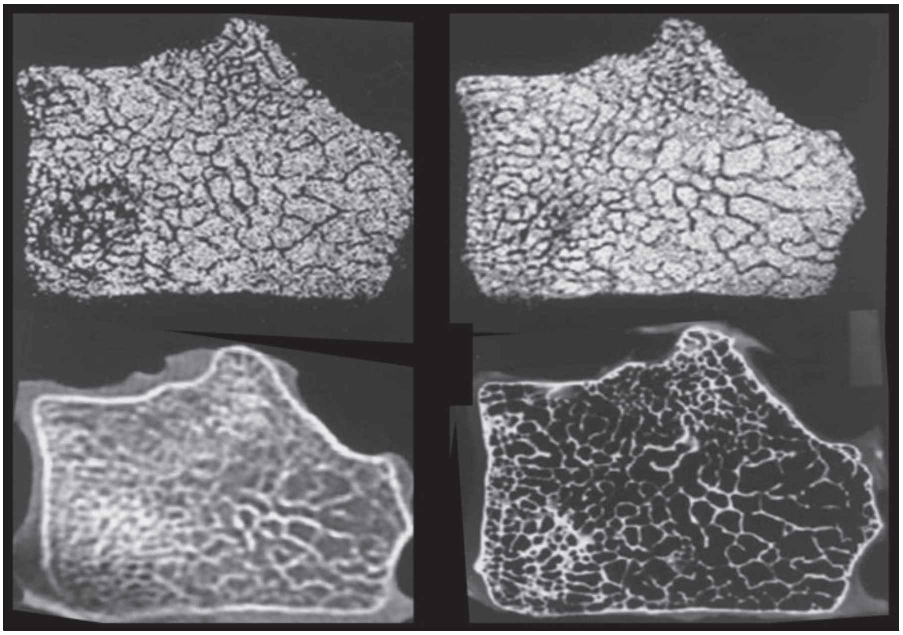


FIGURE 12 Comparison of trabecular structure of a calcaneus specimen imaged with hrMRI, hrCT, and microradiography. (*Top*) 3D hrMRI spin-echo sequence; in-plane pixel size, 150 μm; slice thickness, 300 μm (*Left*) and 900 μm (*Right*). (*Bottom*) Left hrCT plane pixel size, 250 μm; slice thickness, 1000; right contact radiograph of a 1-mm-thick section cut from the calcaneus; slice of plane pixel size, 5 μm (Link *et al*., 2003).

obtained from the radiographs than those obtained from hrCT (Link *et al.,* 2003). However, for contact radiographs a slice thickness of 1 mm was chosen to match the hrCT and hrMRI protocols used in the study. Thin sections to accurately determine histomorphometric parameters were not produced.

Nevertheless, in hrMRI as in hrCT the spatial resolution is still insufficient for an accurate measurement of histomorphometric parameters, and the same limitations as discussed in the section on hrCT above apply. This is also the reason why in hrMRI most work has been done in the appendicular skeleton, namely in the distal forearm and the calcaneus where a higher spatial resolution can be obtained than in the spine or hip. Similar to hrCT considerable resolution dependence was observed for 2D and 3D stereological parameters using hrMRI (Majumdar *et al.,* 1996; Kothari *et al.,* 1998). In a study comparing the analysis of histomorphometric parameters from hrMRI with thin sections, correlations were highest for BV/TV and Tb.Th with r^2 values of about 0.5. The image segmentation parameters used for a binarization had a significant impact on the correlations (Vieth *et al.,* 2001) confirming results from a similar study (Engelke *et al.,* 2001). The effect of thresholding technique and section thickness in hrMRI was also confirmed in another study of femoral specimen. Depending on image-processing technique, correlations between hrMRI and hrCT results varied considerably (Issever *et al.,* 2002; Link *et al.,* 2003).

In order to reduce the effect of partial volume artifacts a number of advanced preprocessing techniques, such as distance transforms (Laib *et al.,* 2002; Saha *et al.,* 2004), subvoxel processing (Hwang *et al.,* 2002), autocorrelation (Hwang *et al.,* 1997; Wald *et al.,* 2007), or adaptive thresholds (Gordon *et al.,* 1997; Vasilic *et al.,* 2005), have been suggested. As an alternative to histomorphometric analysis, several methods to analyze network topology (Gomberg *et al.,* 2000; Boutry *et al.,* 2003; Gomberg *et al.,* 2003; Tabor 2005), 3D morphology (Boehm *et al.,* 2003), anisotropy (Harrigan *et al.,* 1984; Rotter *et al.,* 2001), fractals (Zaia *et al.,* 2006), parameters describing the fuzziness of the network (Carballido-Gamio *et al.,* 2006), or other textural parameters, have also been introduced to hrMRI (Herlidou *et al.,* 2004; Pothuaud *et al.,* 2004; Mueller *et al.,* 2006; Krug *et al.,* 2007).

Several studies obtaining stereological as well as textural parameters from hrMRI images have been carried out to characterize trabecular architecture in various subject groups. For instance, in early studies representative axial sections of the distal radius from normal and osteoporotic subjects clearly depicted the loss of the integrity of the trabecular network with the development of osteoporosis (Majumdar *et al.,* 1994) (Fig. 13), sagittal sections of the calcaneus demonstrated age-related changes (Majumdar *et al.,* 1997; Ouyang *et al.,* 1997) and regional variations of trabecular parameters (Lin *et al.,* 1998). Images of the calcaneus of normal subjects showed that the orientation of the trabeculae also is significantly different in various anatomic regions. Ellipses representing the mean intercept length (MIL) were used to map the degree of anisotropy (DA) of trabecular structure (Majumdar *et al.,* 1994). DA was calculated as the ratio between the maximal and the minimal radius of the MIL ellipsoid.

The use of special-purpose RF coils at the appendicular skeleton can partially offset the problem of limited spatial resolution. Wehrli and colleagues (Jara *et al.,* 1993) and Glüer and colleagues (Kühn *et al.,* 1997; Stampa *et al.,* 2002) have utilized clinical imagers at 1.5 Tesla with coil designs optimized for the phalanges, a convenient anatomical site suitable for obtaining high signal-to-noise images *in vivo* with spatial resolution of $150 \times 150 \times 280\,\mu m^3$. Stampa used these phalangeal images to derive quantitative three-dimensional parameters for defining trabecular rods and plates (Stampa *et al.,* 2002). Others, including Link (Link *et al.,* 1998, 2002), Majumdar (Majumdar *et al.,* 1999; Laib *et al.,* 2002), and Wehrli (Wehrli *et al.,* 2001) have shown the ability to discriminate spine and/or hip fractures by using trabecular structure or textural parameters in vivo.

After 24 months of testosterone treatment in hypogonadal men, in addition to BMD of the spine (+7.4%)

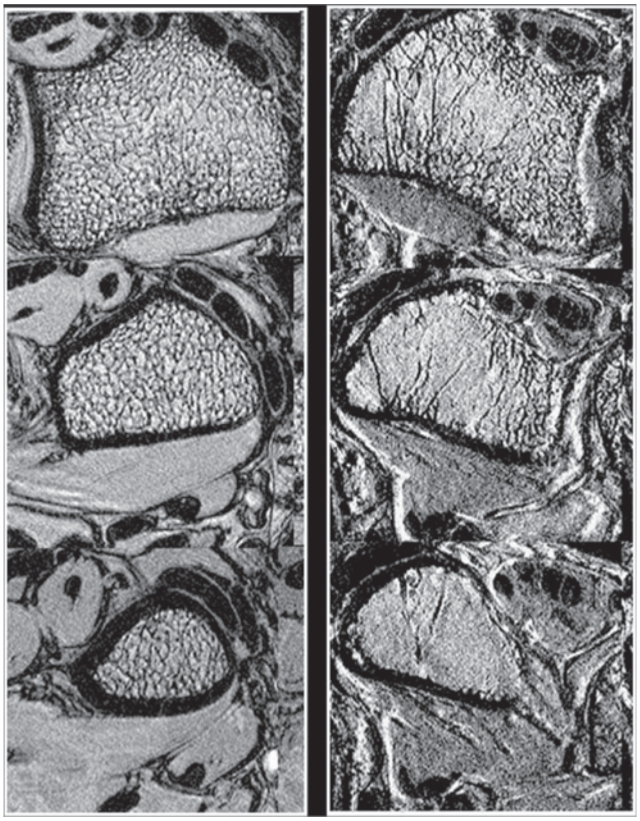


FIGURE 13 hrMRI (~$150 \times 150 \times 500\,\mu m$) of the distal radius of a young woman (*Left*) and an elderly osteoporotic woman (*Right*); axial gradient echo technique. Courtesy of Sharmila Majumdar.

and of the total hip (+7.5%), two advanced architectural parameters assessed by hrMRI significantly changed: the surface-to-curve ratio (+11%) and the topological erosion index (−7.5%) (Benito *et al.,* 2005). In another study with salmon calcitonin, hrMRI imaging at the distal radius but not at the ultradistal radius revealed significant improvement or preservation of appBV/TV, appTb.N, and appTb.Sp in the treated group compared with significant deterioration in the placebo control group (Chesnut *et al.,* 2005). However, in both studies it remained unclear whether results in the structural parameters were adjusted for BMD. A few studies have also indicated a role for monitoring trabecular architecture after heart and kidney transplantations (Link *et al.,* 2000; Link, 2002; Link *et al.,* 2002) and in renal osteodystrophy (Wehrli *et al.,* 2004).

In parallel to the *in-vivo* studies listed earlier, a number of *in-vitro* studies using whole bones or trabecular cubes from the distal radius (Gordon *et al.,* 1998; Link *et al.,* 2004; Hudelmaier *et al.,* 2005), lumbar vertebrae (Link *et al.,* 1998; Majumdar *et al.,* 1998; Beuf *et al.,* 2001; Pothuaud *et al.,* 2002), calcaneus (Majumdar *et al.,* 1998; Link *et al.,* 2004), or proximal femur (Link *et al.,* 1998; Majumdar *et al.,* 1998; Link *et al.,* 2003) addressed a variety of topics. For example, studies confirmed *in-vivo* results of differences of trabecular architecture between gender (Hudelmaier *et al.,* 2005) and among anatomical locations (Majumdar *et al.,* 1998). Majumdar also found good correlation between hrMRI and microscopic optical images for appTb.Sp and appTb.N, but not for appTb.Th (Majumdar *et al.,* 1998). In another study of bone cubes extracted from the vertebrae, traditional morphometric measures of bone structure (appBV/TV; appTb.N, appTb.Sp), including appTb.Th, correlated highly ($0.8 < r < 0.92$) with age, BMD, and stress values (Beuf *et al.,* 2001). In a study comparing hrMRI with hrCT texture parameters derived from femur and spine specimens, both techniques correlated equally poorly with maximum compressive strength (spine: $r^2 = 0.3$ to 0.6; femur $r^2 = 0.1$ to 0.2). For hrMRI, texture measures combined with BMD significantly increased r^2, whereas improvement was less significant for hrCT (Link *et al.,* 1998).

Studies investigating the prediction of biomechanical parameters from trabecular structure show a large range in terms of absolute correlations but agree that trabecular structure can improve the prediction of biomechanical parameters over just using BMD (Gordon *et al.,* 1998; Pothuaud *et al.,* 2002; Link *et al.,* 2003; Hudelmaier *et al.,* 2005). For example, in forearm cadavers, Gordon found that BMD as measured by pQCT explained 50% of the variability with load. Indices relating to the size of the marrow spaces either measured by hrMRI or pQCT explained an additional 25% to 30% (Gordon *et al.,* 1998). In contrast, in a study of bone cubes from vertebral bodies, appBV/TV was found to be a much strong predictor of the mechanical properties, accounting for 89% to 94% of the variability of the elastic moduli and for 69% to 86% of the variability of the ultimate stresses. The inclusion of topological parameters increased these values to 96% to 98% for the variability of the elastic moduli (Pothuaud *et al.,* 2002). The correlations are stronger here because cubes, not whole bones, were used, eliminating the big bone/little bone confounding effect.

Most of the development of hrMRI and the majority of *in vivo* studies, including all those cited earlier, have been carried out on clinical 1.5 T imagers. Recently some initial work has been reported from a small compact 1.0 T device (Handa *et al.,* 2007; Iita *et al.,* 2007) as well as from new clinical 3 T devices. At 1.0 T studies have concentrated on the determination of BV/TV (Kose *et al.,* 2004; Tomiha *et al.,* 2005) and it still needs to be proven that, despite limitations in spatial resolution and signal-to-noise, trabecular structure can also be quantified.

The introduction of new high-field MR scanners to clinical use has triggered the extension of the work of hrMRI to the central skeleton. At higher field strength, either the scan time can be reduced, or the signal-to-noise ratio can be increased, or a combination of both can be achieved. New challenges are increases in susceptibility artifacts that may partially be offset by new acquisition sequences (Banerjee *et al.,* 2005). One study showed that structural parameters measured at 3 T better discriminated spinal fractures than the same parameters measured at 1.5 T (Phan *et al.,* 2006), but susceptibly and partial volume artifacts were still present with 3 T, causing an overestimation of 50% for appBV/TV and by 100% for appTb.Th. appTb.Sp was still underestimated whereas appTb.N was largely unaffected (Sell *et al.,* 2005), confirming Laib's approach to use Tb.N as a starting point when calculating histomorphometric parameters (see section 4.3) (Laib *et al.,* 1999).

First *in-vivo* studies at 3 T have demonstrated the possibilities of the characterization of trabecular architecture in the trochanter of the femur (Krug *et al.,* 2005) as well as improved spatial resolution in the wrist (Ludescher *et al.,* 2005). New imaging sequences and segmentation techniques for trabecular bone are also being developed (Vasilic *et al.,* 2005; Magland *et al.,* 2006).

Micro Magnetic Resonance Imaging

In the literature, hrMRI techniques described in the previous section are often termed μMRI or MR microscopy. However, for a better differentiation we prefer and suggest reserving the term μMRI to techniques with a spatial resolution of better than 100 μm and MR microscopy to techniques with a spatial resolution of better than 1 μm. Similar to μCT, μMRI applications are restricted to *in vitro* specimen work and to *in vivo* investigations of small animals. Obviously, with higher spatial resolution, histomorphometric parameters can be determined more accurately, but the main advantage of MRI versus CT, the absence of radiation, is only important for *in vivo* animal and not for specimen work. Also for μMRI different acquisition protocols and image-processing algorithms are used than for hrMRI. Thus, with respect to the imaging of bone architecture, μMRI has only limited value in improving hrMRI techniques. So, not surprisingly, a rather limited number of studies have been published over the past decade and many of the results, in particular, the relation of bone architecture to bone strength, could have been obtained from μCT studies as well.

In μMRI, special-purpose, small-bore, high-field magnets have been employed to obtain very high resolution and to optimize the relation of signal-to-field strength. Early approaches of μMRI in bone showed good in-plane resolutions, but at a slice thickness of 250 μm, partial volume artifacts were still obvious (Kapadia *et al.,* 1993). Wehrli and colleagues obtained 78-μm isotopic voxels of human and bovine bone cubes using three-dimensional imaging at 9.4 Tesla, and derived anisotropy ellipsoids from the analysis of mean intercept length. They also found good correlation between MR-derived parameters and standard histomorphometric measures (Chung *et al.,* 1993, 1995a, 1995b). Timonen and coworkers investigated the connectivity of the trabecular network in rat femora at 7 Tesla (Timonen *et al.,* 2001). At 8.6 Tesla, BV/TV determined in human bone cubes with μMRI no longer differed significantly from BV/TV ($r^2 = 0.81$) determined from optical imaging of the stained cube surfaces (Hipp *et al.,* 1996).

A comparison of μMRI data obtained at 8.5 Tesla and a voxel size of 66 μm^3 with μCT data obtained at a voxel size of 10 μm^3 showed that network connectivity, orientation, and anisotropy as determined by μMRI were accurate. However, BV/TV and morphometric parameters were still biased relative to their values from the μCT data, although there was a significant correlation between the two modalities (Last *et al.,* 2005). In contrast, in another study carried out at 4.7 T in the rat femur, correlations between μMRI (in-plane voxel size 23 μm^2; slice thickness, 39 μm) and histomorphometry of decalcified 5-μm sections were only moderate ($r^2 \approx 0.5$), but the authors pointed out that the decalcification had potentially distorted the sections (Hopper *et al.,* 2004).

In two *in-vitro* studies in OVX rats μMRI was successfully used to show drug-related effects on trabecular structure. In the first study, it was shown that ERT prevented ovariectomy-induced losses in trabecular BV/TV and structure (Kapadia *et al.,* 1998). In the second study carried out at 9.4 T using an isotropic voxel size of 46 μm^3, it was shown that in a group of rats given alendronate for 2 months starting 2 months after OVX, BV/TV was maintained midway between non-OVX and nontreated (intact) animals. Treatment with prostaglandin E_2 instead of alendronate returned BV/TV to levels observed in intact levels (Takahashi *et al.,* 1999). In hind limp suspended mice imaged at 11.7 Tesla, BV/TV (−53%), Tb.Th (−19%), Tb.Sp (+114%), and Tb.N (−43%) changed significantly compared with a control group (Gardner *et al.,* 2001).

In vivo μMRI measurements of the femoral bone structure of C57Bl/6 mice at 11.7 T have been reported recently. Trabecular and cortical bone properties have been compared with histomorphometry. An optimal correlation with histomorphometry was obtained with gradient-echo sequences. Increases of percent area of marrow and decreases of percent area of trabecular bone and of cortical bone thickness from the epiphyseal growth plate to the diaphysis correlated with the histomorphometric data (Weber *et al.,* 2005).

MULTIMODALITY IMAGING

The assessment of bone structure is not only important in osteoporosis. Another application is the investigation in rheumatoid and osteoarthritis (Pelletier 2004; Schett *et al.,* 2005). A few studies using hrMRI (Beuf *et al.,* 2002; Blumenkrantz *et al.,* 2004; Lahm *et al.,* 2006), μCT (Patel *et al.,* 2003; Botter *et al.,* 2006; Chappard *et al.,* 2006; McErlain *et al.,* 2007), and μMRI (Lammentausta *et al.,* 2007) techniques have been reported. Rheumatoid arthritis (RA) and OA are complicated diseases. In OA, cartilage, synovial membrane, and subchondral bone are involved. Thus, a combination of CT and MRI techniques may simultaneously assess cartilage and subchondral bone (Batiste *et al.,* 2004; Wachsmuth *et al.,* 2004). For example, in Figure 14, μCT and μMR images of male Lewis rats demonstrate the potential of multimodality imaging. μCT is preferable to μMRI for the determination of bone structure, whereas μMRI can depict cartilage and other soft tissue. Figure 15 shows the registration of the two datasets so that a local correlation of bone structure and cartilage defects can be performed more accurately.

Finite-Element Modeling

Finite-element modeling (FEM) is a computer-based simulation of the strains and stresses induced by mechanical loading of an object and is widely used in engineering. The object is described as a connected set of simply shaped elements that are ascribed elastic properties. Bone biomechanics research with this tool was pioneered by Lotz and Hayes (Lotz *et al.,* 1991a, 1991b) and Faulkner (Faulkner *et al.,* 1991). One of its goals is to better predict load conditions that lead to fracture and thus to improve fracture prediction. Currently, the models are typically derived from volumetric QCT scans, and element elastic properties are computed from bone density at the position of the elements (Fig. 16) (Lang *et al.,* 1997; Keyak *et al.,* 2000). Finite-element models integrate mechanically all of the anisotropic, inhomogeneous, and complex geometry of the bone structure examined.

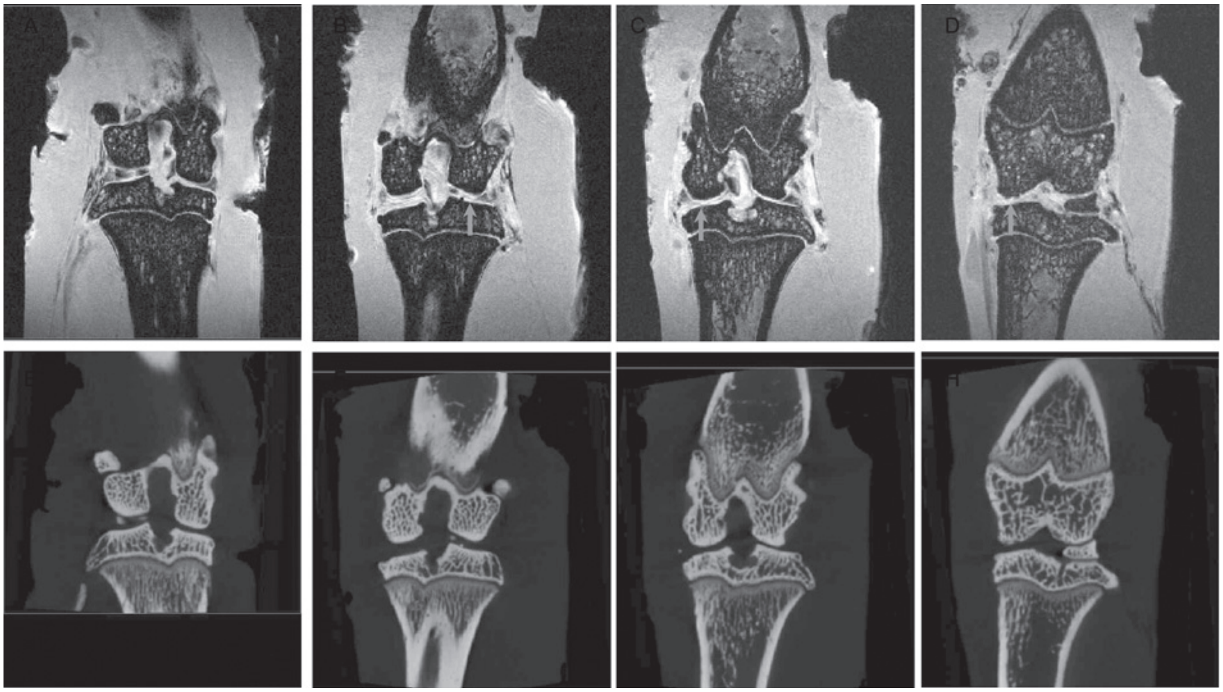


FIGURE 14 µMRI (*Top*) and µCT (*Button*) data for male Lewis rat knees with severed cruciate ligament and removed medial menisci. **A** and **E**, nonoperated contralateral knee; **B–D** and **F–G**, operated knee. In the µCT images the medial subchondral bone shows a higher degree of sclerotization and the trabeculae appear to be thickened compared with the nonoperated knee. In the µMRI images (gradient-echo sequence) the medial cartilage is more homogeneous in the nonoperated knee (Wachsmuth *et al*., 2004).

Finite-element analysis potentially integrates density and geometry and may better account for regional variations than the separation of a bone into subvolumes. Several *in vitro* studies have shown QCT-based finite-element analysis to be a strong predictor of whole-bone strength, with some (Cody *et al.,* 1999; Crawford *et al.,* 2003), though not all (Keyak *et al.,* 1998; Buckley *et al.,* 2007), studies reporting stronger correlations for FEM than for DXA- or QCT-based measures. However, it still has to be demonstrated that FEM provides additional advantages to a combination of densitometric and geometrical QCT parameters. For the hip, it has been shown that such a combination of densitometric and geometric parameters that can be extracted easily and rapidly from a QCT dataset is a better predictor of failure load than single parameters alone (Bousson *et al.,* 2006).

At the spine, Keaveny, Hayes and colleagues found that in healthy subjects the cortical shell does not transfer much of the load (Silva *et al.,* 1997). It has been claimed that voxel-based finite-element model-derived estimates of strength are better predictors of *in vitro* vertebral compressive strength than clinical measures of bone density derived from QCT with or without bone size (Crawford *et al.,* 2003). However, this advantage of FEM may not pertain if more sophisticated parameters than just midvertebral trabecular BMD and bone size are measured. Recently, these parameters have been made available (Mastmeyer *et al.,* 2006) but a comparison with FEM has not been performed. A pilot study in 20 randomly selected postmenopausal women treated with PTH for one year indicates that about half the overall increase in vertebral strength can be attributed to an average increase in bone density, and the remaining effect is due to alterations in the distribution of bone density within the vertebra (Black *et al.,* 2005). Although imaging resolution for FEM is not critical in cross-sectional studies using clinical CT scanners, longitudinal studies that seek to track more subtle changes in stiffness over time should account for the small but highly significant effects of voxel size (Crawford *et al.,* 2003).

In the femur, FEM so far has mostly been used for the optimization of endoprostheses; vQCT-based applications for fracture prediction are still rare. One study of the hip in 51 women aged 74 years (Ciarelli *et al.,* 2000) showed different risk factors for hip fracture during single-limb stance and falls, which agrees with epidemiological findings of different risk factors for cervical and trochanteric fractures. In the *in-vitro* arm of the European femur fracture study with finite-element analysis and QCT (EFFECT), QCT parameters predicted fracture load in fall and stance configurations as well as FEM (Engelke *et al.,* 2006).

With the vast increases of computer power during the past decade and the availability of µCT data, the application of FEM at spatial resolutions that allow modeling of individual trabeculae, which is computationally much more demanding than just using voxels containing average gray values, has become feasible (Fig. 17). Although 2D simulation studies indicate that the amount and thickness of trabeculae strongly determine overall bone strength (Silva *et al.,* 1997), full 3D models were first developed

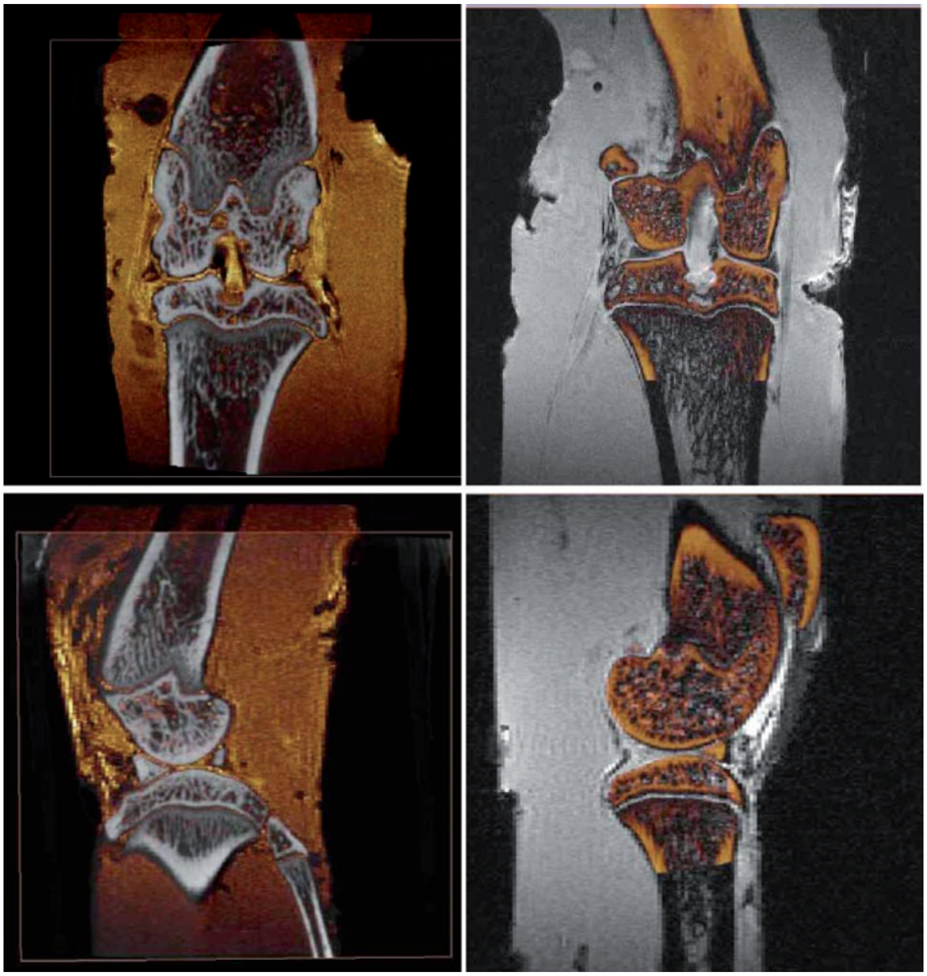


FIGURE 15 Registration of μMRI and μCT datasets from Fig. 14. In the two images on the left the μCT part is gray and the μMRI part is colored; in the two images on the right the color coding is inverted. (See plate section)

by van Rietbergen *et al.* (1995). Prediction of overall bone strength recorded during mechanical testing of small samples of trabecular bone with such models are indeed better than with macroscopic bone density measurements (Ulrich *et al.,* 1997; Crawford *et al.,* 2003).

μCT scanning has only recently reached the resolution to allow conversion of the gray values of the individual pixels to elastic moduli to further improve the accuracy of fracture load prediction (Homminga *et al.,* 2001, 2002). Using this improved technique, Homminga and colleagues showed that, although osteoporotic vertebrae can withstand daily load patterns comparably to normal bone, loading as occurs during forward bending caused much higher stresses in the osteoporotic vertebra (Homminga *et al.,* 2004). μFEM based on μCT data has been used to estimate distal radius failure loads (Pistoia *et al.,* 2002), to calculate load transfer from the biomaterial structure of bone scaffolds (Lacroix *et al.,* 2006), to determine regional variations and age-related changes in mechanical parameters of trabecular bone in the vertebrae (Gong *et al.,* 2007), or to predict mechanical properties of fracture callus during fracture healing (Shefelbine *et al.,* 2005).

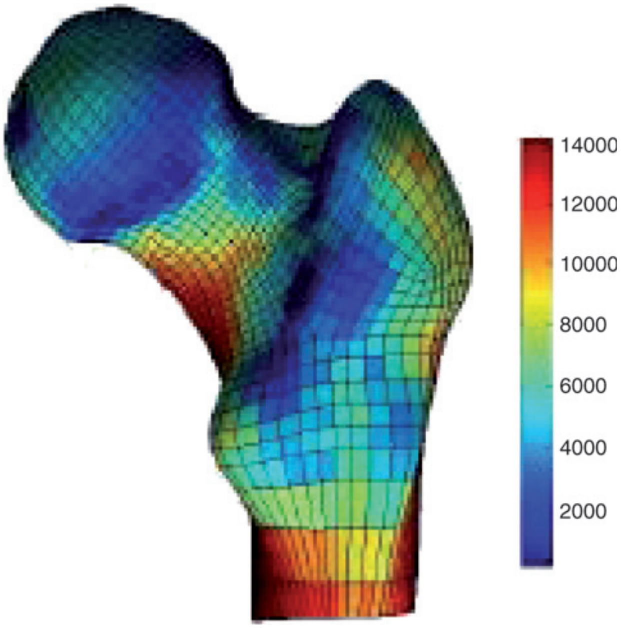


FIGURE 16 Distribution of Young's modulus computed from BMD in the elements of the mesh using vQCT data. Courtesy of David Mitton. (See plate section)

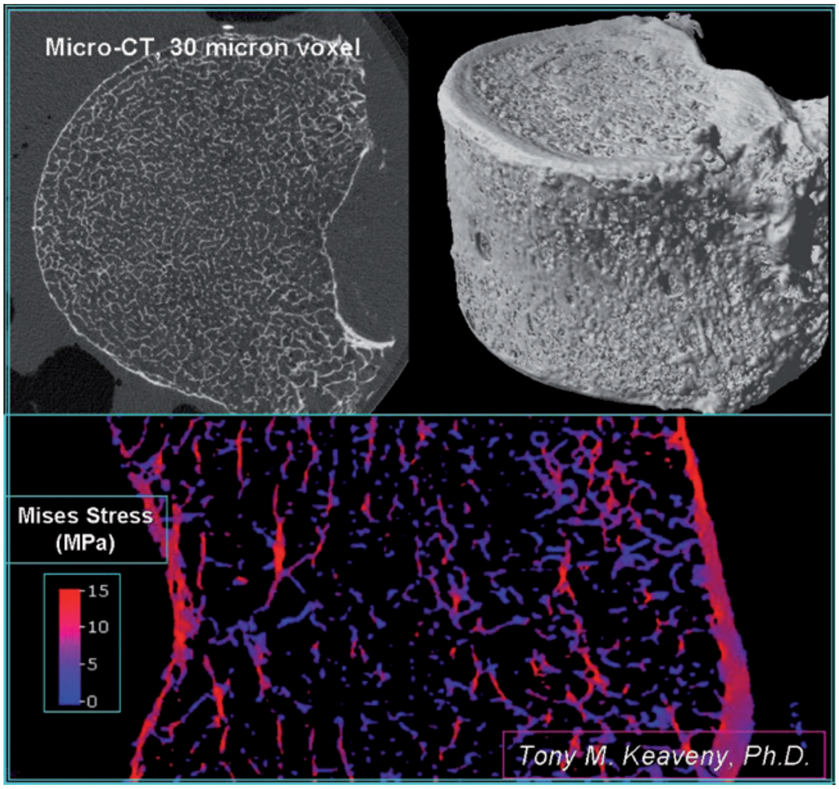


FIGURE 17 Micro CT of vertebral body at 30-μm resolution and generation of micro finite-element model at the trabecular level. Courtesy of Tony Keaveny. (See plate section)

FEM has also been applied to magnetic resonance images. Newitt and coworkers have reported promising results on the application of micro finite-element analyses based on hrMR images of the distal radius *in vivo* (Newitt *et al.*, 2002). In another study structure parameters determined in high-resolution MR images of the proximal femur specimen correlated significantly with bone strength, with the highest correlations obtained by combining DXA BMD and structure measures (Link *et al.*, 2003). The femoral neck trabecular microstructure significantly correlated with biomechanical properties, and its combination with BMD further improved the prediction of bone quality (Jiang *et al.*, 2005).

SUMMARY

For many years the assessment of 3D bone architecture has been an important aim in bone research in order to better understand disease etiology, to facilitate bone strength determination, to improve fracture risk prediction, and to more effectively assess aging and treatment mechanisms. Many studies have indeed shown that changes in bone quality and structural characteristics lead to changes in bone biomechanical competence or individual risk of fracture independently of BMD.

From a clinical perspective, two directions seem to be most relevant. The first is the quantitative characterization *in vivo* of bone macro- and microstructure in humans as information complimentary to BMD. The second is the *in-vivo* investigation of mice and rats that should include bone structure and density. Fueled by the introduction of genetically engineered mice and rat models, most progress has been made in the field of μCT. *In-vivo* investigations of anesthetized animals with spatial resolution down to 50 μm are almost routine procedures today. Obviously, this greatly facilitates preclinical research. By using 3D analysis algorithms to analyze trabecular structure it has been demonstrated that, in μCT, standard parameters such as Tb.N, BV/TV, and Tb.Sp can be measured reproducibly and with adequate accuracy despite remaining partial volume artifacts. However, acceptable limits of radiation exposure that eventually define maximum achievable image quality in terms of noise and spatial resolution still have to be determined for small animals. The measurement of BMD with μCT by using polychromatic radiation emitted from x-ray tubes also still has to be validated.

μCT has also been used successfully for *in vitro* characterization of bone structure. Spatial resolution below 10 μm, which is required to investigate cortical bone structure, is still the domain of synchrotron radiation, although the first such x-ray tube-based scanners have recently become available. While μMRI techniques have not found widespread use for the determination of bone architecture they may become more relevant in order to bypass limitations of spatial resolution owing to radiation exposure in *in-vivo* μCT. For *in-vitro* applications, the domain of μMRI may be limited to soft tissue imaging such as cartilage, however, multimodality applications of μCT and μMRI, in particular for Osteoarthritis, show promising potential.

With respect to *in-vivo* imaging of bone structure in humans, volumetric QCT has significantly improved our capability to determine BMD in dedicated regions of interest, to assess bone geometry and, with some limitations, cortical thickness. Finite-element modeling has shown the potential to integrate BMD and geometry, although it still has to be shown that FEM is superior to a multivariate model of BMD and geometrical parameters directly measured with CT for fracture prediction. The extension of DXA into a more volumetric technique may open new avenues to obtain volumetric BMD information with DXA equipment.

With respect to trabecular structure, however, despite more than ten years of development and impressive technological advances of the equipment, little progress has been made with hrCT or hrMRI. Only a few clinical and pharmaceutical studies have been reported, and even these have been supervised and analyzed by highly skilled researchers at those few sites involved in the development of these techniques. Obviously, the use of these techniques, in particular, the acquisition in hrMRI and image processing in hrMRI and hrCT is still very challenging in the context of multicenter, multinational clinical trials and far too complicated for routine clinical work.

One reason is that the spatial resolution of hrCT and hrMRI is not adequate to measure stereological parameters accurately, and apparent Tb.N, Tb.Sp, and Tb.Th are not independent from the selected image acquisition and processing techniques. This may explain their limited success as additional predictors for fracture discrimination or for health and disease or treatment assessments, which are substantially independent of BMD. Also typically, these structural parameters correlate highly with BV/TV and as a consequence are highly correlated to site-matched BMD. A huge variety of alternate parameters characterizing texture has been introduced but their meaning as well as their relevance often remains obscure, in particular, because two studies have rarely used the same set of parameters. Interestingly, the performance of texture parameters applied to radiographs also seems to be promising.

For theoretical reasons, parameters describing geometry and structure are expected to provide a more accurate prediction of structural strength than a global BMD measurement because they consider the spatial distribution of bone material and the bone architecture rather than bone mass alone. However, the distribution of available bone mass is thought to be subject to an optimization process that provides adequate mechanical strength and is influenced by mechanical stimuli (Huiskes, 2000). Therefore, the strength of the relationships between mechanical competence might be similar for geometric and bone mass measurements.

Of course it has been demonstrated that trabecular networks with different SMI, that is with different structure, still may have identical BMD (Muller *et al.,* 1997), thus BMD and bone strength may be decoupled. However, during normal aging, at least in the spine, decrease of BMD and structural deterioration of the trabecular network seem to occur in parallel. This supports the hypothesis by Huiskes and may explain why structural parameters in many studies have added only a minor value to BMD in improving fracture discrimination (Chevalier *et al.,* 1992; Ito *et al.,* 1995; Gordon *et al.,* 1998; Link *et al.,* 1998; Majumdar *et al.,* 1999; Wehrli *et al.,* 2001; Link, 2002), although structural parameters remained significant after BMD adjustment. Under pharmaceutical treatment this may change, at least it is known that BMD increases do not fully account for fracture reduction. Often this has been explained with systematic errors of DXA, but μCT studies have shown differential effects on structure under different treatment options. Of course, it is speculative that under pharmaceutical treatment, structure and density may be decoupled but it definitely provides further motivation to improve hrCT and hrMRI techniques and to include BMD measurements in μCT analyses. For the forearm and tibia, an advanced high-resolution pQCT scanner with integrated software for structural analysis that offers a spatial resolution of about 100 μm has recently become available commercially, and several studies are currently evaluating the potential of this device; however, for the spine and femur, the most important fracture sites, technical challenges are still waiting to be solved.

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